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Cytotoxic, thrombolytic, membrane stabilizing and antioxidant activities of *Hygrophila schulli*

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Abstract

The crude methanol extract of *Hygrophila schulli* and its petroleum ether, chloroform, ethyl acetate and aqueous soluble Kupchan partitionates were investigated for *in vitro* cytotoxic, thrombolytic, membrane stabilizing and antioxidant activities. In the brine shrimp lethality bioassay, the petroleum ether soluble extract of *H. schulli* showed significant cytotoxicity (LC_{50} = 0.1 µg/mL and LC_{90} = 15 µg/mL) as compared to vincristine sulfate (LC_{50} = 0.4 µg/mL and LC_{90} = 9 µg/mL). Among all the partitionates, chloroform soluble fraction demonstrated the highest thrombolytic activity with 10.5% clot lyses. Moreover, in hypotonic- and heat-induced conditions, the chloroform soluble extractive inhibited hemolysis of human erythrocyte by 113.7% and 14.3%, respectively as compared to 71.9% and 42.2% demonstrated by standard acetylsalicylic acid. On the other hand, in anti-oxidant activity test, chloroform soluble fraction revealed mild antioxidant activity (IC_{50} = 195.1 µg/mL) as compared to standard tert-butyl-1-hydroxytoluene (IC_{50} = 27.5 µg/mL).

Introduction

During the last two or three decades, advances in phytochemistry and identification of new bioactive compounds from plants which are effective against certain diseases, have renewed the popularity of herbal medicines (Babu and Subhasree, 2009). In fact, plant derived drugs are of increasing interest to many pharmaceutical companies mainly due to the current widespread belief that 'Green Medicine' is effective, safer and more reliable than synthetic drugs (Sujatha, 2005). Hence, screening medicinal plants for promising biological activities in order to discover novel drug candidate is a necessity (Chowdhury et al., 2009).

Hygrophila schulli (Buch.-Ham) (Fam.: Acanthaceae) is an unbranched herb with straight curved thorns. It grows naturally in moist places. It is commonly distributed in Indo-China, Myanmar, India, Nepal, Sri Lanka, Pakistan, Tropical Africa and Bangladesh. In Bangladesh, the plant has been reported to be used in dropsy, diarrhea, dysentery, cough, jaundice, and urinogenital diseases. Seed juice of the plant was found to be useful during child birth; leaf paste was reported to be applied externally to lumbago and rheumatism (Asiatic Society, 2010). Previous phytochemical investigations of *H. schulli* led to the isolation of isoflavone glycoside, an alkaloid and small quantities of uncharacterized bases (Nikam et al., 2012). It was found to possess antimicrobial activity in a study (Chandran et al., 2013).

As a part of our ongoing research on medicinal plants of Bangladesh (Haque et al., 2005; Ara et al., 2012; Sikder et al., 2013; Begum et al., 2014), we studied the crude methanol extracts of *H. schulli* as well as its organic and aqueous soluble fractions for cytotoxic, thrombolytic, membrane stabilizing and anti-oxidant activities for the first time and, herein, report the results of our investigations.

Materials and Methods

Plant materials

Healthy and disease free plants of *H. schulli* were collected from Avoynogor, Jessore, Bangladesh in March 2012. The exsiccated plant sample was identified by Bangladesh National Herbarium, Mirpur, Dhaka. A voucher specimen of the plant has been preserved for future reference (Accession number: DACB-39,532).

Extraction and fractionation

The fresh, exsiccated and shade dried plants were powdered using a suitable blender (Noka super blender, China). The powdered materials (300 g) were soxhlet extracted with methanol at a temperature not exceeding 40°C. The methanol (crude) extract thus obtained was concentrated at 40°C with a rotary evaporator (Bibby RE200, Sterilin Ltd., U.K) to get a greenish gummy concentrate of 27.9 g (percent of yield = 9.3%). The crude extract was then stored in a refrigerator at 4°C. The crude extract (6.2 g) was further fractionated by modified Kupchan partitioning protocol (Venwagenen et al., 1993) to afford petroleum ether, chloroform, ethyl acetate and aqueous soluble materials.

Evaluation of cytotoxicity

Brine shrimp lethality bioassay (McLaughlin and Rogers, 1998; Meyer et al., 1982) technique was used to test cytotoxicity of the extracts. Anticancer drug vincristine sulfate (Techno Drugs Ltd, Bangladesh) was used as positive control and dimethyl sulfoxide (DMSO, BioReagent, for molecular biology; Sigma-Aldrich, India) was used as solvent and negative control. In this experiment, 4 mg of each test sample was dissolved in DMSO to obtain solutions of varying concentrations (400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.5625 and 0.78125 µg/mL) by serial dilution technique. DMSO solutions were then applied against *Artemia salina* for 24 hours to calculate percent mortality.LC_{50} and LC_{90} values were determined from the analysis of concentration-mortality data.

