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Phytochemical and *in vitro* biological investigations of methanolic extracts of *Enhydra fluctuans* Lour.

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PEER REVIEW

Peer reviewer

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Comments

This is a valuable research work in which authors have demonstrated three important activities of *E. fluctuans*, antioxidant, anthelmintics and thrombolytic activities. In general, people eat this herb as vegetables. Therefore, the findings of this study will encourage people to use this as medicinal plants in future.
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ABSTRACT

Objective: To study the phytochemical and biological properties (antioxidant, anthelmintic and thrombolytic) of methanolic extracts of *Enhydra fluctuans* Lour., a plant belonging to the Asteraceae family.

Methods: The phytochemical evaluation was carried out by qualitative analysis. *In vitro* antioxidant activity of extract was studied using free radical scavenging assay, ability of reduction, total phenol and total flavonoid contents determination assays. The anthelmintic activity was determined using paralysis and death time of *Pheretima posthuma* (earthworm) and thrombolytic activity by clot disruption assay.

Results: The phytochemical evaluation showed significant presence of flavonoids, triterpenes, carbohydrate, reducing sugars, saponins, phenols, diterpenes, protein and tannin. The antioxidant activity was found significant [IC_{50}=(135.20±0.56) μg/mL] as compared to ascorbic acid [(130.00±0.76) μg/mL]. The reducing power was increased with concentration. Total phenol and total flavonoid contents were (153.08±0.38) mg/mL and (172.04±0.56) mg/mL respectively. The paralysis and death time of earthworms for different concentrations of extract were determined and compared with albendazole. The results showed that 10 mg/mL of the crude extract had similar effect with albendazole. Additionally, the crude extract showed a concentration depended relationship with its anthelmintic property. The clot lysis activity of crude extract was compared to the standard streptokinase's clot lysis (40.13%) activity and found significant (31%).

Conclusions: The study proves that the crude methanolic extract of *Enhydra fluctuans* Lour. has significant antioxidant, anthelmintic and thrombolytic activity containing wide range of phytochemicals.

KEYWORDS

Enhydra fluctuans Lour., Phytochemicals, Antioxidant, DPPH radical scavenging, *Pheretima posthuma*, Streptokinase

1. Introduction

Nature has been a supply of medicative agents for thousands of years and a formidable variety of recent medications were isolated from natural sources, several of that supported their use in ancient medication. In sight of this, our attention has been targeted significantly to *Enhydra fluctuans* Lour. (*E. fluctuans*) (Family: Asteraceae), native name (helencha), edible semi aquatic nonwoody vegetable plant with separate leaves. It is native to India, Bangladesh, Burma, Srilanka and a number of other places in South East Asia[1]. This plant may be a

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prostate, spreading, annual herb. The stems are somewhat fleshy, thirty centimeters or a lot of long, branched development at the lower nodes, and somewhat bushy. The leaves are sessile, linear-oblong, three to five centimeters long, pointed or blunt at the tip, sometimes truncate at the bottom[1]. The leaves are slightly bitter, cure inflammation, skin diseases and small pox. It possesses biological value and its fuel extract has been reportable to own analgesic, cytotoxic, antimicrobial, hepatoprotective, hypotensive, CNS depressant, antidiarrheal activity[2-7].

The role of free radical reactions in disease pathology is well established. The free radicals are responsible for not only in support of aging but also many age-related diseases[8]. Free radical damage within cells has been connected to a range of disorders including cancer, arthritis, atherosclerosis, Alzheimer's disease, and diabetes[9-11]. There has been some proof to suggest that free radicals and some reactive nitrogen species trigger and increase cell death mechanisms such as apoptosis and in extreme cases necrosis[12,13]. Scientists recommend that antioxidant can reduce the activity of free radicals including their so called side effects and thus increase the cell survival times effectively[14].

Thrombosis is the fundamental patho-physiological process that underlies the acute coronary disorders which are the main causes of morbidity and mortality in developed countries. Portal vein thrombosis frequently caused by thrombus formation in vein leads to the constricting of portal vein followed by portal hypertension. Cerebral epithelial duct occlusion may be a common disorder that in the midst of vital morbidity and mortality. Clot buster medication like tissue proteinase, alteplase (Activase), reteplase (Retavase), tenecteplase (TNKase), streptokinase, urokinase *etc.* play an important role in the management of patients with cerebral epithelial duct occlusion[11].

