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Phytochemical and Pharmacological Potential of *Enhydra fluctuans* Available in Bangladesh

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Authors' contributions

This work was carried out in collaboration among all authors. Authors SK and MHS designed the study, performed the statistical analysis and wrote the protocol. Author SK wrote the first draft of the manuscript. Authors SRR, SS and FRL managed the analyses of the study, literature searches and review of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Objectives: The possible phytochemical constituents, thrombolytic and membrane stabilizing activities of the crude ethanolic extract of *Enhydra fluctuans* (CE) were investigated along with the antimicrobial, antioxidant and cytotoxic potentials of its petroleum ether (PESF), carbon tetrachloride (CTCSF), chloroform (CSF) and aqueous (AQSF) soluble fractions.
Materials & Methods: The coarse leaf powder was extracted at room temperature with ethanol. Solvent-solvent partitioning was done to obtain the four soluble fractions. Anticoagulant potential was determined by the *in vitro* thrombolytic model, membrane stabilization method was used to assess *in vitro* anti-inflammatory activity, the disc diffusion method was used for anti-microbial screening, antioxidant potential was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method and brine shrimp lethality bioassay method was used for cytotoxic activity determination.
Results: Chemical screening of the crude extract evidenced the presence of alkaloids, saponins, tannins, flavonoids, reducing sugars and gums. It showed significant clot lysis property of 46.91%. It also significantly inhibited heat and hypotonic solution induced lysis of the human red blood cell

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membrane with values of 71.80% and 47.60%, respectively. CTCSF and PESF showed mild antimicrobial activity. AQSF showed most prominent antioxidant activity with IC_{50} value of 12.27 µg/mL. CTCSF showed LC_{50} value of 0.84 µg/mL, with most potent cytotoxic activity.
Conclusion: Significant thrombolytic, membrane stabilizing, antioxidant and *in vitro* cytotoxic activities of the ethanolic plant extract were observed in this study. *In vivo* activities and isolation of active compound(s) from this extract are yet to be investigated.

Keywords: Enhydra fluctuans; thrombolytic; membrane stabilizing; antioxidant; cytotoxic.

1. INTRODUCTION

Medicinal plants have played an important role in treating various diseases since ancient times. Increased drug resistance and side effects of existing drugs have escalated the research on traditionally available medicinal plants.

Myocardial or cerebral infraction and other atherothrombotic diseases are consequences of thrombus formed in blood vessel [1,2]. Fibrinolytic drugs such as tissue plasminogen activator (t-PA), urokinase, streptokinase and others dissolve thrombin in acutely occluded coronary arteries and restore blood supply to ischemic myocardium, to limit necrosis and improve prognosis [3]. Yet all the available thrombolytic agents have significant deficiencies, including the necessity of large doses to be maximally effective, limited fibrin specificity and a significant associated bleeding tendency. Therefore, studies are going on to develop improved thrombolytic drugs in order to minimize deficiencies of the available drugs.

Inflammation is a pathological disorder, in which inflammatory cells produce a complex mixture of growth and differentiation cytokines as well as physiologically active arachidonate metabolites, also generate reactive oxygen species (ROS) that can damage cellular biomolecules which in turn augment the state of inflammation [4]. Compounds that possess radical scavenging ability may therefore expect to have the therapeutic potentials against inflammation [5]. Moreover, stabilization of lysosomal membrane limits inflammatory response by inhibiting the release of lysosomal constituents of activated neutrophil such as bactericidal enzymes and proteases, which are responsible for further tissue inflammation and damage [6].

Currently antibiotic resistance has become a major clinical and public health problem for most people [7,8]. This multidrug resistance (MDR) is clearly related to the misusage of different antibiotics [9,10]. The increasing clinical importance of drug resistant bacterial pathogens necessitates the search for additional antibacterial therapy. The antibacterial screening which is the first stage of antibacterial research is performed to ascertain the susceptibility of various bacteria to any agent.

