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“Preliminary Phytochemical Screening and *In Vitro* Thrombolytic Potential of The Methanolic Extract of *Enhydra Fluctuans* Lour (Leaves)”.

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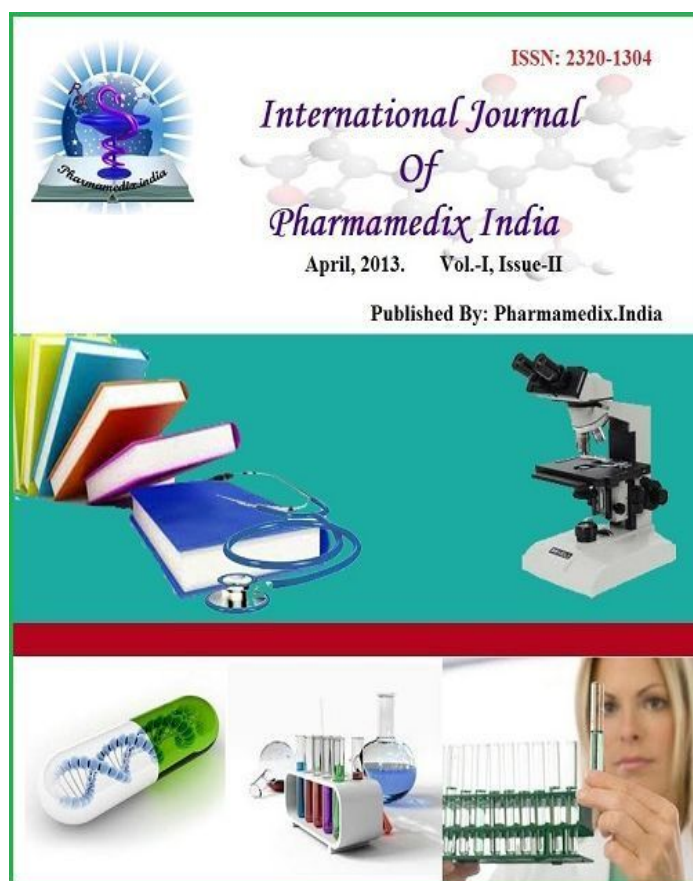
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Abstract:

Investigation with methanol extract of *Enhydra fluctuans* Leaves was carried out to evaluate its thrombolytic potential and to find out possible phytochemical nature. Preliminary phytochemical analysis of methanolic extract showed the presence of alkaloids, carbohydrates, saponins, phenols, tannin, proteins and amino acids, gum and mucilages and triterpenes. A quick & rapid methodology (*In-vitro* Thrombolytic model) was applied to find out their thrombolytic potential where streptokinase and distilled water were employed as positive and negative controls, respectively. The thrombolytic nature of the plant was found significant ($p < 0.001$) when compared with the negative control (water) at different doses. The plant showed significant clot lysis, i.e. $15.98 \pm 2.88\%$, $21.56 \pm 3.32\%$, $24.78 \pm 1.59\%$, $28.67 \pm 4.51\%$, and $31.25 \pm 1.96\%$ at 2, 4, 6, 8, and 10 mg/ml concentrations respectively, while the standard (streptokinase) and negative control (distilled water) showed $40.13 \pm 1.65\%$ and $2.56 \pm 1.23\%$ clot lysis respectively. Through our study, it was found that *E. fluctuans* possess thrombolytic property that could lyse blood clots *in vitro*; however, *in vivo* clot dissolving properties and active component(s) of this extract for clot lysis are yet to be discovered.

Keywords: *Enhydra fluctuans*, thrombolytic potential, phytochemical analysis, clot lysis, *in vitro*.

Introduction:

Atherothrombotic diseases for instance myocardial or cerebral infarction are severe consequences of the thrombus formed in blood vessels^[1,2]. Tissue plasminogen activator, urokinase, streptokinase (SK), etc. are used as thrombolytic agents to dissolve the already formed clots in the blood vessels^[3-5]. However, these drugs have certain limitations which cause severe and sometimes fatal disorders including hemorrhage, severe anaphylactic reaction, lacked specificity, etc.

Moreover, consequently of immunogenicity multiple treatments with SK in a specified patient are restricted^[6].

Agents from plant source are likely to be less antigenic and inexpensive. Significant efforts have been focused towards the finding and progress of natural products from various plant and animal sources which have antiplatelet^[7,8], anticoagulant^[9-11], antithrombotic and thrombolytic activities^[12]. Epidemiologic studies have provided indication that foods with experimentally

showed antithrombotic effect could lessen risk of thrombosis. Herbs showing thrombolytic activity have been premeditated and some momentous observations have been reported^[11].

