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Biological investigations of medicinal plants of *Heliotropium indicum* indigenous to Bangladesh

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ABSTRACT

Objective: To investigate the clot lysis, antimicrobic and membrane stabilizing potentials of ethanolic extractives of the leaves of *Heliotropium indicum* (*H. indicum*).

Methods: Crude ethanolic extracts of *H. indicum* leaves were partitioned successively using solvents of different polarity and subjected to determine qualitatively and quantitatively for the presence of different bioactive constitutes and fractions which were assessed for their possible clot lysis, antimicrobic, and membrane stabilizing activities as compared with the known drugs.

Results: For the thrombolytic activity, the petroleum ether soluble fraction showed the highest percent of clot lysis (35.4%) among all fractions, while streptokinase and water resulted in 65.15% and 3.77% clot lysis, respectively. With respect to the membrane stabilizing activity, carbon tetrachloride soluble fraction of *H. indicum* profoundly interdicted the hemolysis of erythrocytes brought about by osmotic induction (39.24%) or by heat (40.20%). The other fractions exhibited less significant membrane stabilizing effect. By contrast, acetylsalicylic acid resulted in 72.25% ± 0.30% inhibition of osmotically induced hemolysis and showed a lower level of protection of heat induced hemolysis (42.56%). Crude ethanolic extracts were moderately sensitive against known pathogenic microbes. Since pharmacological activities of *H. indicum* are due to the presence of bioactive compounds, we detected and quantified the presence of significant levels of flavonoid and tannin substances.

Conclusions: The outcomes of this research show that the leaves of *H. indicum* have the potential to be used as a remedy for thrombosis, inflammatory diseases and against few important bacterial pathogens.

1. Introduction

Different medicinal plants and their various parts are mostly used for mitigation of numerous ailments throughout the world. The study of different plants for their traditional use is the most effective way to determine the upcoming lead compounds. The significance of study of different medicinal plants for the preclusion and cure of human diseases cannot be underlined. Nowadays, medicinal plants are one of the biggest source for "lead compounds". They provide new substances while the existing grow resistance in some cases *e.g.* antibiotics. Medicinal plants contain many constituents like alkaloids, cardiac glycosides, quinines, phenols, flavonoids, saponins *etc.* that have many biological functions like antiseptic, antipyretic, analgesic, antimicrobial, antitumor, antiviral, anti-inflammatory, cardio tonic, wound healing and many more[1]. *Heliotropium indicum* (Family: Boraginaceae) (*H. indicum*) is a perennial herb, indigenous to Asia particularly in Bangladesh, India, Sri Lanka, Nepal, Thailand, tropical Asia and also found in some parts of Africa. The plant is a perennial, straight, cleft plant with 15–50

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cm tallness. The shrubberies are always irregular or alternate, slightly hirsute[2,3]. This plant is widely used as folk medicines in many illnesses especially infusion of the flowers taken in small doses which regulates menstruation cycles, whereas large doses are abortive, and juice of the leaves is antiseptic and anti-inflammatory. In India, *H. indicum* is used for the treatment of skin diseases, venom bites, stomachache and nerve disorders as well[4]. In some African countries, *H. indicum* is used for the treatment of malaria. The infusion of the flower is taken orally by females for the treatment of menorrhagia in Jamaica while in Philippines and Senegal, it is used orally as diuretic and for the treatment of kidney stone[5,6]. Our current research project on curative plants leaves extractives and its dissimilar resolvable fractions were undertaken for the potential estimation of membrane stabilizing, clot lysis and antimicrobic potentials.

2. Materials and methods

2.1. Sources of leaves

Plant leaves of *H. indicum* were brought from Sharinagor, Munshiganj, Bangladesh, in April 2016. A voucher specimen (access number: 43319) of leaves was preserved in Bangladesh National Herbarium, Dhaka, Bangladesh for easy future references.

2.2. Extract preparation

The leaves were dehydrated at 37 ˚C for 12 h for easy crushing to find even particle size powder. An amount of 410 g of the powder was dipping in 1 500 mL of 90% ethyl alcohol. After 14 days, the powder in solvent was sieved and the filtrate was condensed by a vacuity revolving evaporator at 40 ˚C to recover solvent. Concentrated aqueous ethanolic extractive was fractionated by Kupchan method and obtained fractions of soluble ethanol, petroleum ether, carbon tetrachloride and chloroform.

2.3. Phytochemical screening

For the qualitative determination, different organic extractives were estimated for the manifestation of several classes of biochemicals. The presence of these phytochemicals was ensured by the properties of color variation by previously described suitable methods[7].