Thrombolytic activity

In vitro thrombolytic activity was determined by following established protocol (Rahman et al., 2013). Lyophilized altepase (streptokinase) vial (Beacon Pharmaceutical Ltd) of 15,00,000 IU and distilled water were used as positive and negative control, respectively. Venous blood drawn from healthy volunteers was incubated in pre weighed sterile microcentrifuge tubes (1 mL/tube) at 37°C for 45 min. After clot formation, the serum was completely removed and clot weight was determined for each tube. 100 µL aqueous solution of different partitionates along with the crude extract was added separately to each microcentrifuge tube containing pre-weighed clot. All the tubes were then incubated at 37°C for 90 min and observed for clot lyses. Percent of clot lyses were determined with the following equation:

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

Membrane stabilizing activity

Membrane stabilizing activity in hypotonic solution- and heat-induced solution was studied by previously described method (Shinde et al., 1999). Absorbance values of the supernatants were measured at 540 nm using Shimadzu UV spectrophotometer.

Hypotonic solution-induced hemolysis

The extracts and standard acetyl salicylic acid were taken in different centrifuge tubes. In each tube 0.50 mL erythrocyte (RBC) suspension, 5 mL of hypotonic solution (50 mM NaCl) and 10 mM sodium phosphate buffer saline (pH 7.4) were taken. The mixtures were incubated at room temperature for 10 min and then centrifuged for 10 min at 3,000 rpm. After centrifugation the soluble supernatant of each tube was

Table I

Brine shrimp lethality bioassay and antioxidant activity of *H. schulli* extracts

Test samples	Brine shrimp lethality bioassay		Antioxidant assay
	LC_{50} (µg/mL)	LC_{90} (µg/mL)	IC_{50} (µg/mL)
Vincristine sulfate	0.4	9.0	-
tert-butyl-1-Hydroxytoluene (BHT)	-	-	27.5
Methanol (crude) extract	2.8	65.5	233.5
Petroleum ether extract	0.1	15.0	348.3
Chloroform extract	1.0	120.6	195.1
Ethyl acetate extract	15.1	3226.5	233.1
Aqueous extract	1.7	1904.6	321.9

Table II

Thrombolytic activity and membrane stabilixing activity of *H. schulli* extracts

Test samples	% clot lyses	% Inhibition of hemolysis	
		Hypotonic solution-induced	Heat-induced
Acetylsalicylic acid	-	71.9	42.2
Streptokinase	66.9	-	-
Methanol extract	8.9	25.7	1.9
Petroleum ether extract	0.7	-	-
Chloroform extract	10.5	113.7	14.3
Ethyl acetate extract	0.5	-	-
Aqueous extract	3.9	-	-

decanted and filtered. Then the absorbance for each tube was measured. The percentage inhibition of hemolysis or membrane stabilization was calculated using the following equation:

% Inhibition of hemolysis = $100 \times \{(OD_1 - OD_2)/ OD_1\}$

Where, OD_1 = Optical density of hypotonic-buffered saline solution alone (control) and

OD_2 = Optical density of test sample in hypotonic solution.

Heat induced hemolysis: Aliquots of 5 mL isotonic buffer, 1.0 mg/mL of an extract and 30 µL erythrocyte suspension were taken in two different sets of centrifuge tubes. The same aliquot without the extract was taken in two other centrifuge tubes. The tubes were mixed gently by inversion. One pair of the tubes was incubated at 54°C for 20 min in a water bath while the other was maintained at 0-5°C in an ice bath. The reaction mixtures were centrifuged for 3 min at 1,300 rpm and the absorbance of the supernatants were measured. The percentage inhibition or, acceleration of hemolysis in tests was calculated according to the following equation:

% Inhibition of hemolysis = $100 \times [1 - (OD_2-OD_1/ OD_3-OD_1)]$

Where, OD_1 = test sample unheated, OD_2 = test sample heated & OD_3 = control sample heated

Anti-oxidant activity

Antioxidant activity was assessed by the previously developed method (Brand-Williams et al., 1995). Tert-butyl-1-hydroxytoluene (BHT) (Jayson Pharmaceuticals Ltd., Bangladesh) was used as positive control. In this study, 2.0 mL of a methanol solution of the sample (extractives/control) at different concentrations (500, 250, 125, 62.5, 31.25, 15.625, 7.813, 3.906, 1.953 and 0.977 µg/mL) were mixed with 3.0 mL of a DPPH methanol solution (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 with a UV-visible spectrophotometer-1240 (Shiamdzu, Japan). Solutions of varying concentrations of extractives and control were obtained from serial dilution of their respective mother solutions (1000 µg/mL). Inhibition of free radical DPPH in percent *(1%)* 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). IC_{50} was calculated from the graph plotted inhibition percentage against extract concentration.