Helminthiasis, a macro-parasitic disease, is observed in humans and animals which reflects serious social and economic problems throughout the world, especially in the third world countries. In this disease, a part of the body is infested with parasitic worms like roundworms (nematodes), tapeworms (cestodes) or flukes (trematodes)[15]. In the medical field, helminthes have been a matter of concern for centuries and they still cause considerable problems to human and other animals. World Health Organization estimates that about two billion of people throughout the world are affected by parasitic worm infection and the reason for it is associated with poor management practices and inadequate control measures[16]. Although numerous advances were made in understanding the mode of transmission and the treatment of the helminthes during the last few decades, there is still no potential product which can control specific helminthes[17].

However, indiscriminate use of several anthelmintic, antioxidant, clot buster medication has emerged problems, leading to the development of resistance as well as chemical residue and toxicity problems[18]. For these reasons, phytochemical screening of medicinal plants for their antioxidant, anthelmintic and clot lysis activity has become a matter of great scientific interest though synthetic chemicals are extensively used in modern clinical practices worldwide[19]. On the contrary, our traditional system of medicine and folklore are using the whole medicinal plant or a part of it for the treatment of all types of disease successfully including antibacterial, anthelmintic, anti-inflammatory *etc.* since the time immemorial[20]. This is because the traditional medicines act as an easily available and effective source of medicines to people with a broad spectrum of action like high percentage of cure with single therapeutic dose, cost effective and free from toxicity[21].

Thus the native use of *E. fluctuans* as medicament prompted us to research the phytochemical analysis, antioxidant, anthelmintic and clot buster activity of *E. fluctuans* that has not been explored so far. The methanolic extracts of *E. fluctuans* were evaluated for phytoconstituents, total phenolic content, total flavonoid content, the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity, ability of reduction, the anthelmintic and clot lysis activity in the present study.

2. Materials and methods

2.1. Chemicals

Lyophilized *S-Kinase*™ (streptokinase) vial (1 500 000 IU) was purchased from Popular Pharmaceuticals Ltd., Bangladesh; Batch No: VEH 09. DPPH (1, 1-diphenyl 2-picryl hydrazyl), trichloroacetic acid, gallic acid, ferric chloride and quercetin were obtained from Sigma Chemical Co. Ltd, (St. Louis, MO, USA). Ascorbic acid was obtained from SD Fine Chem. Ltd., India. All other chemicals and reagents were of analytical grade.

2.2. Plant materials

Whole plant of *E. fluctuans* was collected from Lakshmipur district, Bangladesh in July 2012. After collection whole plant were thoroughly washed with water. The plant was identified and authenticated by taxonomist of Bangladesh National Herbarium, Mirpur, Dhaka, Bangladesh (accession number-37925). The collected plant parts were separated carefully. The separated samples were then dried at room temperature in the shade and away from direct sunlight for 5 d and finally kept in hot air oven for 3 d.

2.3. Preparation of crude extract

After drying, the total plants were coarsely fine-grained (120 g) and extracted by dissolving with methanol (500 mL)

for 15 d concomitant occasional shaking and stirring. The sediments were filtered and also the filtrates were dried at 40 °C during a water bathtub. The solvent was utterly removed by filtering with Whatman paper (Bibby RE200, Sterilin Ltd., UK) and obtained dried crude extract was used for experiment.

2.4. Phytochemical screening

Testing of various chemical compounds within the extract, represents the preliminary phytochemical studies. Little amount of methanolic extracts of *E. fluctuans* was subjected to preliminary quantitative phytochemical investigation for detection of phytochemicals like alkaloids, carbohydrates, viscous glycosides, phytosterols, proteins, flavonoids, tannins, saponins, phenols, terpenes *etc.* exploiting the quality ways[22-24].

