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In vitro Free Radical Scavenging and Thrombolytic Activities of Bangladeshi Aquatic Plant *Aponogeton undulatus* Roxb

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Abstract: Investigation with the crude methanolic extract of *Aponogeton undulatus* was carried out to evaluate its possible antioxidant and thrombolysis activity. In DPPH free radical scavenging assay, the extract exhibited potent antioxidant activity with a IC_{50} values of 2.43±1.06 µg/ml while in ascorbic acid, the value become 2.14±0.11 µg/ml. In thrombolytic activity using *in vitro* clot lysis assay method, the crude methanolic extract was found to have significant ($p<0.001$) thrombolytic activity at a dose of 10 mg/ml with a miximum effect of 20.23±1.56% while the standard streptokinase showed 46.13±3.87%. The extract was also investigated for its antibacterial and toxic potentiality using agar diffusion and Brine Shrimp lethality bioassay, respectively. The highest antibacterial effect was shown against *Bacillus cereus* (zone of inhibition 12±0.65 mm) followed by *Escherichia coli* (zone of inhibition 10±0.71 mm). In this bioassay the extract showed significant toxicity to Brine Shrimp nauplii with the LC_{50} value of 2.24±0.98 µg/ml. The study clearly indicated that the extract possesses good antioxidant and thromolytic activity along with broad spectrum antibacterial and toxic potentiality.

Key words: Free radical · Thrombolysis · *Aponogeton undulatus* · Toxicity

INTRODUCTION

Free radicals cause depletion of immune system antioxidants, change in gene expression and induce abnormal proteins and contribute to more than one hundred disorders in humans including atherosclerosis, arthritis, ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, cancer and AIDS [1, 2]. Undoubtedly, *in vivo* suppression of free radicals is important for the human body to eliminate the toxicity induced by free radicals. For several years, many researchers have been investigated powerful and nontoxic antioxidants from natural sources, especially edible or medicinal plants to prevent the free radical related disorders in human, which replaces the synthetic compounds because of their probable carcinogenic activity which are harmful to the lungs and liver [3]. Cerebral venous sinus thrombosis (CVST) is a common disorder which accompanied by significant morbidity and mortality [4, 5]. Heparin, an anticoagulating agent, is the first line of treatment for CVST, because of its efficacy, safety and feasibility [6]. Thrombolytic drugs like tissue plasminogen activator (t-PA), urokinase, streptokinase etc. play a crucial role in the management of patients with CVST [7].

Plants produce wide array of bioactive principles and constitute a rich source of medicines. In many developing countries, traditional medicine is one of the primary health care systems [8, 9]. Large scale evaluation of the local flora exploited in traditional medicine for various biological activities is therefore necessary. Isolation and characterization of the bioactive principles ultimately leading to new drug development. In view of this, our attention has been focused particularly

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Aponogeton undulatus Roxb. belongs to the family "Aponogetonaceae". The rootstock of the plant is an important food item for the low-income people in this deeply flooded area. The nutrient composition of rootstock of *Aponogetan undulatus* Roxb. shows that it can provide an adequate supply of carbohydrate, protein and some minerals. This food can be exceptionally useful as a nutrient supplement in many areas whereas the purchasing power is limited because of low incomes. The nutrient composition of the rootstock of *Aponogeton undulatus* Linn. shows that it can provide an adequate supply of carbohydrates (42.8g/100g), protein (8.3g/100g), fats (0.7g/100g), iron (18.2g/100g), calcium (37.2g/100g) [10]. The literature review revealed that the leaf pastes are used with hot water to treat cuts and wounds and in Ayurveda, the plant is claimed to be effective against cough, tuberculosis, acne, cancer, diarrhoea, dysentery, jaundice etc [11]. Based on these reports our studies have been designed to examine whether the methanolic extract *Aponogetan undulatus* exerts *in vitro* free radical scavenging and thrombolytic activity. In addition, we investigated whether these extract have any antimicrobial and toxic effect.

MATERIALS AND METHODS

Plant Material: The whole plant of *A. undulatus* was collected from the lake, Chalanbil, Nator the North-West district of Bangladesh during the month of January 2010. The plant material was taxonomically identified by the National herbarium of Bangladesh whose voucher specimen no. 32072 is maintained in our laboratory for future reference.