Results

In the brine shrimp lethality bioassay, the petroleum ether soluble fraction revealed the highest cytotoxicity (LC_{50} = 0.1 µg/mL and LC_{90} = 15 µg/mL) as compared to vincristine sulfate (LC_{50} = 0.4 µg/mL and LC_{90} = 9 µg/mL) (Table I).

In the *in vitro* thrombolytic activity test, chloroform solu-ble fraction showed the highest thrombolytic activity with 10.5% clot lyses (Table II).

In the assay for membrane stabilizing activity, chloroform soluble extractive inhibited hemolysis of human erythrocyte by 113.7% and 14.3% as compared to 71.9% and 42.2% demonstrated by the standard acetylsalicylic acid in the hypotonic solution- and heat-induced conditions, respectively (Table II).

Table I also shows the results of anti-oxidant activity test of the extractives and standard BHT. Among all the test samples, chloroform soluble fraction demonstrated the highest anti-oxidant activity (IC_{50} = 195.1 µg/mL) as compared to BHT (IC_{50} = 27.5 µg/mL).

Discussion

In the study, aqueous and chloroform soluble fraction of methanolic extract showed LC_{50} and LC_{90} at a low concentration indicating that the extracts are potent in cytotoxicity and hence can be used to treat cancer. But anticancer agents sometimes are toxic to normal cells, more specifically, rapidly growing cells (Priestman, 2008). As a result, the cytotoxic activity of these two extracts may be tested against various cancer cell lines as well as normal cell lines. The extracts are also worth chemical investigation to isolate specific anticancer agents.

Thromboembolic disorders such as pulmonary emboli, deep vein thrombosis, strokes and heart attacks are the main causes of morbidity and mortality in developed countries (Nicolini et al., 1992). On the other hand, the first generation thrombolytic agents (streptokinase and urokinase) show relatively weak substrate specificity and can cause systemic fibrinolysis and associated bleeding complications (Marder, 1993). Hence, attempts are underway to develop improved recombinant variants of these drugs (Wu et al., 2001). The present study was also aimed to investigate the thrombolytic activity of different extractives of *H. schulli*. Crude methanolic extract and chloroform soluble extract showed mild thrombolytic activity.

Erythrocyte membrane resembles to lysosomal membrane. So, it can be extrapolated that the drugs which stabilizes erythrocyte membrane, stabilizes lysosomal membrane (Omale and Okafor, 2008) and thus interfere with the release and/or action of mediators like histamine, serotonin, prostaglandins, leukotrienes etc. (Shinde et al., 1999) and hence show anti-inflammatory activity. In the present study, chloroform soluble partitionate showed significant inhibition of erythrocyte hemolysis in hypotonic solution- and heat-induced conditions and hence anti-inflammatory activity which justifies the folkloric use of *H. schulli* plant in rheumatoid arthritis.

Synthetic antioxidants such as BHT, butylated hydroxyanisole (BHA), propyl gallate (PG) and tert-butylhydroquinone (TBHQ) used as food additives to increase shelf life are known to have not only toxic and carcinogenic effects (Ito et al., 1986; Wichi, 1988), but abnormal effects on enzyme systems. Therefore, the interest in natural antioxidant, especially of plant origin, has greatly increased in recent years (Jayaprakasha and Jaganmohan, 2000). Thus, the purpose of this study was to evaluate different extractives of *H. schulli* as new potential sources of natural antioxidants and phenolic compounds. In the investigation, the extracts and BHT showed a linear increase in anti-oxidant activity at lower concentrations until they reached a plateau at higher concentrations. IC_{50} values for both test samples and standard fell within the linear region of the curve. Using the values of the linear region, the chloroform soluble fraction revealed the highest anti-oxidant activity (IC_{50} = 195.1 µg/mL as compared to BHT with IC_{50} 27.5 µg/mL.

Conclusion

The present study demonstrated that *H. schulli* can be a very potential source of cytotoxic, thrombolytic, antioxidant and anti-inflammatory agents.

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Conflict of Interest

Authors declare no conflict of interest

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