Original Research Article

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

ABSTRACT

Objectives: The possible phytochemical ~~nature~~constituents, thrombolytic and membrane stabilizing ~~activity~~ activities of the crude ethanolic extract of *Enhydra fluctuans* (CE) were investigated along with the anti-microbial, 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 ~~by the modified Kupchan method~~. 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 evidences the presence of alkaloids, saponins, tannins, flavonoids, reducing sugars and gums. It showed significant clot lysis property of 49.29%. It also significantly inhibited heat and hypotonic solution induced lysis of the human red blood cell membrane with values of71.67~~%~~ and 47.93%, 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 cytotoxic activities of the plant was found from this study. *In vivo* activities and isolation of active compound(s) from this extract are yet to be investigated.

Keywords: *Enhydra fluctuans*, thrombolytic ~~activity~~, membrane stabilizing ~~activity~~, antioxidant ~~activity~~ and cytotoxic ~~activity~~.

INTRODUCTION

Medicinal plants have played an important role in treating various diseases since ancient times. Increased drug resistance and side effects of existing pharmaceutical drugs have escalated the research based study on traditionally available plants. Myocardial or cerebral infraction and other atherothrombotic diseases are consequences of thrombus formed in blood vessel[1,2]. Fibrinolytic drugs ~~like~~ such as tissue plasminogen activator (t-PA), urokinase, streptokinase ~~etc.~~and others

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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].

Bacteria are responsible for many infectious diseases in 21st century[7]. Antibiotic resistance has become a major clinical and public health problem for most people now-a-days[8,9]. This ~~Multidrug~~ multidrug ~~Resistance~~ resistance (MDR) is clearly related to the misusage of different antibiotics[10,11]. The increasing clinical importance of drug resistant bacterial pathogens necessitates emergence of 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[12,13]. Antioxidants protect body by delaying or reducing the oxidation of the substrate in turn neutralize or terminate the chain reaction before vital molecules within the body are damaged[14].Currently available synthetic antioxidants like butylated hydroxyl anisole (BHA), butylatedhydroxy toluene (BHT), tertiary butylated hydroquinone and gallic acid esters show low solubility and moderate antioxidant activity with suspected negative health effects[15]. BHA and BHT are suspected of being responsible for liver toxicity and carcinogenesis[16,17]. Investigations to screen out naturally occurring antioxidants are now mostly demanding.

Bioactive compounds are always toxic to living body at some higher doses and it justifies the statement that 'Pharmacology is simply toxicology at higher doses and toxicology is simply pharmacology at lower doses'. Brine shrimp lethality bioassay implies cytotoxicity as well as a wide range of pharmacological activities such as antimicrobial, antiviral, ~~pesticidal~~pesticides and anti-tumor ~~etc,~~and others of the compounds[18,19].

Enhydra fluctuans Lour (Family: Asteraceae) is a small genus of marsh herb, available in tropical and subtropical regions~~; like~~namely Bangladesh, India, Malaysia, China and the rest of South East Asia and Tropical Africa[20,21]. 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[22]. Traditional uses indicate that the leaves of the plant are good as laxative. The expressed juice of the leaves is an excellent demulcent in ~~gonorrhoea~~gonorrhea and also used in the treatment of skin and nervous system[23]. These activities can be attributed mainly to the presence of phytochemicals such as flavonoids, alkaloids, saponins, tannins, phenols and carbohydrates. But

there are insufficient records in literature of this plant, regarding its pharmacological activities and phytochemical characteristics. Thus the present study focuses on screening of the ethanolic extract of *Enhydra fluctuans* Lour for its phytochemical and pharmacological potential.

MATERIALS AND METHODS

Instrumentation

The extract was ~~condensed~~ 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~~ 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~~ 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.

Collection and Identification

~~For this investigation,~~ *Enhydra fluctuans* leaves were collected from Narayangonj, Bangladesh on February 2015. Plant was identified by experts. ~~After collection~~The leaves were thoroughly washed with clean water, ~~then shade~~ dried in the shade for several days and oven dried for 24 hours at not more than 40~~-~~°C ~~for better grinding~~. 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 40°C 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 Wagenenet 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)[24].

Phytochemical Screening

Approximately 5 mg of CE was diluted in ethanol to perform the following tests for identification of different chemical groups[25,26].