2.5. Antioxidant activity

2.5.1. DPPH radical scavenging activity

The free radical scavenging activity of the extract, based on the scavenging activity of the stable DPPH free radical, was analyzed by the method described by Braca *et al*[25]. About 2.0 mL of a methanol solution of the extract at different concentration (500 to 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 by UV spectrophotometer. The percentage inhibition activity was calculated from

Inhibition (%)=$[(A_0-A_1)/A_0]\times100$

Where, A_0 is the absorbance of the control, and A_1 is the absorbance of the extract/standard. Then the inhibition curves were prepared and IC_{50} values were calculated. BHT was used as positive control.

2.5.2. Reducing power

Reducing power of crude extract was analyzed by the method described at Srinivas *et al*[26]. The different concentrations of extract (125, 250, 500 and 1 000 μg/mL) in 1 mL of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 mol/L, pH 6.6) and potassium ferricyanide-$K_3Fe(CN)_6$ (2.5 mL, 1% w/v). The mixture was incubated at 50 °C for 20 min. A portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3 000 r/min for 10 min. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1% w/v) and the absorbance was measured at 700 nm. The increased absorbance of the reaction mixture indicates increased reducing power. Ascorbic acid was used as the reference and phosphate buffer (pH 6.6) was used as blank solution.

2.5.3. Total phenol content

Total phenolic content of methanolic extract of *E. fluctuans* was measured applying the method involving Folin-Ciocalteu reagent as oxidizing agent and gallic acid as standard[27,28]. Different gallic acid solutions were prepared having a concentration ranging from 50 μg/mL to 0 μg/mL. A volume of 2.5 mL of Folin-Ciocalteau reagent (diluted 10 times with water) and 2.0 mL of Na_2CO_3 (7.5% w/v) solution was added to 0.5 ml of gallic acid solution. The mixture was incubated for 20 min at room temperature. After 20 min, the absorbance was measured at 760 nm. After plotting the absorbance in ordinate against the concentration in abscissa, a linear relationship was found which was used as a standard curve for the determination of the total phenolic content of the test samples. In 0.5 mL of extract solution (conc. 2 mg/mL), 2.5 mL of Folin-Ciocalteu reagent (diluted 10 times with water) and 2.0 mL of Na_2CO_3 (7.5% w/v) solution was added. The mixture was incubated for 20 min at room temperature. After 20 min, the absorbance was measured at 760 nm by UV-spectrophotometer and using the standard curve prepared from gallic acid solution with different concentration, the total phenolic content of the sample was determined. The phenolic contents of the sample were expressed as mg of (gallic acid equivalent)/g of the extractive.

2.5.4. Total flavonoids content

The total flavonoids content was determined following a method demonstrated by Kumaran *et al.* using quercetin as a reference compound[29]. A volume of 1 mL of the plant extract in methanol (200 μg/mL) was mixed with 1 mL aluminium trichloride in methanol (20 mg/mL) and a drop of acetic acid, and then diluted with methanol to 25 mL. The absorbance was measured at 415 nm after 40 min. Blank samples were prepared from 1 mL of plant extract and a drop of acetic acid, and then diluted to 25 mL with methanol. The total flavonoid content was determined using a standard curve of quercetin (12.5-100 μg/mL) and expressed as mg of quercetin equivalent (QE/mg of extract).

2.6. Anthelmintic activity

The anthelmintic assay was carried out as per the method of Malvankar *et al.* with minor modifications[30]. Adult earthworms (*Pheretima posthuma*) were used to study the anthelmintic activity due to its anatomical and physiological resemblance with the intestinal roundworm parasite of human beings. Because of availability of earthworms, they are widely used as effective tools for anthelmintic study[31]. After collection, earthworms were washed with saline water. Different concentrations of test sample (10-80 mg/mL) were prepared. About 150 mg of albendazole was measured by weighing machine and dissolved in 10 mL water to make

a concentration of 15 mg/mL standard solution. A control group was established with distilled water to ensure that the test was a validate one. Earthworms were divided into eleven groups each containing three earthworms. Five groups were used to the five concentrations of methanolic extract of *E. fluctuans* One group was applied to reference standard and another to control group. For each concentration, triplets (three Petri dishes) were used, each Petri dish containing equal sized earthworm. Continuous observation was made to notice any physical change in the earthworms. The time of paralysis was recorded when no movement of any sort could be observed except the worms were shaken vigorously. Time for death of worms were recorded after ascertaining that the worms neither moved when shaken vigorously nor when dipped in warm water at 50 °C.