Preparation of Plant Extract: The shade dried plant materials were pulverized into coarse powder using mechanical grinder, seiving through sieve #40 and stored in an air tight container. The dried powdered material (650 g) was exhaustively extracted with methanol for 72 h in a Soxhlet apparatus. The solvent was distilled under reduced pressure and the resulting semisolid mass was dried in vacuo using rotary flash evaporator to yield a solid residue (18.42% w/w). The preliminary phytochemical analysis was performed to identify the phytoconstituents present in the extract [12].

Chemicals: 1,1-diphenyl- 2-picryl-hydrazyl (DPPH), ascorbic acid, purchased from Sigma Chemical Co. Ltd, (St. Louis, MO, USA). Lyophilized Streptokinase was purchased from Polamin Werk GmbH, Herdecke, Germany. All other chemicals and reagents were of analytical grade.

Test Microorganisms: Strains of both fungi and bacteria (Gram positive and Gram negative) were obtained from International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). *Bacillus cereus* ATCC 14579, *Bacillus megaterium* ATCC 13578, *Bacillus subtilis* ATCC 6059, *Staphylococcus aureus* ATCC 6538, *Sarcina lutea* ATCC 9341, *Escherichia coli* ATCC 25922, *Pseudomonus aeruginosa* ATCC 27853, *Salmonella paratyphi* ATCC 9150, *Salmonella typhi* ATCC 13311, S*higella boydii* ATCC 9234, *Shigella dysenteriae* ATCC 9361, *Vibrio mimicus* ATCC 33653, *Vibrio parahemolyticus* ATCC 17802, *Candida albicans* ATCC 90028, *Aspergillus niger* ATCC 1004 and *Sachoromyces cerevacae* ATCC 60782 were used as test microorganism. All these bacterial and fungal species are recommended by ATCC for their susceptibility assay. The strains are maintained and tested on Nutrient Agar media (NA) for bacteria and Sabourand dextrose agar media (SDA) for fungi.

Free Radical Scavenging Activity Measured by 1,1-diphenyl-2-picryl-hydrazyl (DPPH): The free radical scavenging activity of MeOH extract, based on the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical, was determined by the method described by Braca *et al*. [13]. Plant extract (0.1 ml) was added to 3ml of a 0.004% MeOH solution of DPPH. Absorbance at 517nm was measured after 30 min and the percentage inhibition activity was calculated from $[(A_0–A_1)/A_0]$ x 100, where A_0 is the absorbance of the control and A_1 is the absorbance of the extract/ standard. IC_{50} value was calculated from the equation of line obtained by plotting a graph of concentration (µg/ml) versus% inhibition.

***In vitro* Thrombolysis Activity:** Phosphate buffered saline (PBS) (5 ml) was added to the commercially available lyophilized streptokinase vial (15, 00,000 I.U.) and mixed properly. This suspension was used as a stock from which appropriate dilutions were made to observe the thrombolytic activity. Experiments for clot lysis were carried as reported earlier [14]. In brief, 2 ml venous blood drawn from healthy volunteers was distributed in three different pre weighed sterile microcentrifuge tube (0.5 ml/tube) and incubated at 37°C for 45 minutes. After clot formation, serum was completely removed (aspirated out without disturbing the clot formed) and each tube having clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube – weight of tube alone). To each microcentrifuge tube containing pre-weighed clot, 100 µl of MeOH extract (10 mg/ml) of was added. As a positive control, 100 µl of

streptokinase and as a negative non thrombolytic control, 100 µl of distilled water were separately added to the control tubes numbered. All the tubes were then incubated at 37°C for 90 minutes and observed for clot lysis. After incubation, fluid released was removed and tubes were again weighed to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis.