Free radicals are highly toxic and reactive; and cause diseases like aging, atherosclerosis, cancer, diabetes, liver cirrhosis, cardiovascular disorders, etc. by attacking different macro-molecules including lipids, proteins and DNA resulting in the cellular damage [11-13]. Currently available synthetic antioxidants like butylatedhydroxy anisole (BHA), butylatedhydroxy toluene (BHT), tertiary butylated hydroquinone and gallic acid esters show low solubility and moderate antioxidant activity with suspected negative health effects [14-16]. Investigations to screen out naturally occurring antioxidants are now mostly demanding.

Bioactive compounds are always toxic to living body at some higher doses. Brine shrimp lethality bioassay implies cytotoxicity as well as a wide range of pharmacological activities such as antimicrobial, antiviral, pesticidal and anti-tumor activities [17,18].

Enhydra fluctuans Lour (Family: Asteraceae) is a small genus of marsh herb, available in tropical and subtropical regions namely Bangladesh, India, Malaysia, China and the rest of South East Asia and Tropical Africa [19,20]. The herb is relatively glabrous sometimes pubescent glandular. Stems are usually 0.3 to 0.6 m, elongated simple or divaricating rooting at the nodes [21]. Traditional uses indicate that the leaves of the plant are good as laxative. The expressed juice of the leaves is an excellent demulcent in gonorrhea and also used in the treatment of skin and nervous system [22]. Different phytoconstituents such as flavonoids, isoflavone glycosides, terpenoids like sesquiterpene lactones, saponins etc have already been isolated from *E. fluctuans* [23-26].

There are reports on antioxidant property of the methanolic extract, antimicrobial property of the toluene, chloroform and methanolic extract of *E. fluctuans*, moreover CNS depressant activity, anti-inflammatory activity of *E. fluctuans* have also been reported [27-30]. But there are insufficient records in literature, regarding pharmacological activities and phytochemical characteristics of *E. fluctuans* Lour available in Bangladesh which are known to differ with plant's geographical location. Thus the present study focuses on screening of the ethanolic extract of *Enhydra fluctuans* Lour to identify its phytochemical and pharmacological potential.

2. MATERIALS AND METHODS

2.1 Instrumentation

The extract was concentrated in rotary vacuum evaporator (Heidolph Instruments GmbH & Co. KG, Germany). Samples were centrifuged on a bench top centrifuge machine (HERMLE Labortechnik GmbH, Germany). The absorbance was recorded by a UV-Visible spectrophotometer (Analytic Jena AG, Germany). Ketoconazole was obtained from Beximco Pharmaceuticals Ltd., Bangladesh. Streptokinase vial (Beacon Pharmaceutical Ltd) was purchased from local pharmacy. Trolox and BHA were obtained from Calbiochem, Merck, Germany and VWR International Ltd., England, respectively. Nutrient agar and potato dextrose agar and kanamycin 30 µg standard disc were purchased from HiMedia Laboratories Pvt. Ltd., India. All other reagents were purchased from Sigma-Aldrich, USA and all other solvents used were of analytical grade and purchased from Active Fine Chemicals Ltd., Bangladesh.

2.2 Collection and Identification

Enhydra fluctuans leaves were collected from Narayangonj, Bangladesh on February 2015. Plant was identified by taxonomist. The leaves were thoroughly washed with clean water, dried in the shade for several days and oven dried for 24 hours at not more than 40ºC. The dried leaves were then ground to a coarse powder. The powder (140 g) was subjected to cold maceration by soaking in 1200 mL of ethanol in a clean, amber colored reagent bottle. The container was kept for a period of 10 days with occasional shaking and stirring. The whole mixtures were then filtered through a fresh cotton plug and finally with a Whatman No.1 filter paper. The filtrate was concentrated at 40ºC under reduced pressure to evaporate approximately 70% of the solvent to obtain the crude extract, CE. 5.0 g of CE was dissolved in 10% aqueous ethanol. Solvent-solvent partitioning of this solution was done using the protocol designed by Kupchan and modified by Van Wagenen et al, (1993) with petroleum ether, then with carbon tetrachloride and finally with Chloroform to obtain three fractions (PESF, CTCSF and CSF) and the remaining part was named as aqueous soluble fraction (AQSF)[31]. Before carbon tetrachloride and chloroform extraction, water was added to the remaining aqueous extract to increase the polarity difference for separation.

