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SCREENING OF BIOCHEMICAL ACTIVITIES OF A POTENTIAL MEDICINAL HERB GROWING IN BANGLADESH

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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 a medicinal herb, Polygonum viscosum (leaves). 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 extracts were a rich source of phytochemicals. In DPPH free radical scavenging test, the the Chloroform soluble fraction showed the highest free radical scavenging activity with IC_{50} value 15.09µg/ml while compared to that of the reference standards ascorbic acid. Polygonum viscosum was also found as a good source of total phenolic contents. Morever, the extracts revealed good 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, Polygonum viscosum.

INTRODUCTION

A potential medicinal herb, Polygonum viscosum belonging to the family Polygonaceae, common name-‘‘Bishkatali”, is an annual herb native to Nepal and widely distributed in Bangladesh, northeast India, Japan and China. [1] The genus Polygonum, comprising about 45 genera (300 species), is distributed all over the world, mostly in north temperate regions. [2] This genus is well known for producing a number of bioactive compounds and also for its use in the oriental traditional medicine systems for the treatment of various ailments such as fever, pain, infections, inflammation, cancer and tumour. [3] Thus, use of medicinal plants as traditional health remedies is very popular [2] because they are relatively safer than the synthetic alternatives, they are easily available and cheaper. [4] Different plants have been used as a source in the development of novel drug [4]. Morever, in many developing countries almost 80% of available drugs come from medicinal plants and in developed countries plants make up the raw material for processes, which synthesize pure chemical derivatives. [2, 5] Report shows that about 80% of the world’s population uses plants for various medical purposes. [6] It is now believed that phytochemicals obtained from medicinal plants serve as pilot molecules in the modern medicines. [7]

According to our knowledge, there are very few studies are presently available that documents the biological activities of Polygonum viscosum (P. viscosum). So, in this study, our main goal was to evaluate possible chemical groups, and biochemical activities such as antioxidant, antimicrobial and thrombolytic activities of P. viscosum to validate its use in folkloric treatments. Plants contain various types of chemical groups which are responsible for showing a variety of pharmacological actions in human body. [8] Medicinal plants containing antioxidant activity are being considered for use in antioxidant formulations. Antioxidants minimize oxidative damage, thus decreasing the risk of free radical-induced diseases. [9] A proper balance between antioxidation and oxidation is essential for maintaining a healthy biological system. [10] Now a day, much attention has been put toward natural antioxidants because synthetic antioxidants may have adverse effects on human body. [11] Medicinal plants also contain antimicrobial property. Antimicrobial assay procedures for plant extracts provide a measure of antibiotic activity. [12] Thrombosis is a patho-

physiological process which is characterized by the development of a blood clot (thrombus) in the circulatory system of the human body. This condition is responsible for the development of coronary disorders such as strokes and heart attacks which are the causes of morbidity and mortality in developed countries. [13] Therefore, anticoagulation therapy is essential in the management of thrombosis patients. Streptokinase and urokinase are commonly used as thrombolytic agents because of their cost effectiveness as compared to other thrombolytic drugs. [13] Recently, The thrombolytic potency of some medicinal plants have been studied using an in vitro model and significant thrombolytic property were found for some plants. [14]

MATERIAL AND METHOD

Plant materials

For this present investigation, the P. viscosum barkswere collected from Comilla in December 2011. The plant was identified and authenticated by the help of expert botanist of Bangladesh National Herbarium, Mirpur, Dhaka (DACB Accession No. 38305).

Chemicals and Reference drug

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

Extraction of plant materials

For methanolic extraction, about 400 gm of air dried, powdered sample were immersed in 2500 ml of 80% methanol (Merck KGaA, Darmstadt, Germany) in a clean and sterilized glass container. The glass container with its contents was sealed and kept for maceration for 25 days accompanying occasional shaking and stirring. At the end of 25th day, the whole mixture was filtered carefully using filter cloth and Whatman® filter paper (Sargent-Welch, USA). The resultant filtrate was then allowed to evaporate in water bath maintained about 45^0C to dryness and thus a greenish black semisolid mass of the extract was obtained (yield 26 gm). This gummy semisolid mass was designated as crude extract of methanol.

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

Phytochemical screening: Small quantity of prepared methanolic extract and different fractions of leaves of P. viscosum were subjected to preliminary phytochemical screening 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]

Antioxidant activity: There are few 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 carefully for investigating the antioxidant activity of P. viscosum.