Tests for tannins

Ferric ~~Chloride~~ chloride Test: About 5 mL of the solution was taken in a test tube~~-~~ ,followed by addition of 1 mL of 5% ferric chloride solution ~~was added to it~~. Greenish black precipitate ~~will~~ indicates the presence of tannins.

Potassium ~~Dichromate~~ 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 ~~will~~ suggest the presence of tannins.

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Lead ~~Acetate~~ acetate Test: About 1 mL of 10% lead acetate solution was added to 5 mL of extract solution. The ~~P~~presence of tannins ~~will be~~were indicated by yellow precipitate.

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.

Test for Saponins

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

Tests for Gums

About 5 mL solution of the extract was ~~taken and then~~added to molisch reagent and sulfuric acid ~~were added~~. Red violet ring at the junction of the two liquids evidences presence of gums.

Tests for Steroids

About 1 mL concentrated ~~sulphuric~~ sulfuric acid was added to 1 mL ~~solution~~ of ~~the~~ chloroform extract. Red color in the lower layer will indicate the presence of steroids.

Tests for alkaloids

Mayer's test: About 2 mL solution and 0.2 mL of dilute hydrochloric acid were taken in a test tube. Then 1 mL of Mayer's reagent was added. Yellowish buff colored precipitate ~~is indicative~~ indicates ~~of~~ 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. Then 1 mL of Dragendroff's reagent was added. Orange brown precipitate evidences the presence of alkaloids.

Hager's test**:** **About** 2 mL ~~solution~~ 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 presence of alkaloids.

Test for ~~Reducing~~ reducing ~~Sugar~~sugar

About 2 mL of aqueous extract ~~of the plant material~~ was added to 1 mL of a mixture of equal volumes of Fehling's solutions A and B, boiled for few minutes. Brick red colored precipitate will indicate presenceof reducing sugar.

Thrombolytic activity

~~As a part of exploration of cardio protective drugs from natural resources the e~~The extract of *E. fluctuans* was assessed for thrombolytic activity using *in vitro* thrombolytic model[27]. 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 37 °C 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. ~~Then the~~The soluble supernatant was decanted and filtered. Approximately 100 µL of the solution was added to a vial containing pre-weighed clot. Lyophilized

~~Streptokinase~~ streptokinase (SK) vial of 15,00,000 I.U. was reconstituted with 5 ~~ml~~ 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 clot separately. All the three vials were then incubated at 37~~.~~°C 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 ~~shown below~~the following:

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

Membrane stabilization activity

To assess the anti-inflammatory activity, membrane stabilization potential of CE was evaluated by measuring the heat and hypotonic solution induced ~~haemolysis~~hemolysis of erythrocyte following standard protocol[28].

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.

Heat induced ~~haemolysis~~hemolysis

CE was dissolved in isotonic solution. Isotonic buffer containing aliquots (5 mL) of the extract at 1.0 mg/mL was taken in ~~6~~ six centrifuge tubes in three sets of two. Two sets of control tubes ~~were taken~~ containing 5 mL of the vehicle control and 5 mL of 0.1 mg/mL of acetyl salicylic acid (ASA), respectively. Erythrocyte suspension was added to each tube and mixed gently by inversion. One pair of the tubes was incubated at 56°C 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:

~~%~~ 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

Hypotonic solution induced ~~haemolysis~~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 (ASA) at 0.1 mg/mL ~~concentration~~ was taken as negative and positive controls respectively in separate centrifuge tubes. Erythrocyte suspension (0.5 mL) was added to each of the tube~~s~~ 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 ~~haemolysis~~hemolysis or membrane stabilization was calculated using the following equation:

Inhibition of ~~haemolysis~~hemolysis ~~(%)~~ = $100 [1-\{(OD_2-OD_1)/(OD_3-OD_1)\}]$

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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

Antimicrobial assay

The disc diffusion technique[29] was used for preliminary screening of antimicrobial activity. Two Gram Positive bacterial strains (*Bacillus megaterium*, *Staphylococcus aureus*), two Gram Negative bacterial strains (*Escherichia coli*, *Pseudomonas aeruginosa*) and two fungal strains (*Aspergillus niger* and *Aspergillus flavus*) were collected as pure cultures. 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~~ 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 determined by measuring the diameter of zone of inhibition expressed in ~~millimeter~~mm.