Antimicrobial Activity: Crude methanolic extract was dissolved in 10% DMSO to get a concentration of 400 µg/ml and sterilized by filtration by 0.45 µm Millipore filters. Standard antibacterial agents Ciprofloxacin (30µg/disc) were prepared. Antimicrobial tests were then carried out by modified agar diffusion method [15, 16] using 100 µl of suspension containing 108 CFU/ml of bacteria, 106 CFU/ml of yeast and 104 spore/ml, spread on nutrient agar (NA) and subourand dextrose agar (SDA), respectively [16]. Bacteria were cultured overnight at 37°C and fungi at 28°C for 72 hour used as inoculums. Nutrient agar (20 ml) was dispensed into sterile universal bottles. These were then inoculated, mixed gently and poured into sterile petri dishes. After setting a number 3-cup borer (6 mm) diameter was properly sterilized by flaming and used to make three to five uniform cups/wells in each Petri dish. A drop of molten nutrient agar was used to seal the base of each cup. The cups/wells were filled with 50µl of the extract at a concentration of 400 µg/ml and were allowed for diffuse (45 minutes). The plates were then incubated at 37°c for 24 hours for bacteria. The above procedure was followed for fungal assays and the media used was sabourand dextrose, incubated at 25°C for 48 hours. The zones of inhibition were measured with antibiotic zone scale in mm and the experiment was carried out in triplicate.

Determination of Relative Percentage Inhibition: The relative percentage inhibition with respect to positive control was calculated by using the following formula [17]. Relative percentage inhibition of the test extract = [{100 x (a - b)}/(c - b)]. Whereas, a: total area of inhibition of the test extract; b: total area of inhibition of the solvent; c: total area of inhibition of the standard drug. The total area of the inhibition was calculated by using area = πr^2; where, r = radius of the zone of inhibition.

Brine Shrimp Lethality Bioassay: The toxic potentiality of the plant crude extract was evaluated using Brine Shrimp lethality bioassay method [18] whereas 6 graded doses (viz,. 10, 20, 40, 80, 160 and 320µg/ml) were used. Brine shrimps (*Artemia salina* Leach) nauplii Ocean 90, USA were used as test organisms. For hatching, eggs were kept in brine with a constant oxygen supply for 48 hours. The mature nauplii were then used in the experiment. DMSO was used as a solvent and also as a negative control. The median lethal concentration LC_{50} of the test sample after 24 hours was obtained by a plot of percentage of the dead shrimps against the logarithm of the sample concentratin. Tamoxifin, a well known anticancer drug was used as a reference standard in this case.

Statistical Analysis: All the *in vitro* experimental results were given as mean±SEM of three parallel measurements and data were evaluated by using student's t test. *P* values<0.001 were regarded as significant.

RESULTS

Phytochemical Analysis: The result showed that the extract of leaves contain reducing sugar (carbohydrate), tannins, saponins and alkaloids. (Table 1).

DPPH Radical Scavenging Activity: The percentage (%) scavenging of DPPH radical was found to be concentration dependent i.e. concentration of the extract between 1-100 µg/ml greatly increasing the inhibition activity and statistically significant ($p<0.001$) (Figure 1). The IC_{50} value of the extract was 2.43±1.06 µg/ml as opposed to that of ascorbic acid (IC_{50} 2.14±0.11 µg/ml), which is a well known antioxidant.

Thrombolytic Activity: Figure 2 shows the effect of the extract on clot lysis activity. The percentage (%) clot lysis was statistically significant ($p<0.001$) when compared with vehicle control. The plant extract showed moderate colt lysis activity (18.27±1.07%, 20.23±1.56% and 17.82±1.97% for volunteer 1, 2 and 3, respectively) whereas standard streptokinase showed 46.13±3.87% clot lysis activity.

Table 1: Result of chemical group tests of the methanol extract of *Aponogetan undulatus* leaf

Extract	Carbohydrate	Tannin	Flavonoid	Saponin	Gum	Steroid	Alkaloid
ME of *A. undulatus*	+++	++	-	+	-	-	+++

ME: Methanolic extract; (+): Present; (-): Absent; (+ + +): Reaction intensity is high; (+ +): Reaction intensity is medium; (+): Reaction intensity is normal;

Table 2: *In vitro* antimicrobial activity of MeOH extract of the leaves of the *A. undulatus* on various bacterial and fungal strains by agar diffusion method