2.7. Thrombolytic activity

In vitro clot lysis activity of *E. fluctuans* was carried out according to the method illustrated by Dewan and Das with minor modification[32]. In the commercially available lyophilised streptokinase vial (1 500 000 IU) 5 mL sterile distilled water was added and mixed properly. This suspension was used as a stock solution from which appropriate dilution was made. Five milliliter of venous blood was drawn from the healthy volunteers (n=10) without the history of oral contraceptive or anticoagulant therapy and was distributed (0.5 mL/tube) to each 10 previously weighed sterile micro centrifuge tube and incubated at 37 °C for 45 min to form the clot. After the formation of clot, serum was completely removed 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 properly 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 separately added to the control tubes numbered. Then all the tubes were incubated again at 37 °C for 90 min and observed for clot lysis. After incubation, the obtained fluid was removed from the tubes and they were again weighed to observe the difference in weight after clot disruption. At last, difference obtained in weight was calculated and the result was expressed as percentage of clot lysis following the underneath equation.

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

2.8. Statistical analysis

All the results obtained by *in vitro* experiment were expressed as mean±SEM of three measurements following paired t-test analysis where $P<0.05$ was considered as statistically significant.

3. Results

The phytochemical screening of methanolic extract of *E. fluctuans* indicated the qualitative presence of flavonoids, triterpenes, carbohydrate, reducing sugar, saponins, phenols, diterpenes, proteins and tanins (Table 1).

Table 1

Phytochemical constituents identified in the methanolic extract of *E. fluctuans*.

Phytochemicals	Methanolic extract of *E. fluctuans*
Alkaloid	−
Flavonoids	+
Triterpenes	+
Cardiac glycoside	−
Carbohydrate	+
Reducing sugars	+
Saponins	+
Phenols	+
Diterpenes	+
Proteins	+
Tanin	+
Phytosterols	−

+: Presence, −: Absence.

The DPPH free radical scavenging activity depends on the ability of DPPH, a stable free radical, to be decolorized in the presence of antioxidants[33]. DPPH radical scavenging activity of *E. fluctuans* was found to increase with increasing concentration of the extract (Figure 1). IC_{50} value of DPPH scavenging activity was (135.20±0.56) μg/mL as compared to ascorbic acid (130.00±0.76 μg/mL). The extract also demonstrated significant reducing power which was found to increase with the increasing concentration (Figure 2). During the determination of total phenol and flavonoid content, it was observed that the amount of phenols and flavonoids was significant. Total phenol and total flavonoid contents were (153.08±0.38) mg/mL and (172.04±0.56) mg/mL respectively. The methods and results for determination of antioxidant activity were compared with other studies[34-36].

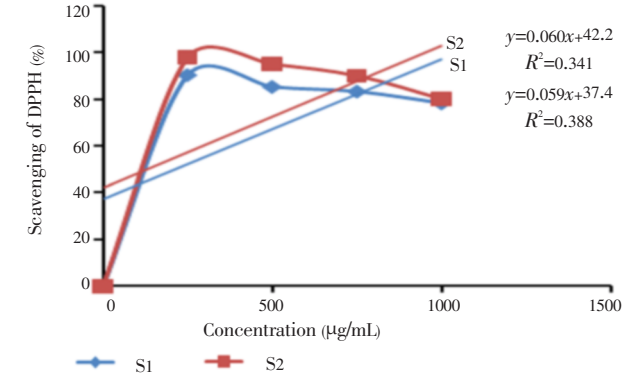

Figure 1. The free radical scavenging activity of *E. fluctuans* and ascorbic acid by DPPH.

S1=%Scavenging of *E. fluctuans* extract, S2=%Scavenging of ascorbic acid. The results are expressed as mean±SEM of three consecutive experiments.