Enhydra fluctuans Lour. (Family: Asteraceae) is a small genus of marsh herb, available in tropical and subtropical regions. Mainly the plant is originated in Eastern Bengal, Assam and Sylhet^[13]. The herb is relatively glabrous sometimes pubescent glandular. Stems are usually 0.3-0.6m, elongated simple or divaricating rooting at the nodes^[14]. Survey of literature showed that the leaves of the plant are used as laxative. Leaves are also used in the treatment of the skin and nervous system. The expressed juice of the leaves is an excellent demulcent in gonorrhoea^[15]. But there are insufficient records in literature of this plant, regarding its pharmacological activities and physicochemical characteristics. Thus the present study focuses on screening of methanolic extract of *Enhydra fluctuans* Lour. Leaves, for its clot lysis property (thrombolytic activity) by using an in vitro assay method.

MATERIALS AND METHODS:

Collection and extraction

For this present investigation, *Enhydra fluctuans* leaves were collected from Noakhali, Bangladesh on September, 2012. After collection leaves were thoroughly

washed with water. The plant was identified by expert of Bangladesh National Herbarium, Mirpur, Dhaka, Bangladesh. (Accession number: DACB 37925).

500 mg of the dried and powdered sample was soaked in 2500 ml of 99.8% methanol). After 10 days the solution was filtered using filter cloth and Whatman® filter paper No. 1. The resulting filtrates were then evaporated in water bath below 40°C to dryness and thus a concentrated semisolid mass of the extract was obtained.

Streptokinase (SK)

Commercially available lyophilized Altepare (Streptokinase) vial (Beacon pharmaceutical Ltd) of 15, 00,000 I.U., was collected and 5 ml sterile distilled water was added and mixed properly. This suspension was used as a stock from which 100µl (30,000 I.U) was used for in vitro thrombolysis^[16].

Specimen

Whole blood (7 ml) was drawn from healthy Bangladeshi human volunteers (n = 5) (aged 20-23 years) without a history of oral contraceptive or anticoagulant therapy (using a protocol approved by Institutional Ethics Committee). 1 ml of blood was transferred to each of the 7 previously weighed sterilized micro-centrifuge tubes to form clots.

Herbal Preparation

Five different test solutions were used to evaluate the thrombolytic activity of the plant extract. The plant extract was dissolved in methanol and shaken vigorously on a vortex mixer to prepare different concentrations (2, 4, 6, 8 and 10 mg/ml respectively) of the test sample. The suspension was kept overnight and decanted to remove the soluble supernatant, which was filtered through a 0.22 micron syringe filter. 100 µl of the methanolic preparations of the plant were added to the micro-centrifuge tube containing the clots to check thrombolytic activity^[16].

Phytochemical evaluation

Small quantity of freshly prepared methanolic extract of *Enhydra fluctuans* leaves were subjected to preliminary quantitative phytochemical investigation for the detection of phytochemicals such as alkaloids, carbohydrates, glycosides, phytosterols, proteins, flavonoids, tannins, saponins, phenols, gums and mucilages, fats & fixed oils using the following standard methods^[17-20].

i. Detection of alkaloids

Both the extracts were dissolved individually in dilute Hydrochloric acid and the solutions were filtered.

a) Mayer's Test: Filtrates were treated with Mayer's reagent (Potassium Mercuric Iodide). Formation of a yellow colored precipitate marked the presence of alkaloids.

b) Hager's Test: Filtered solutions were taken in a test tube and Hager's reagent (saturated picric acid solution) was added with it. Presence of alkaloids was confirmed by the formation of yellow colored precipitate.

ii. Detection of carbohydrates

Extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were evaluated for the presence of carbohydrates.

a) Benedict's test: Filtrates were treated with Benedict's reagent and heated gently. Orange red precipitate pointed the presence of reducing sugars.

b) Fehling's Test: Filtered solutions were hydrolyzed with dil. HCl, neutralized with alkali and heated with Fehling's A & B solutions. Formation of red precipitate specified the presence of reducing sugars.

iii. Detection of glycosides

Extracts were hydrolyzed with dil. HCl, and then subjected to test for glycosides.

a) Legal's Test: Extracts were mixed with sodium nitropruside in pyridine and sodium hydroxide. Formation of pink to blood red color indicated the presence of glycosides.

b) Modified Borntrager's Test: Extracts were treated with Ferric Chloride solution and immersed in boiling water for about 5 minutes. The mixture was cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink color in the ammoniacal layer showed the presence of glycosides.

iv. Detection of saponins

a) Froth Test: Extracts were diluted with distilled water to 20 ml and this was shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam expressed the presence of saponins.