2.4. Determination of total flavonoid content

Kumaran and Karunakaran technique[8] was used to estimate the total flavonoid content utilizing quercetin as a mentioned standard. For the purpose of total flavonoid content, 1 mL ethanol extract (250 μg/mL) was added with 1 mL aluminum chloride in ethanol (20 mg/mL) and a drop of acetic acid followed by up to 25 mL of ethanol was added for dilutions. After 40 min, the optical absorbance was measured at a wavelength of 415 nm. A blank sample was prepared also. The standard curve was extrapolated by taking optical absorbance using different concentrations of quercetin solution at similar conditions.

2.5. Estimation of total tannin content

The total tannin content of extracts of *H. indicum* leaves was assessed by Folin-Ciocalteu technique[9]. Concisely, 0.3 mL (300 μL) of the crude ethanolic extract (CEE) and its fractions were taken into a volumetric flask (10 mL) having 2.7 mL of Folin-Ciocalteu (1:10) phenol mixture. Five minutes later, 2 mL of 7.5% sodium carbonate solution was poured to every test tube and shaken followed by preserving at room temperature for 30 min in shady place after warming at 45 ˚C. Series of mentioned standard solutions of tannic acid were arranged similarly without extract. Absorbance for test and standard solutions were estimated against the blank at 725 nm with a UV/observable spectrophotometer. The total tannin content was estimated from extrapolation of tannic acid standard curve.

2.6. In vitro clot lysis actions

Extracts (2 mg) were separately dissolved in 1 mL of purified water and agitated strongly on a sonicator for 10 min to get solutions. The prepared solution was retained for 12 h. Clear solutions were filtered by a Whatman filter paper (0.3 μm) to get a residue free clear solution which was utilized for thrombolytic activity *in vitro* assessment.

2.7. Clot lysis analysis

In clot lysis actions, various extract fractions were evaluated as described by Prasad *et al.*[10]. A stock solution of streptokinase (SK) (lyophilized altepase, Beacon Pharmaceuticals Limited) was prepared by adding 5 mL of sterile distilled water to a vial containing 1.5 million units of freeze-dried SK and mixed well by repeated inversion. A volume of 100 μL (30 000 IU) was used as the standard for *in vitro* evaluation of clot lysis potential. A voulme of 6 mL of blood was taken from healthy male persons. Thrombolytic study was conducted according to international protocol. A remount Professor included in the ethics committee. They have given written approval regarding experiment. One

milliliter of blood was poured into six sterile, pre-weighed 1 mL vials and gestated for 45 min at 25 ˚C to allow it to form coagulation. The serum was totally withdrawn without agitating the coagulation and every vial was then re-weighed to examine the coagulation weight. Each vial was labeled to indicate the type of extracted fraction to be added to it. An aliquot of 100 µL of the appropriate extracted fraction (prepared as described above) was poured to each vial. Later, 100 µL of SK was added to a similarly prepared clot containing vial as a positive control. In addition, an identical vial to which 100 µL of isotonic sodium chloride solution was added served as a negative (non-thrombolytic) control. Vials were preserved at room temperature for 90 min and then all the liquid in the vial (including any fluid released as a result of thrombolysis) was separated followed by weighing once more and the weight variance was taken to indicate the weight of clot dissolved (lysed).

2.8. Membrane stabilization analysis

The membrane stabilizing potential of different fractions was evaluated by assessing their capability to reduce the breakdown of human red blood cells (RBCs) prompted osmotically using a hypotonic solution or by heat following the method of Omale and Okafor[11].

2.9. Osmotically prompted breakdown of RBCs

Examined sample contained RBCs (0.5 mL) with 5 mL buffered hypotonic solution and fraction of the ethanolic extract at a final concentration of 2 mg/mL or acetylsalicylic acid (ASA) at a final concentration of 0.1 mg/mL. ASA was chosen as the mentioned standard followed by centrifuging for 10 min at 3 000 r/min, and gestated for 10 min at a warm temperature of 25 ˚C. The optical absorbance of the clear portion was taken at a wavelength of 540 nm by a UV spectrophotometer (Dynamica Halo VIS-10).

2.10. Hemolysis by heat

Two 5 mL samples of the buffered 0.9% NaCl solution each having 2 mg/mL of one of the ethanol soluble fraction (ESF) were placed in centrifuge tubes[12]. Also, two sets of control tubes were prepared, one containing only 5 mL isotonic solution and the other containing 5 mL of buffered 0.9% NaCl solution plus ASA at a concentration of 0.1 mg/mL. Nearly 30 µL of RBCs was poured to each tube and suspended softly by ups and downs. Two tubes were gestated in a water bath at 54 ˚C for 20 min. The other two tubes were kept in an ice bath (0–5 ˚C), and the samples were centrifuged for 10 min at 3 000 r/min and the optical absorbance was taken at a wavelength of 540 nm. Triplicate samples were used for each test and the mean ± SD values were calculated.