The anthelmintic activity of methanolic extract of *E. fluctuans* on earthworms (*Pheretima posthuma*) was

determined by different concentration of extract compared with standard (albendazole) and negative control. Crude methanol extracts of *E. fluctuans* inhibited earthworms in a significant dose-dependent manner (Figure 3). The paralysis time of earthworms for extract at different concentrations, including 10 mg/mL, 20 mg/mL, 40 mg/mL, 60 mg/mL and 80 mg/mL was 33.66, 32.33, 29.00, 23.33 and 21.33 min respectively, whereas death time was 70.33, 67.66, 63.66, 36.66 and 33.33 min respectively compared with albendazole (35.33 min and 71.33 min respectively).

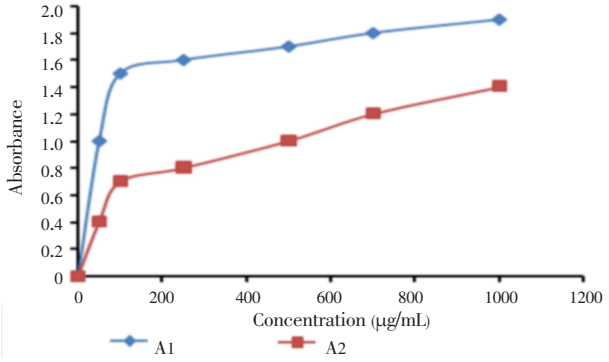

Figure 2. Reducing capacity of *E. fluctuans* extract.
A1=Absorbance of ascorbic acid, A2=Absorbance of extract. The results are expressed as mean±SEM of three consecutive experiments.

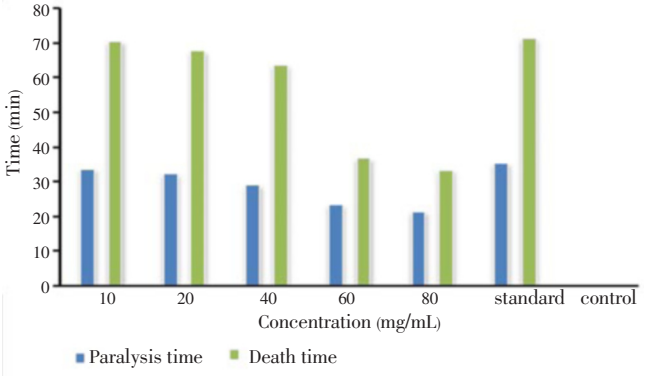

Figure 3. Paralysis and death time of methanolic extract of *E. fluctuans*.
The results are expressed as mean±SEM of three measurements.

The clot lysis activity of methanolic extract of *E. fluctuans* increased with the increase of concentration as compared to standard streptokinase's clot lysis (40.13%) activity. The concentration 10 mg/mL methanolic extract of *E. fluctuans* exhibited the highest (31%) thrombolytic activity (Figure 4). The percentage (%) of clot lysis was significant (P<0.05) when compared with control.

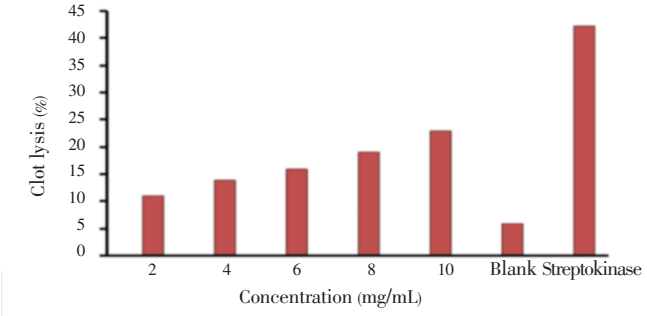

Figure 4. Clot lysis activity of different concentrate *E. fluctuans*.
The results are mean±SEM of three consecutive experiments.

4. Discussion

The phytochemical screening of methanolic extracts of *E. fluctuans* shows the presence of flavonoids, triterpenes, saccharide, reducing sugar, saponins, phenols, diterpenes, proteins and tanins. The biological activities of this plant may be due to the presence of those various groups of chemical compounds[37,38].