2.3 Phytochemical Screening

Approximately 5 mg of CE was diluted in ethanol to perform the following tests for identification of different chemical groups [32,33].

2.3.1 Tests for tannins

Ferric chloride test: About 5 mL of the extract solution was taken in a test tube, followed by addition of 1 mL of 5% ferric chloride solution. Greenish black precipitate indicates the presence of tannins.

Potassium dichromate test: About 1 mL of 10% potassium dichromate solution was added with 5 mL of the extract solution in a test tube. Yellow precipitate suggests the presence of tannins.

Lead acetate test: About 1 mL of 10% lead acetate solution was added to 5 mL of extract solution. Yellow precipitate formation is indicative of presence of tannins.

2.3.2 Test for flavonoids

A few drops of concentrated hydrochloric acid were added to a small amount of CE. Immediate red color formation is caused by flavonoids.

2.3.3 Test for saponins

About 1 mL of the solution was diluted with 20 mL distilled water and shaken in a graduated cylinder for 15 minutes. One-centimeter layer of foam formation is indicative of the presence of saponins.

2.3.4 Tests for gums

About 5 mL solution of the extract was added to a test tube containing molisch reagent and sulfuric acid. Red violet ring at the junction of the two liquids evidences presence of gums.

2.3.5 Tests for steroids

About 1 mL concentrated sulfuric acid was added to 1 mL of chloroform extract. Red color in the lower layer indicates the presence of steroids.

2.3.6 Tests for alkaloids

Mayer's test: About 2 mL solution and 0.2 mL of dilute hydrochloric acid were taken in a test tube followed by addition of 1 mL of Mayer's reagent. Yellowish buff colored precipitate indicates the presence of alkaloids.

Dragendroff's test: About 2 mL solution and 0.2 mL of dilute hydrochloric acid were taken in a test tube followed by addition of 1 mL of Dragendroff's reagent. Orange brown precipitate evidences the presence of alkaloids.

Hager's test**:** About 2 mL of the extract and 0.2 mL of dilute hydrochloric acid were taken in a test tube. Then 1 mL of picric acid solution or Hager's reagent was added. Yellowish precipitate evidences the presence of alkaloids.

2.3.7 Test for reducing sugar

About 2 mL of aqueous extract was added to 1 mL of a mixture of equal volumes of Fehling's solutions A and B followed by boiling for few minutes. Brick red colored precipitate is indicative of the presence of reducing sugar.

2.4 Thrombolytic Activity

The extract of *E. fluctuans* was assessed for thrombolytic activity using *in vitro* thrombolytic model [34]. Taking account of all ethical considerations, and aseptic precautions, 20 mL of venous blood was drawn from healthy human volunteer without a history of oral contraceptive or anticoagulant therapy. This withdrawn blood was then distributed in pre-weighed sterile vials (1 mL/tube) and incubated at 37ºC for 45 minutes. After clot formation, the serum was completely removed without disturbing the clot and clot weight was determined (clot weight = weight of clot containing vial – weight of vial alone). About 100 mg of CE was dissolved in 10 mL of ethanol and kept overnight. The soluble supernatant was decanted and filtered. Approximately 100 µL of the solution was added to a vial containing pre-weighed blood clot. Lyophilized streptokinase (SK) vial of 15,00,000 I.U. was reconstituted with 5 mL sterile distilled water. About 100 µL (30,000 I.U) of the solution was added as positive control and 100 µL of distilled water was added as a negative non thrombolytic control to the vial containing pre-weighed blood clot separately. All the three vials were then incubated at 37ºC for 90 minutes and observed for clot lysis. After incubation, the released fluid was removed and vials were again weighed to observe the weight difference after clot disruption. This difference of weight before and after clot lysis was expressed as the percentage of clot lysis as the following [34]:

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

2.5 Membrane Stabilization Activity

To assess the anti-inflammatory activity, membrane stabilization potential of CE was evaluated by measuring the heat and hypotonic solution induced hemolysis of erythrocyte following standard protocol [35].