Total phenolic content determination

The amount of total phenolics in extracts was measured with the Folin-Ciocalteu reagent. [7] Here, gallic acid (as a standard) was used and the amount of total phenolics were designated as mg/g of gallic acid equivalents (GAE). Concentration of 6.25, 12.5, 25, 50, and 100 µg/ml of gallic acid and 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 nicely with para-film and allowed to stand for about 30 min at room temperature before the absorbance was read at 760 nm spectrophotometrically (UV-1800, Shimadzu, Japan). Here, all determinations were performed nicely in triplicate. [7] Thus, total phenolic content was measured as mg of gallic acid equivalent per gram using the equation obtained from a standard gallic acid calibration curve.

***DPPH scavenging activity*:** The 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 about 15 seconds. Then the solutions were allowed to stand at dark place (at room temperature) for 30 min for reaction to occur. After 30 min, absorbance was measured against a blank at 517 nm with a double beam UV spectrophotometer. The percentage of DPPH radical-scavenging potency of each plant extract was calculated as:

DPPH radical-scavenging activity (%I),

$$= \frac{A_0 - A}{A_0} \times 100$$

Where,

A_0 = absorbance of the control solution (containing all reagents except plant extracts);

A = absorbance of the DPPH solution containing plant extract.

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

Antimicrobial activity

Test Organisms

Here, 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 potency. These organisms were subcultured properly in nutrient broth and nutrient agar. They were collected from the, department of microbiology, Chittagong Veterinary and Animal Sciences University, Bangladesh.

Study design

For the determination of antimicrobial activity, disc diffusion method is widely acceptable. [18] In this method, antibiotics were diffused from a 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 applied on nutrient agar medium consistently seeded with the test microorganisms. Here, 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 about 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 potency inhibited microbial growth in plates and thereby yielded a clear, distinct zone defined as zone of inhibition. Thus, the antimicrobial activity of the test sample was determined nicely by measuring the zone of inhibition expressed in millimeter. [7]

Thrombolytic activity

In vitro clot lysis activity of P. viscosum was carried out according to the method of Prasad et al., 2007 [18] with minor modifications

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 was added and mixed properly. Then the solution was diluted up to 300000IU and used as a stock from which 100 µl (30,000 I.U) was used for in vitro thrombolysis assay. [19]

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. Then, 500 µl of blood was transferred to each of the previously weighed micro centrifuge tubes to form clots. [19]

Preparation of sample

The prepared 0.9% NaCl solution was used carefully to make different concentrations of plant extract: 5, 10 and 20 mg/mL.

Study design

Venous blood drawn from healthy volunteers (n = 10) was immediately citrated consciously using 3.1% sodium citrate solution and then was transferred in the previously weighed different sterile micro centrifuge tube (500 µl/tube). Two hundred microlitre of 2% calcium chloride was then added to 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.

% of clot lysis = (wt. of released clot /clot wt.) × 100

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).

RESULTS

Phytochemical screening

The phytochemical screening of **Polygonum viscosum** bark extracts of methanol, pet ether, carbon tetra chloride and chloroform solvent showed varied results (Table-1).

Table 1: Preliminary Phytochemical screening of methanolic extract and different fractions of leaves of Polygonum viscosum

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

Key: (+) = Present and (−) = Absent

From the above performed tests it is clear that Polygonum viscosum leaves neither contain any reducing sugar nor glycoside but all type of extracts of this plant part contain steroids. Both methanolic and pet ether extracts contain tannins and flavoniods. Terpenoid is only found in chloroform extract while in saponins is found in methanolic extract only.

Total phenolic content determination

The methanolic crude extracts of leaves of Polygonum viscosum 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 and compared with the standard solutions of gallic acid equivalents (Table 2 and figure 1). Here, total phenolic content of the samples are expressed as mg of gallic acid equivalent (GAE)/ gm of dry extract.

Polygonum viscosum was also 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 12.35µg of GAE / mg, 11.4µg of GAE / mg, 19.83µg of GAE / mg and 21.55µg of GAE / mg extracts respectively

Table 02 : Results of Total Phenolic Content of Polygonum viscosum leaves Extracts

Polygonum viscosum leaves	Absorbance at 760 nm	Total Phenolic Content (mg of GAE / gm) of Extracts
Methanolic Crude Extract(250 µg/ml)	.536	12.35
Pet Ether Extract(250 µg/ml)	.498	11.4
Chloroform Extract(250 µg/ml)	1.125	27.075
Carbon tetra Chloride Extract (250 µg/ml)	.835	19.83