Antioxidant scavenging activity

Antioxidant potential was studied using DPPH radical scavenging activity with slight modification ~~of the method~~as described by Brand-William *et al*[30]. Here, 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:

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

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

Cytotoxic activity

Brine shrimp lethality bioassay technique was applied for the determination of general toxic properties of the plant ~~extractives~~ extracts against *Artemiasalina*[19,31]. The test samples were dissolved in dimethyl sulfoxide (DMSO) following serial dilutions to obtain 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. Vincristine ~~sulphate~~ sulfate was used as positive control in this assay to compare the cytotoxicity of the test samples.

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RESULTS AND DISCUSSION

Phytochemical screening

Preliminary phytochemical screening evidences the presence of alkaloids, saponins, tannins, flavonoids, reducing sugar and gums (**Table1**).

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.

Thrombolytic activity

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[32,33]. Addition of 100µL SK solution, a positive control to the clots and subsequent incubation resulted in 66.67% lysis of clot. On the other hand, negative control exhibited a negligible lysis of clot (8.33%). When clots were treated with the test sample significant clot lysis activity (46.91%) was observed. When compared with the negative control (water) the mean of percentage (%) of clot lysis was significant ($p < 0.001$). Figure 1 shows?????????

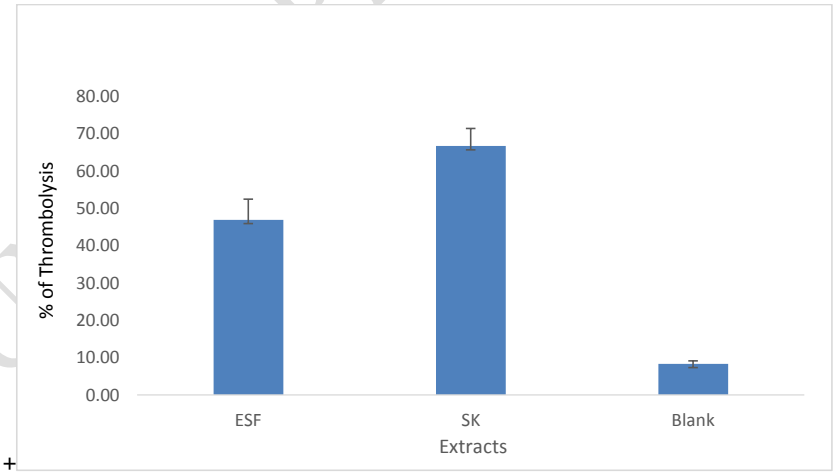

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Figure 1: Thrombolytic activity (in terms of % of clot lysis) of CE with standard deviation error bar where n=3.

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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[34]. The ethanol extracts of *E. fluctuans* at concentration 1.0 mg/mL significantly protected the lysis of human erythrocyte membrane by hypotonic solution and heat induced ~~haemolysis~~hemolysis compared to the standard acetyl salicylic acid (0.10 mg/ml). For heat induced condition CE demonstrated 71.80% inhibition of ~~haemolysis~~hemolysis of RBCs, whereas acetyl salicylic acid inhibited 77.20%.On the other hand, during hypotonic solution induced ~~haemolysis~~hemolysis, CE inhibited 47.60% ~~haemolysis~~hemolysis of RBCs as compared to 71.90% produced by acetyl salicylic acid.

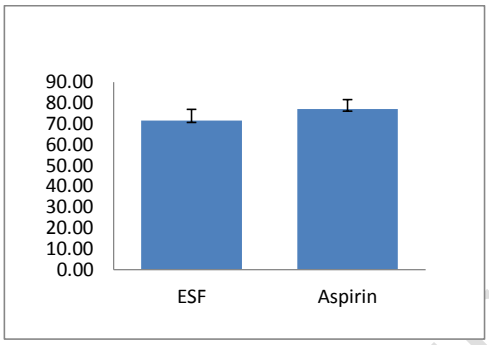

Figure 2: Heat--induced ~~haemolysis~~hemolysis with standard deviation error bar.