[a]Zone of inhibition in diameter (mm) (n=3)		
Fraction	MeOH	Ciprofloxacin[b]
Gram Positive		
Bacillus cereus	12±0.65 (45.34%)	42±0.65
Bacillus megaterium	8±0.15 (19.01%)	41±0.25
Bacillus subtilis	8±0.25 (19.01%)	41±0.55
Staphylococcus aureus	7±0.45 (16.34%)	42±0.16
Sarcina lutea	9±0.26 (21.23%)	42±0.75
Gram Negative		
Escherichia coli	10±0.71 (32.56%)	42±0.75
Pseudomonas aeruginosa	8±0.32 (19.01%)	42±0.75
Salmonella typhi	7±0.53 (16.34%)	41±0.35
Salmonella paratyphi	6±0.29 (14.39)	42±0.35
Shigella boydii	NA	41±0.85
Shigella dusenteriae	9±0.42 (21.23%)	41±0.15
Vibrio mimicus	9±0.32 (21.23%)	42±1.75
Vibrio parahemolyticus	NA	41±1.35
Fungi		
Candida albicans	11±1.12 (41.56%)	42±0.45
Aspergillus niger	10±0.02 (32.56%)	42±0.25

[a] Values of the observed diameter zone of inhibition (mm) excluding cap diameter. Incubation conditions for bacteria – 24 hours at 37°C and for fungi – 48 hours at 25°C. Assay was performed in triplicate and results are the mean of three values±Standard Deviation. Relative percentage of inhibition are indicated at parenthesis. [b] Reference standard

NA- Zone of inhibition < 5 mm consider as no activity.

Antibacterial Activity: Table 2 expresses the antibacterial and antifungal activity (zone of inhibitions) of extract of the leaves of *A. undulatus*. The zone of inhibition range for Gram positive bacteria was found to be 7±0.45 to 12±0.65 mm in which the highest activity was shown against *Bacillus cereus* (zone of inhibition 12±0.65 mm). For Gram negative bacteria, *Escherichia coli* was to be more susceptible (zone of inhibition 10±0.71 mm) whereas *Salmonella paratyphi* showed more resistance to the extract (zone of inhibition 6±0.29 mm). The methanolic extract of *A. undulatas* showed remarkable antifungal activities against all the tested fungi and the range of zone of inhibition was to be 10±0.02 to 11±1.12 mm.

Results of relative percentage inhibition are reported in table 2. The Methanolic extract of *A. undulates* showed the maximum relative percentage inhibition against *Bacillus cereus* (45.34%) for bacteria and *C. albicans* (41.56%) for fungi whereas, lowest relative percentage inhibition against *S. paratyphi* (19.43%) for bacteria and S. *cerevaceae* (19.20) for fungi.

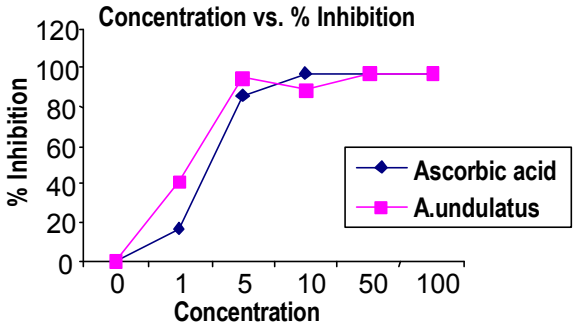

Fig. 1: Free radical scavenging activity of different concentrations of crude extract of *A. undulatus* and ascorbic acid by DPPH radicals. Results are mean±SEM of three parallel measurements.

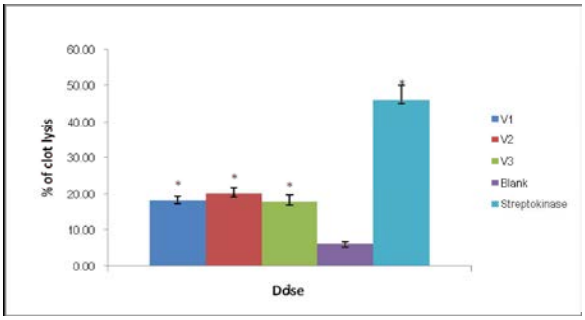

Fig. 2: Thrombolysis activity of crude extract of *A. undulatus* and Streptokinase by clot lysis activity. Results are mean±SEM of three parallel measurements.