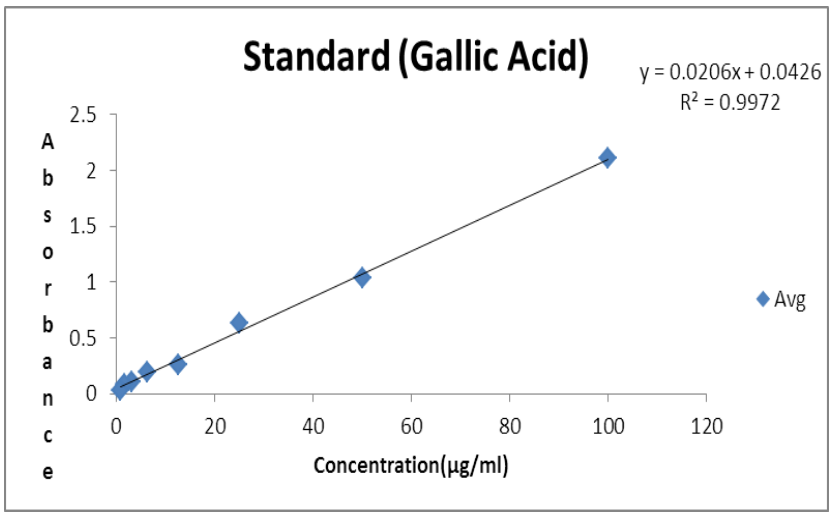

Figure 01: Total Phenolic Content of Gallic Acid (Standard)

DPPH scavenging activity: DPPH free radical scavenging activity of crude extracts of bark of P. viscosum and their different soluble fractions were found to be increased with the

increase of concentration of the extract (Table 3). Different partitions of methanolic extract of P. viscosum were subjected to free radical scavenging activity where ascorbic acid was used as reference standard. In this investigation, the Chloroform soluble fraction showed the highest free radical scavenging activity with IC_{50} value 15.09µg/ml. At the same time the Pet ether soluble fractions, aqueous and crude methanolic extract also exhibited moderate antioxidant potential having IC50 value 126.22µg/ml, 516.57µg/ml and 242.18µg/ml respectively. Also carbon tetra chloride soluble fractions of methanolic extracts showed IC_{50} value 31.86.

Table-3: Comparative DPPH radical scavenging activity of different extracts of the leaves of P. viscosum 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	0.96	17.97	0.76	38.23	1.23	41.65
10	6.34	31.24	9.84	52.61	4.96	49.45
20	10.15	42.38	15.28	53.42	11.32	54.28
40	19.51	51.91	21.49	55.23	14.36	56.86
60	24.56	60.31	29.64	60.23	19.69	59.02
80	31.24	73.24	39.61	64.87	26.34	65.45
100	34.71	75	48.56	69.23	32.48	72.28
250	49.96	79.32	69.31	78.23	43.76	83.95
500	69.69	83	75.54	92.23	53.43	97.41
IC_{50} (µg/ml)	**242.18**	**31.86**	**126.22**	**15.09**	**516.57**	**14.51**

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 which gives clear zone of inhibition. After incubation, the antimicrobial activities of the test materials such as Methanolic crude extract of P. viscosum (MEPV), Pet. Ether fraction of P. viscosum crude extract (PEPV), Chloroform fraction of P. viscosum crude extract (CPV), Carbon tetra Chloride fraction of P. viscosum crude extract (CTPV) and Aqueous fraction of P. viscosum crude extract (AEPV) were determined by measuring the diameter of the zones

of inhibition in millimeter with a transparent scale (Table 4). Standard antibiotic disc of ciprofloxacin (5µg/disc) was used for the comparison.

Table 04: Results of in vitro antimicrobial screening of Polygonum viscosum

Types of micro organism		Samples of Polygonum viscosum Inhibition zone diameter (mm / sample)					Ciprofloxacin
		MEPV	PEPV	CLPV	CTPV	AEPV	
Gram Positive Bacteria	Bacillus subtilis	9	-	14	-	8	34
	Staphylococcus aureus	10	-	15	-	9	30
Gram Negative Bacteria	Pseudomonas aeruginosa	7	-	12	-	7	36
	Salmonella enterica	6	-	9	-	6	25
	Escherichia coli	9	-	10	-	8	28
Fungi	Candida albicans	6	-	7	-	4	32
	Aspergillus alliaceus	7	-	7	-	3	30

The chloroform soluble fraction of the methanolic extract exhibited significant activity against the growth of the most of the test organisms (Table 4), while the methanolic crude extract exhibited moderate inhibitory activity against most of the microorganisms and aqueous extract exhibited less than moderate inhibitory activity against most of the microorganisms (Table 4). The zones of inhibition produced by chloroform soluble fraction of the methanolic extract were ranged from 7-14 mm, while the methanolic crude extract showed 7.0-10 mm and aqueous extract showed 3-8 mm at a concentration of 400 µg/disc.