2.5.1 Preparation of erythrocyte suspension

Fresh whole blood (20 mL) was collected intravenously in syringe containing 3.1% sodium citrate solution as anti-coagulant from healthy human volunteer without history of non steroidal anti-inflammatory drugs (NSAIDs) therapy for 2 weeks prior the experiment. The blood cells were washed three times with isotonic buffer solution, 154 mM NaCl in 10 mM sodium phosphate buffer (pH 7.4) through centrifugation at 3000 g for 10 min.

2.5.2 Heat induced hemolysis

CE was dissolved in isotonic solution. Isotonic buffer containing aliquots (5 mL) of the extract at 1.0 mg/mL was taken in six centrifuge tubes in three sets of two. Two sets of control tubes containing 5 mL of the vehicle control and 5 mL of 0.1 mg/mL of acetyl salicylic acid (Aspirin), respectively. Erythrocyte suspension was added to each tube and mixed gently by inversion. One pair of the tubes was incubated at 56ºC for 30 min in a water bath, while the other pair was maintained at 0 to 5°C in an ice bath. The reaction mixture was centrifuged for 5 min at 2500 g and the absorbance of the supernatant was measured at 560 nm. The percentage inhibition or acceleration of hemolysis was calculated according to the equation [35]:

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

Where,

OD_1= optical density of unheated test sample;
OD_2= optical density of heated test sample;
OD_3= optical density of heated control sample.

2.5.3 Hypotonic solution induced hemolysis

About 5 mL hypotonic solution (distilled water) containing 1.0 mg/mL of CE were put in centrifuge tube in triplicates. About 5 mL of hypotonic solution and 5 mL of acetyl salicylic acid (Aspirin) at 0.1 mg/mL were taken as negative and positive controls respectively in separate centrifuge tubes. Erythrocyte suspension (0.5 mL) was added to each of the tube and mixed gently. The mixture was incubated for 10 min at room temperature and then centrifuged for 10 min at 3000 g. The absorbance of the supernatant was measured at 540 nm. The percentage inhibition of hemolysis or membrane stabilization was calculated using the following equation [35]:

$$\text{Percentage of inhibition of hemolysis} = 100 \times [1-\{(OD_2-OD_1)/(OD_3-OD_1)\}]$$

Where OD_1 = absorbance of test sample in isotonic solution; OD_2 = absorbance of test sample in hypotonic solution; OD_3 = absorbance of control hypotonic sample.

2.6 Antimicrobial Assay

The disc diffusion technique [36] was used for preliminary screening of antimicrobial activity. Two Gram Positive bacterial strains (*Bacillus megaterium* ATCC 13578, *Staphylococcus aureus* ATCC 25923), two Gram Negative bacterial strains (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27833) and two fungal strains (*Aspergillus niger* and *Aspergillus flavus*) were freshly cultured in nutrient agar media for bacteria and potato dextrose agar media for fungi. PESF, CTCSF, CSF, AQSF fractions of the crude extracts were dissolved separately in specific volume of dichloromethane or methanol depending on their solubility. The diluted samples were applied on the sterile discs at a concentration of 500 µg/disc. Kanamycin (30 µg/disc) and ketoconazole (30 µg/disc) were used as standard antibiotics for antibacterial and antifungal screening, respectively. Solvent was used as negative control. The antimicrobial activity of the test agent was checked after 18 hrs of incubation for bacteria at 37ºC and 48 hrs of incubation for fungi at 28ºC. The result was determined by measuring the diameter of zone of inhibition expressed in mm.