2.11. Evaluation of antimicrobic actions

Extracts were evaluated for their antimicrobic actions by the standard disc diffusion techniques. Nine known pathogenic bacteria were included in the current experiment, where six Gram negative and three Gram positive bacteria were included to determine the antibacterial activity. The Gram negative strains contained *Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Salmonella paratyphi, Vibrio mimicus* and *Shigella dysenteriae*. The Gram positive bacteria species contained *Bacillus cereus*, *Bacillus subtilis* and *Staphylococcus aureus*. The bacterial strains were cultured on broth medium at 4 ˚C for 12 h.

Extracts were subjected for their antimicrobic actions, compared to the kanamycin standard (30 µg/disc) by the disc diffusion methods[13] by the several known bacterial strains. Broth medium was sanitized at 121 ˚C for 15 min, and cooled then placed in sterilized Petri dishes. One milliliter of plant extract was added in the paper discs containing 100 µg/mL extract. Dried separated paper disc was placed into the agar surface to ensure complete contact. The agar plates had before been inoculated with the known bacteria[14]. Petri dishes were placed at 4 ˚C for 12 h. After that, plates were gestated at 37 ˚C for 16 h to favor bacterial growth. The inhibition of growth round every disc was examined in millimeter and analysis was done in triplicate for every fraction sample.

3. Results

3.1. Identification of bioactive constituents

Phytochemical screening with specific reagents, the CEE and it's fractions of *H. indicum* leaves showed the positive test for important secondary biochemical compounds such as alkaloid, steroid, flavonoid, saponin, tannin and reducing sugar and negative test is shown by glycoside and gum.

3.2. Total flavonoid and tannin content

In these studies, carbon tetrachloride soluble fraction (CTCSF) exhibited the maximum flavonoid content [(97.37 ± 0.25) mg/g] though petroleum ether soluble fraction (PESF) was found the minimum flavonoid content [(33.44 ± 0.47) mg/g]. The total tannin content showed highest in PESF [(62.47 ± 0.33) mg/g]

though ethanolic extract showed the smallest [(26.97 ± 0.49) mg/g] in quantity (Table 1).

Table 1
Total flavonoid and tannin content of extracts of *H. indicum*.

Extracts	Total flavonoid content (mg of QAE/g) of dry extract	Total tannin content (mg of TAE/g) of dry extract
ESF	37.36 ± 0.24	27.08 ± 0.20
CTCSF	97.37 ± 0.25	35.09 ± 0.11
PESF	33.44 ± 0.47	62.47 ± 0.33
Ethanolic extract	–	26.97 ± 0.49

Values are expressed as mean ± SD (n = 3). Values are statistically significant (P < 0.05). QAE: Quercetin antioxidant equivalents; TAE: Tannic acid equivalents.

3.3. Thrombolytic activity

In case of *in vitro* clot lysis actions, test showed that adding of 100 µL SK drugs as a standard (30 000 IU) to the coagulation containing tubes trailed by gestation for 90 min at room temperature presented 65.15% breakdown of clot whereas sterile water (negative control) exhibited 3.77% clot lysis. Whereas, ethanolic extract, petroleum ether, carbon tetrachloride and chloroform showed 23.78%, 35.40%, 32.48% and 18.95% clot lysis correspondingly.

3.4. Membrane stabilizing activity

In this study exposed, 2 mg/mL of CEE and fractions of CEE of *H. indicum* pointedly inhibited the breakdown of RBCs membrane provoked by hypotonic and heat induced solution associated to the reference drug (acetyl salicylic acid). In case of hypotonic solutions, standard ASA exhibited 72.25%, and ESF, PESF, CTCSF and chloroform soluble fraction (CSF) showed 35.15%, 37.37%, 39.24% and 34.56% correspondingly though in heat, ASA, ESF, PESF, CTCSF and CSF showed 42.56%, 32.59%, 27.39%, 40.20%, and 23.12% correspondingly. But by heat induced method, CTCSF of *H. indicum* inhibited significant percentage (39.24%) of breakdown of RBCs prompted by hypotonic solution and (40.20%) induced by heat, respectively among all fractions. The study of membrane stabilizing activity of *H. indicum* showed the following result.