The pet. ether and carbon tetra chloride soluble fraction of the methanolic extract exhibited no inhibitory activity against tested bacteria and fungi.

Thrombolytic potential

Streptokinase (30000 and 15000 I.U.) as a positive control to the clots along with 90 minutes of incubation at 37 ^{0}C showed 47.22 and 24.73% clot lysis respectively. Clots when treated with 100 microlitre of 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 of crude methanolic extract, petroleum ether extract of P. viscosum in the concentration of 20, 10, 5

mg/ml showed also varying clot lysis i.e., 12.49, 9.54, 6.69% and 17.07, 13.81, 7.92 % 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 P. viscosum and appropriate control are shown in fig.2.

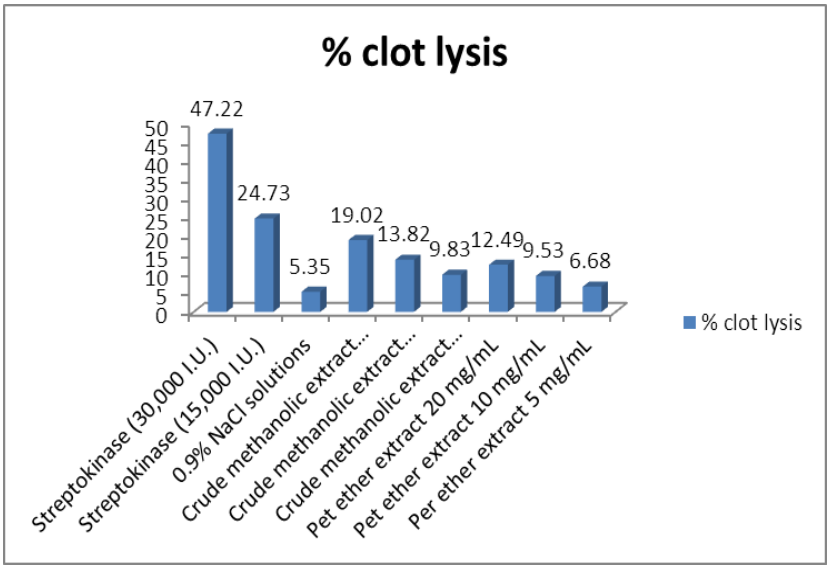

Fig.2: clot lysis of streptokinase, Nacl and various concentrations of P. viscosum. Maximum clot lysis was observed in a clot treated with streptokinase. Crude methanolic extract and pet ether extract of P. viscosum at 20,10 and 5 mg/ml showed 19.02, 13.82, 9.83, 12.49, 9.53, 6.68 % respectively

DISCUSSION

Medicinal plants contain phytochemicals.For this reason, they show a variety of pharmacological actions in human body [20] and in our investigation, preliminary phytochemical screening showed the presence of various phytochemicals.

The presence of polyphenolic compounds (such as flavonoids and tannins) are responsible for antioxidant activity of the plant extracts. [21] In this investigation, the plant extract showed moderate antioxidant activities with an IC_{50} which were compared with the values of standard drugs used. Antioxidant activities of different extracts of the leaves of P. Viscosum was found to increase with the increasing concentration. [22] This plant contains a good amount of total phenolic content. It also contains moderate DPPH scavenging activity although chloroform soluble fraction showed highest free radical scavenging activity.The effect of antioxidants of any plant on DPPH is thought to be due to their hydrogen donating ability. [22] Therefore, the extracts of this plant could be used 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, phytosterol and alkaloids, that singly or in combination may be responsible for the defense mechanism against microorganisms and insects. [7] For this reason, methanolic extract of this plant contains antimicrobial activity.

Platelets play a nice role in the process of formation of thrombus on the endothelial surface [13]. Several types of 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 fatal consequences. [23] In our present study, the extracts of this plant showed significant thrombolytic activity compared to negative control. This thrombolytic activity may be due to the fact that the extracts are a nice sources of alkaloids, flavonoids, tannins and terpenoids which are said to exert clot lysis. [24, 25]

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