2.7 Antioxidant Scavenging Activity

Antioxidant potential was studied using DPPH radical scavenging activity with slight modification of the method described by Brand-William et al. [37]. Here, sample solution of varying concentrations such as 200 µg/mL, 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.125 µg/mL, 1.5625 µg/mL and 0.78125 µg/mL were obtained by serial dilution technique in test tubes, where 2 mL of each of the test sample solution was mixed with 2 mL of a DPPH-methanol solution (20 µg/mL) to obtain the above mentioned concentrations. Test tubes were allowed to stand for 20 minutes in dark for the reaction to occur. The absorbance was determined at 517 nm and percentage of inhibition was calculated by using the following equation [37]:

$$\text{Percentage of radical inhibition} = [1- (ABS_{sample} /ABS_{control})] \times 100.$$

Then % inhibitions were plotted against respective concentrations used and from the graph IC_{50} was calculated. Here, ascorbic acid (vit c), BHA and trolox were used as the positive controls.

2.8 Cytotoxic Activity

Brine shrimp lethality bioassay technique was applied for the determination of general toxic properties of the plant extracts against *Artemia salina* [18,38]. The test samples were dissolved in dimethyl sulfoxide (DMSO). Serial dilution technique is used to obtain sample concentrations of 400, 200, 100, 50, 25, 12.5, 6.25 and 3.125 µg/mL in separate test tubes containing 10 shrimps in simulated brine water (5 mL). The test tubes were incubated at room temperature for 24 hours. The LC_{50} of the test samples was determined by a plot of percentage of the shrimp mortality against the logarithm of the sample concentrations.

3. RESULTS AND DISCUSSION

3.1 Phytochemical Screening

Preliminary phytochemical screening evidenced the presence of alkaloids, saponins, tannins, flavonoids, reducing sugars and gums (Table 1).

3.2 Thrombolytic Activity

Addition of 100 µL SK solution, a positive control to the blood clots and subsequent incubation resulted in 66.67% lysis of clot. On the other hand, negative control exhibited a negligible lysis of blood clot (8.33%). When blood clots were treated with the test sample significant clot lysis activity (46.91%) was observed. When compared with the negative control (water) the mean percentage (%) of blood clot lysis was significant ($p < 0.001$) as shown in Fig. 1. Blood clot or fibrin lysis in thrombosis occurs by plasmin produced from plasminogen which in turn activated by plasminogen activator [39]. The plant extract thus may act as plasminogen activator to produce clot lysis activity almost similar to standard which must be proved through more specific test.

3.3 Membrane Stabilizing Activity

Flavonoid rich ethyl acetate extract of *E. fluctuans* collected from West Bengal, India showed significant anti-inflammatory activity in carrageenan and histamine induced paw edema in rats [23]. In our study the ethanolic extracts of *E. fluctuans* at concentration 1.0 mg/mL significantly protected the lysis of human erythrocyte membrane by hypotonic solution and heat induced hemolysis compared to the standard aspirin (0.10 mg/mL). For heat induced condition CE demonstrated 71.80% inhibition of hemolysis of RBCs, whereas aspirin inhibited 77.20%. On the other hand, during hypotonic solution induced hemolysis, CE inhibited 47.60% hemolysis of RBCs as compared to 71.90% produced by aspirin as shown in Fig. 2. The erythrocyte membrane resembles to lysosomal membrane and as such, the effect of drugs on the stabilization of erythrocyte could be extrapolated to the stabilization of lysosomal membrane to exhibit anti-inflammatory activity [40,41].

3.4 Antimicrobial Assay

Table 2 shows antimicrobial activity of the tested samples. PESF showed mild activity against Gram negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*. CTCSF exhibited mild activity against both Gram positive and Gram negative bacteria. All the fractions were inactive against fungal strains. There are reports of mild to moderate antibacterial activity of the methanolic, ethanolic, acetonic and chloroform extract of the plant against *Staphylococcus aureus*, *Staphylococcus saprophyticus*, *Bacillus cereus*, *Escherichia coli*, *Salmonella typhi*, *Shigella sonnei*, *Shigella shiga* [42-44]. As CTSF exhibited most prominent activity as compared to the other fractions, further study is necessary to isolate phytoconstituent with antimicrobial property from the chloroform extract of *E. fluctuans*.