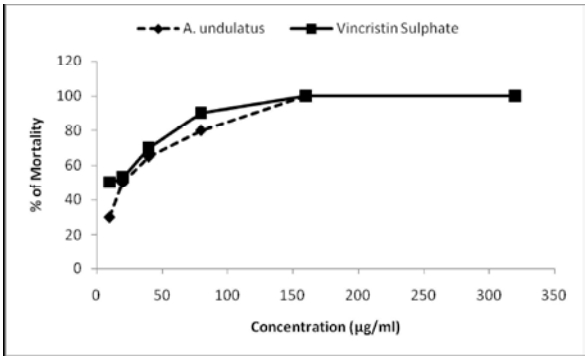

Fig. 3: Toxicity assay of crude extract of *A. undulatus* by Brine shrimp lethality bioassay. Results are mean±SEM of three parallel measurements.

Toxicity Assay: In general Brine shrimp lethality bioassay, used to measure the toxicity of plant extract and also an indicative of toxicity, antibacterial activities, pesticidal effects and various pharmacologic actions. In toxicity studies, the extract was found to be highly effective and showed an LC_{50} value of 2.24 µg/ml whereas 0.25µg/ml was observed for the standard vinvristine sulfate. Results of toxic activity are presented in figure 3.

DISCUSSION

Increased intracellular generation of reactive oxygen species leading to tissue injury with a variety of pathological processes like ischaemia, inflammation, atherosclerosis and thrombosis [19]. The vascular endothelium is the principal site of action of cardiovascular risk factors and early atherogenesis [20]. The imbalance between prooxidants and antioxidants play a vital role for the development of atherosclerosis and has prompted the investigation of antioxidants as a possible therapy [21]. The screening of the antioxidant activity of this plant has revealed its capacity to scavenge the free radical (DPPH) at low concentration may be due to the presence of tannin in its crude extract.

Platelets play an important role in the process of atherothrombosis by adhering to the damaged regions (caused by reactive oxygen species) of the endothelial surface. The activated platelets form platelets to platelets bonds, binds also to leucocytes bringing them into a complex process of plaque formation and growth [22]. Plasmin, natural fibrinolytic agent, lyses clot by breaking down the fibrinogen and fibrin contained in a clot. Streptokinase forms a 1:1 stochiometric complex with plasminogen that can convert additional plasminogen to plasmin [23]. Moreover, phlorotannin, isolated from marine brown algae, have a unique property in promotion of dissolution of intravascular blood clot via antiplasmin inhibition [24]. Since phytochemical analysis showed that the crude extract contains tannin, alkaloid and saponin could be participated for it's clot lysis activity.

Phytoconstituents such as alkaloid, sesquiterpine, phenolic compounds and glycosides have been reported to inhibit bacterial growth and to be protective to plants against bacterial and fungal infections [25, 26]. So, the antimicrobial activity showed by the methanlic extract of *A. undulatus* may be due to presence of alkaloids, saponin and tannin.

The brine shrimp lethality bioassay (BSLA) has been used routinely in the primary screening of the crude extracts to assess the toxicity towards brine shrimp, which could also provide an indication of possible toxicity of the test materials. A number of novel antitumor and pesticidal natural products have been isolated using this bioassay [18]. The variation in BSLA results (Table 5) may be due to the difference in the amount and kind of cytotoxic substances (e.g. Alkaloids, tannins, flavonoids) present in the crude extracts. Moreover, this significant lethality of the crude plant extracts (LC_{50} values less than 100 ppm or µg/mL) to brine shrimp is indicative of the presence of potent cytotoxic and probably insecticidal compounds which warrants further investigation. BSLA results may be used to guide the researchers on which crude plant extracts/fractions to priority for further fractionation and isolation of these bioactive compounds. Other cytotoxicity tests and specific bioassays may be done on the isolated bioactive compounds are in progress.

In conclusion, the results of the present study indicated that the MeOH extract exhibits interesting antioxidant properties, as well as significant thrombolysis, broad spectrum antibiotic and toxicity effect which may be due to the presence of tanin and alkaloid in the extract. The plant also used to discover bioactive natural products that may serve as leads for the development of new pharmaceuticals that address hither to unmet needs. Furthermore, active plant extracts can be subjected to various chemical evaluation by several methods such as GC-MS, NMR, HPLC, Mass Spectroscopy, etc, for the isolation of the therapeutic antimicrobials.

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