b) Foam Test: 0.5 gm of extract was shaken with 2 ml of water. Foam was produced which remained for 10 minutes and pointed the presence of saponins.

v. Detection of phytosterols

a) Salkowski's Test: Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of conc. sulphuric acid, shaken and allowed to stand. Appearance of golden yellow color showed the presence of triterpenes.

b) LibermannBurchard's test: Extracts were mixed with chloroform and filtered. The filtrates were treated with few drops of acetic anhydride, boiled and cooled and then conc. sulphuric acid was added. Formation of

brown ring at the junction confirmed the presence of phytosterols.

vi. Detection of phenols

Ferric Chloride Test: Extract solutions were taken in test tubes and 3-4 drops of ferric chloride solution were added to them. Formation of bluish black color indicated the presence of phenols.

vii. Detection of tannins

Gelatin Test: To the extract, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate confirmed the presence of tannins.

viii. Detection of flavonoids

a) Alkaline Reagent Test: Extracts were treated with 4-5 drops of sodium hydroxide solution. Formation of intense yellow color, which becomes colorless on addition of dilute acid, indicated the presence of flavonoids.

b) Lead acetate Test: 4-5 drops of lead acetate solution was added to the extract solutions. Formation of yellow color precipitate marked the presence of flavonoids.

ix. Detection of proteins and amino acids

a) Xanthoproteic Test: The extracts were treated with 4-5 drops of conc. Nitric acid. Formation of yellow color indicated the presence of proteins.

b) Ninhydrin Test: To the extract, 0.25% w/v ninhydrin reagent was added and boiled for few minutes. Formation of blue color indicated the presence of amino acid.

x. Detection of fixed oils and fats

A few drops of 0.5N alcoholic potassium hydroxide were added to a small quantity of extract along with a drop of phenolphthalein. The mixture was heated on a water bath for 1-2 hours. Formation of soap or partial neutralization of alkali pointed the presence of fixed oils and fats.

xi. Detection of gums and mucilages

1 ml of the extract was hydrolyzed using dil. HCl (3ml). Then Fehling's solution was added drop by drop till the appearance of red. Test for mucilages were carried out by treating 1 ml of extract with 2 ml of ruthenium red solution to get red coloured solution.

Thrombolytic assay

In vitro clot lysis activity of *Enhydra fluctuans* was carried out according to the method of Prasad *et al.*, 2007 with minor modifications. 7 ml of venous blood was drawn from healthy volunteers (n = 5) and transferred to different pre weighed sterilized micro-centrifuge tube (1 ml/tube). The micro-centrifuged tubes were exposed to incubation at 37°C for 45 minutes. After the formation of clot, serum was completely discarded from

the tubes (carried out without disturbing the clot formed) and each tube having clot was again weighed to determine the weight of the clot (clot weight = weight of clot containing tube – weight of tube alone).

Each micro-centrifuge tube containing clot was appropriately labeled and 100 µl of the plant extract with various concentrations (2, 4, 6, 8 and 10 mg/ml respectively) was added to the tubes accordingly. As a positive control, 100 µl of streptokinase and as a negative non thrombolytic control, 100 µl of sterilized distilled water were distinctly added to the control tubes numbered. After that the tubes were incubated again at 37°C for 90 minutes and observed for clot lysis. After the following incubation, the obtained fluid was discarded from the tubes and they were again weighed to observe the difference in weight after clot disruption. Finally, difference obtained in weight was calculated and the result was expressed as percentage of clot lysis following the underneath equation.

$$\% \text{ of clot lysis} = (\text{wt. of released clot} / \text{clot wt.}) \times 100$$

Statistical analysis

The significance of percent clot lysis by herbal extracts (with different concentrations) and streptokinase was compared with water using paired t-test analysis. Data are expressed as mean $\pm$ standard deviation (SD).

A p value < 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

Preliminary phytochemical screening of the methanolic extract of *E. fluctuans* showed the presence of Alkaloids, Carbohydrates, Saponins, Phenols, tannin, Proteins and amino acids, Gum and mucilages and triterpenes (Table 1).