Table 1. Results of chemical group tests

Tested groups	Alkaloids	Steroids	Saponin	Tannins	Flavonoids	Reducing Sugars	Gums
Ethanolic Extract of *Enhydra fluctuans*	+	-	+	+	+	+	+

Note: + = Indicates the presence of the tested group, - = Indicates the absence of the tested group

Table 2. Antimicrobial activity of test samples of *E. fluctuans*

Test organisms	Diameter of zone of inhibition (mm)				
	PESF 100 µg/disc	CTSF 100 µg/disc	CSF 100 µg/disc	AQSF 100 µg/disc	Kanamycin/ Ketoconazole (30 µg/disc)
B. megaterium	−	7	−	−	40
S. aureus	−	8	−	−	28
E. coli	7	8	−	−	26
P. aeruginosa	7	7	−	−	27
A. niger	−	−	−	−	26
A. flavus	−	−	−	−	36

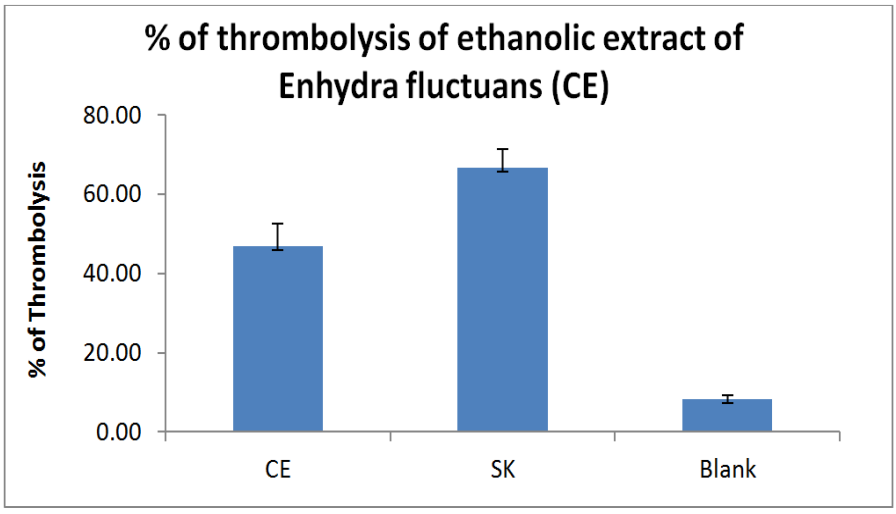

Fig. 1. Thrombolytic activity (in terms of % of clot lysis) with standard deviation error bar
where n=3. "CE"-Ethanolic crude extract of E. fluctuans, "SK"-Positive control

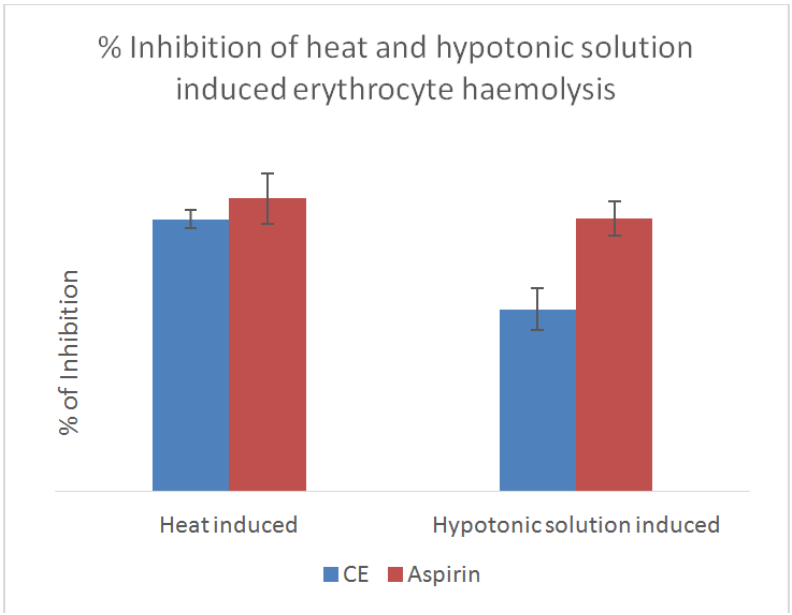

Fig. 2. Membrane stabilizing activity (in terms of % inhibition of erythrocyte hemolysis) with standard deviation error bar
where n=3. "CE"-Ethanolic crude extract of E. fluctuans, "Aspirin"-Positive control