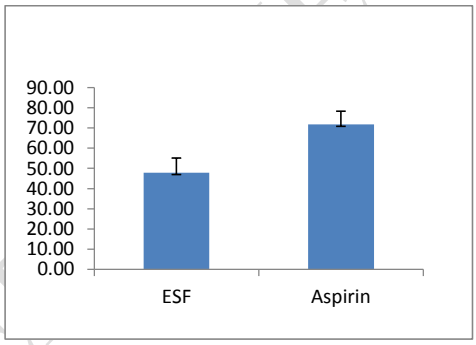

Figure 3: Hypotonic solution induced ~~haemolysis~~hemolysis with standard deviation error bar.

Antimicrobial assay

Table 2 shows antimicrobial activity of the tested samples. PESF showed mild activity against ~~gram~~ Gram negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*. CTCSF exhibited mild activity against both ~~gram~~ Gram positive and ~~gram~~ Gram negative bacteria. All the fractions were inactive against fungal strains. This result is consistent with some antimicrobial screening reports on the plant[35].

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

Test organisms	Diameter of zone of inhibition(mm)				
	PESF 100µg/disc	CTSF 100µg/disc	CSF 100µg/disc	AQSF 100µg/disc	Ketoconazole 30µg/disc
~~Gram positive Bacteria~~					
Bacillus megaterium	–	7 ~~mm~~	–	–	40 ~~mm~~
Staphylococcus aureus	–	8 ~~mm~~	–	–	28 ~~mm~~
~~Gram negative Bacteria~~					
Escherichia coli	7 ~~mm~~	8 ~~mm~~	–	–	26 ~~mm~~
Pseudomonas aeruginosa	7 ~~mm~~	7 ~~mm~~	–	–	27 ~~mm~~
~~Fungal Strain~~					
Aspergillus niger	–	–	–	–	26 ~~mm~~
Aspergillus flavus	–	–	–	–	36 ~~mm~~

Antioxidant assay

The antioxidant activity was expressed from the IC_{50} value of all samples. Comparison of IC_{50} values of different fractions and extracts with standard was shown in ~~figure~~ **Figure 4**. 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 suggest significant antioxidant property of ethyl acetate extract[36]. Also there has been report of isolation of antioxidative carbohydrate polymer from *E fluctuans* which is the most consistent with our finding[37].

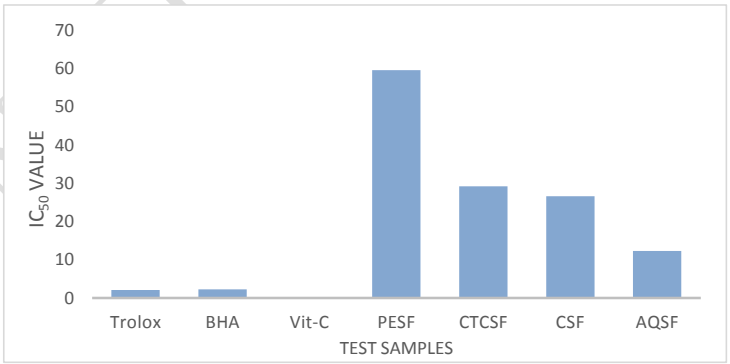

Figure 4: Comparison of LC_{50} value of different extracts of *E. fluctuans* & standards.

Cytotoxicity assay

~~In case of brine shrimp lethality bioassay, the~~The lethality of the PESF, CTCSF, CSF and AQSF fractions against *A. salina* were shown in **Table 3**. Lethality assay is proved to be suggestive of different pharmacological properties by isolation of cytotoxic, antimalarial or insecticidal compounds from the cytotoxic plant extracts[38]. Flavonoids isolated from *E. fluctuans* exhibited anticancer activity tested *in vitro* in mice[39]. There is also report of *E. fluctuans* leaves stimulating cell-mediated immune system by increasing neutrophil's phagocytic activity[40].Here, CTCSFfraction was most potent with the lowest LC_{50} value which is consistent with the existing reports on *E. fluctuans*.

Table 3: Lethal 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

Preliminary chemical screening and *in-vitro* bioactivity study data suggests *Enhydra fluctuans* potential pharmacological activity. It showed significant cytotoxic activity with good thrombolytic and anti-inflammatory potential. Further higher and detailed experiments for isolation of bioactive compounds from *Enhydra fluctuans*.

Conflict of Interest

There is no conflict of interest to declare by the authors.

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