Antioxidants are useful to cut back and forestall injury from radical reactions by donating electrons that neutralize the unconventional while not forming another. For example, vitamin C will lose associate degree negatron to a radical and stay stable itself by passing its unstable negatron round the inhibitor molecule[39]. The methanolic extract of *E. fluctuans* has been tested for the determination of inhibitor activity. The reducing activity of a compound depends on the presence of reductors that has been exhibited antioxidative activity by breaking the radical chain, donating a chemical element atom. The screening of the inhibitor activity of this plant has proven its capability to scavenge the radical (DPPH) at low concentration, which may be due to the presence of phenol and alternative phytoconstituents in its crude extract.

The anthelmintic activity shows that methanolic extracts of *E. fluctuans* possess potent anthelmintic activity in dose dependent manner. The activity shown by this extract contains a very significant importance. The extract of *E. fluctuans* shows the highest activity that is nearly adequate to the consequences shown by albendazole solution. Albendazole acting by inhibiting fibre bundle transmission in worm could also be by acting like GABA; the repressive neurochemical in nematodes. This permits host body to simply take away out the harmful organisms. The time taken for the induction of dysfunction and death in each albendazole and *E. fluctuans* was nearly same. The extent of activity shown by crude extracts was found to be dose dependent.

Platelets play a significant role in the formation of clot by adhering to the broken regions of the epithelial tissue surface. Most thrombolytic agents work by regulating the enzyme plasminogen, which cuts the cross-linked fibrin mesh. This makes the clot soluble and leads to further proteolysis by other enzymes, and thus causes blood flow over occluded blood vessels. Thus thrombolytic agents are useful for the treatment of myocardial infarction, thromboembolic strokes, deep vein thrombosis *etc*[40]. The comparison of the positive management (streptokinase) with negative management clearly showed that clot dissolution didn't occur when water was added to the clot. Having the results of the positive management, we tend to compare five totally different concentrations of the test sample within the same manner with the negative management and discovered vital clot lysis activity. It was conjointly discovered from the study that the proportion of clot lysis is proportional to the concentration. Since phytochemical analysis showed that the crude extract contains flavonoids, triterpenes, carbohydrate, reducing sugar, saponins, phenols, diterpenes, proteins and tanins; it can be predicted that these phytoconstituents

could also be liable for its clot lysis activity[37,38].

Taking into consideration all of the findings from the current study, it will build a general comment that the methanolic extracts of *E. fluctuans* has significant antioxidant, anthelmintic and thrombolytic activity with wide range of phytoconstituents[41]. Since, this study was conducted by crude extract, any advanced studies ought to be allotted for compound isolation and it's necessary to look at that compounds are literally answerable for specific effects.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

Herbs contain various phytochemical constituents and biological properties that used to treat numerous diseases of human and animals without any side effects. As such *E. fluctuans* is an indigenous herbs in Bangladesh used as vegetables but it is not probably unexplored as medicinal plants till this study.

Research frontiers

The present research work depicts various properties of *E. fluctuans*, native name (helencha). The present study revealed various photochemical constituents in this herb and showed antioxidant, anthelmintics and thrombolytic activities *in vitro*. These properties could be tested *in vivo* animal model in future.

Related reports

Extract of *E. fluctuans* possesses high antioxidant properties which increased with the increasing concentration of extract. Paralysis and death of earth worm were revealed based on concentration of extract. Moreover, properties of thrombolytic activity would be an indication in reduction of myocardial infarction, thrombo-embolic strokes, and deep vein thrombosis *in vivo* study.

Innovations and breakthroughs

E. fluctuans is an available herbs in Bangladesh generally used as vegetables. In the present study, the authors have studied the phytochemical constituents and their action as antioxidant, anthelmintics and thrombolytic activity *in vitro*.

Applications

From the literature survey it has been found that *E. fluctuans* is safe for human health. This scientific study supports and suggests the use of this plant as vegetables would be beneficial for health.

Peer review

This is a valuable research work in which authors have demonstrated three important activities of *E. fluctuans*, antioxidant, anthelmintics and thrombolytic activities. In general, people eat this herb as vegetables. Therefore, the findings of this study will encourage people to use this as medicinal plants in future.

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