As a part of discovery of cardio protective drugs from natural resource the extract of *E. fluctuans* was assessed for thrombolytic activity and the results are presented in Table 2. Addition of 100 µl SK, a positive control (30,000 I.U.), to the clots and subsequent incubation for 90 minutes at 37°C, showed 40.13 % lysis of clot. On the other hand, distilled water was treated as negative control which exhibited a negligible percentage of lysis of clot (2.56%). When clots were treated with 100 µl each of different concentrations (2, 4, 6, 8 & 10 mg/ml respectively) of the test sample significant clot lysis activity, i.e., 15.98 %, 21.56 %, 24.78 %, 28.67% and 31.25 % respectively, was observed and when compared with the negative control (water) the mean of percentage (%) of clot lysis was significant (p < 0.001). Percentage of clot lysis after treatment with different concentrations of the methanolic extract and proper controls is shown in Figure 1. The aim of the present study was to find if the herbal preparation of *E. fluctuans* possess clot lysis

potentiality or not. The evaluation of the positive control (streptokinase) with negative control clearly demonstrated that clot dissolution does not occur when water was added to the clot. Encouraged by the result of the positive control, we compared five different concentrations of the test sample in the same manner with the negative control and observed significant thrombolytic activity.. Since phytochemical analysis showed that the crude extract contains tannin, alkaloid and saponin; it could be predicted that these phytochemicals may be responsible for its clot lysis activity^[21].

Conclusion:

This is an important finding which may have important inferences in cardiovascular health. In addition, this finding may indicate the possibility of developing novel thrombolytic compounds from methanolic extract of *E. fluctuans*. Further studies are ongoing to isolate and characterize the compounds responsible for thrombolytic activity.

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Table-1: Preliminary phytochemical screening of the methanolic extract of *E.fluctuans* leaves

Sl. No	Group of phytoconstituents	Methanolic extract
1.	Alkaloids	+
2.	Carbohydrates	+
3.	Glycosides	-
4.	Saponins	+
5.	Phytosterols	-
6.	Phenols	+
7.	Tannins	+
8.	Flavonoids	-
9.	Proteins and amino acids	+
10.	Fats & fixed oils	-
11.	Gum and mucilages	+
12.	triterpenes	+

Table 2: Effect of different concentrations of the test sample and the controls on in vitro clot lysis

Treatment	% Clot lysis (mean $\pm$ S.D)	P value (Two-tailed) when P value (Two-tailed) when compared to negative control (water)
Extract (2 mg/ml)	15.98 $\pm$ 2.88	< 0.001
Extract (4 mg/ml)	21.56 $\pm$ 3.32	< 0.001
Extract (6 mg/ml)	24.78 $\pm$ 1.59	< 0.001
Extract (8 mg/ml)	28.67 $\pm$ 4.51	< 0.001
Extract (10 mg/ml)	31.25 $\pm$ 1.96	< 0.001
Streptokinase (standard)	40.13 $\pm$ 1.65	< 0.001

Statistical representation of percent clot lysis by different treatments, and negative control (sterile distilled water) done by paired t-test analysis; clot lysis % is represented as mean $\pm$ S.D.

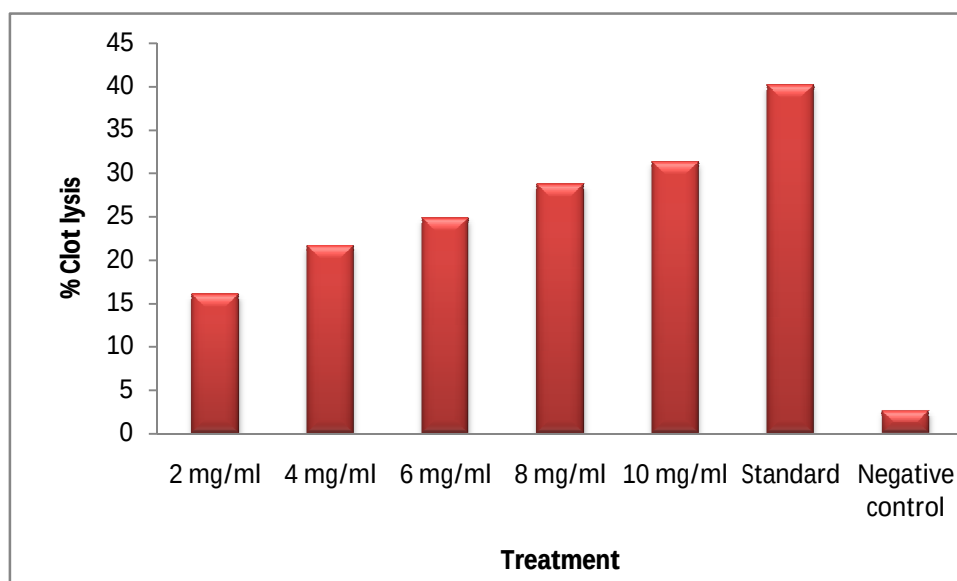


Figure 1: Thrombolytic activity of methanolic extract of *E. fluctuans* Leaves negative control and Streptokinase by clot lysis activity.

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