3.5 Antioxidant Assay

AQSF was found to be most potent with lowest IC_{50} value. IC_{50} values of PESF, CTCSF, CSF, AQSF, trolox, BHA, Vitamin C are 59.59 µg/mL, 29.28 µg/mL, 26.62 µg/mL, 12.27 µg/mL, 2.04 µg/mL, 2.21 µg/mL and 0.17 µg/mL respectively. Previous report of *E. fluctuans* collected from West Bengal, India suggested significant antioxidant property of ethyl acetate extract [45]. Also there has been report of isolation of antioxidative carbohydrate polymer from aqueous extract of *E fluctuans* which is most consistent with our finding [46].

3.6 Cytotoxicity Assay

The lethality of the PESF, CTCSF, CSF and AQSF fractions against *A. salina* were shown in Table 3. Lethality assay is proved to be

Table 3. Concentration for 50% mortality (LC_{50}) of different extracts of *E. fluctuans*

Sample	PESF	CTCSF	CSF	AQSF
LC_{50} value (µg/mL)	0.97	0.84	1.43	1.16

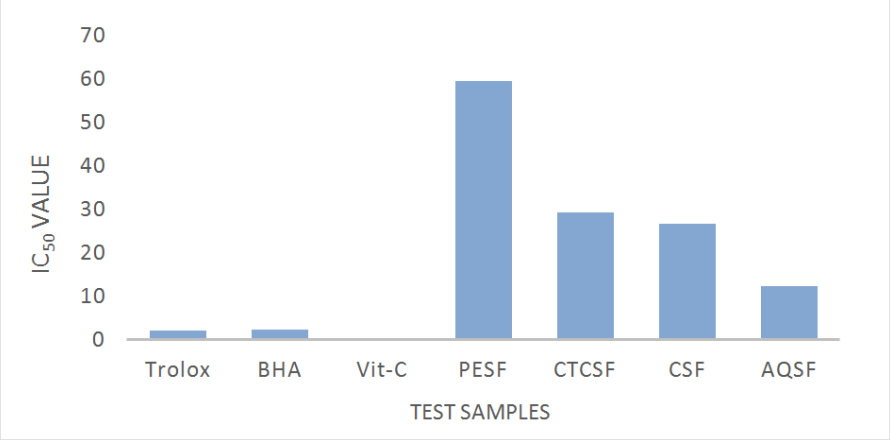

Fig. 3. Comparison of IC_{50} value of different fractions of *E. fluctuans* ethanolic extract and standards

suggestive of different pharmacological properties by isolation of cytotoxic, antimalarial or insecticidal compounds from the plant extracts [47]. Previous reports had suggested cytotoxic potential of the crude methanolic and ethanolic extracts of *E. fluctuans* [44]. Flavonoids isolated from ethyl acetate fraction of *E. fluctuans* exhibited anticancer activity tested *in vitro* in mice [48]. There is report on cell-mediated immune system stimulation by *E. fluctuans* leaves through increasing neutrophil's phagocytic activity [49]. In this study all the fractions exhibited significant cytotoxic activity in the brine shrimp lethality bioassay of which CTCSF fraction was most potent with the lowest LC_{50} value. Presence of several phytoconstituents such as saponins, tannins, flavonoids in the ethanolic extracts of *E. fluctuans* have been proved in our study which may facilitate these potent activities. So further investigation on its different fractions is necessary to isolate bioactive metabolites and specify their pharmacological activities.

4. CONCLUSION

Preliminary chemical screening and *in-vitro* bioactivity study data suggests *Enhydra fluctuans's* potential pharmacological activity. It showed significant cytotoxic activity with good thrombolytic and anti-inflammatory potential. The study necessitates further higher and detailed experiments for isolation of bioactive compounds from *Enhydra fluctuans* which may act as lead compounds for new or improved drug development.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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