3.5. Antimicrobic actions

Antimicrobic actions of *H. indicum* examined samples were screened against three known Gram positive and six Gram negative microbes and results were matched with standard antibiotic kanamycin. The result of antimicrobic actions (Table 2) having inhibition of growth fluctuated from 8 to 18 mm. *H. indicum* showed more activity to Gram positive bacteria than Gram negative bacteria.

Table 2
Antimicrobial potential of *H. indicum*.

Bacterial strains		Inhibition of growth (mm)	
		Crude extract	Kanamycin (30 µg/disc)
Gram positive bacteria	*Bacillus cereus*	18	42
	Staphylococcus aureus	11	40
	Bacillus subtilis	13	41
Gram negative bacteria	*Escherichia coli*	9	42
	Salmonella typhi	8	45
	Pseudomonas aeruginosa	12	40
	Salmonella paratyphi	9	44
	Vibrio mimicus	8	40
	Shigella dysenteriae	9	38

The extractives presented moderate antimicrobic actions against *Bacillus cereus* having 18 mm zone of inhibition of growth and moderate activity against *Bacillus subtilis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* having 13 mm, 11 mm and 12 mm zone of inhibition, correspondingly. It also exhibited mild antimicrobic actions against the other known bacteria.

4. Discussion

The leaves of *H. indicum* have been copiously used in local traditional medicine since primitive times. In the current experimental protocol, we studied the potential health benefits of various fractions of the ethanolic extract of these leaves. Outcomes of the research indicate that the extracts of *H. indicum* have the potential to be used as a remedy for thrombosis and inflammatory diseases and also against few important bacterial pathogens.

Extractives showed positive effect for flavonoid and tannin in the initial test. We have decided to quantify the content of these phytochemicals in the various fractions using the aluminum chloride colorimetric method, which takes advantage of the formation of a flavonoid-aluminum complex. The ethyl acetate soluble fraction exhibited the lowest flavonoid content while the PESF found to possess the highest flavonoid content. The tannin content was determined by Folin-Ciocalteu chemical and stated in relations of tannic acid equivalents. The total tannin content was the highest in the PESF (62.47 ± 0.33) and lowest in the CEE (26.97 ± 0.49).

The fibrinolytic enzyme SK which we used as reference resulted in a greater clot lysis than distilled water. Among the extract fractions, the negative control and CSF had the lowest percent of clot lysis. The remaining fractions resulted in significantly higher percentage. Thrombolytic activity of the various extract fractions was significant compared to the effect of distilled water. SK is a fibrinolytic drug acting as plasminogen activator. Once

it binds to human plasminogen, it results in its activation and the formation of plasmin. Standard SK has been used as an active and reasonable thrombolytic agent for heart attack and pulmonic thrombosis also. So from the pragmatic results, it can be assumed that the prevention of breakdown of RBC properly by *H. indicum* leaves could be the possible thrombolytic activity. Our findings indicate that the thrombolytic activities of at least three of extract fractions (ESF, PESF, and CSF) compare favorably with that of SK.

In our study, for membrane stabilizing activities, all extract fractions provided significant levels of protection against osmotically induced and heat prompted breakdown of RBCs. The PESF exhibited the highest membrane stabilizing effect and the negative control showed the lowest. The reference drug, ASA, resulted in a membrane stabilization. A similar trend was observed with heat induced hemolysis.

The antimicrobic actions of the ethanolic leaves extracts of *H. indicum* are used against known Gram positive and Gram negative bacteria and matched with the standard kanamycin. Our research exposed that fluctuated in growth of inhibition by extracts against known microbes. The Gram positive species *Bacillus cereus* was the strain which was profound to CEE and exhibited the highest growth of inhibition. No antibacterial activity was shown by the aqueous soluble fraction. We can privilege that the leaves of *H. indicum* could be used against known bacteria. Our observations indicate the presence of antimicrobic active ingredients in the leaves of *H. indicum* moderate effective against both Gram positive and Gram negative species. The plant leaf has the potential to serve as a pharmaceutical source of antibiotics.

Qualitative and quantitative bioactive screening of the various fractions of *H. indicum* leaves indicated the existence of a significant amount of phenolic compounds. The results of the study indicate that the leaves possess clot lysis, membrane stabilizing and antimicrobic potentials. These activities may be attributed mainly to the plant's content of phenolic compound. It is safe to conclude that *H. indicum* leaves are the potential source of pharmaceutical agents that may be safe and effective remedy as antimicrobial, thrombolytic and membrane stabilizing agents.

Conflict of interest statement

We declare that we have no conflict of interest.

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