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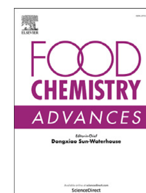

Enhydra fluctuans Lour. aqueous extract inhibited the growth of calcium phosphate crystals: An *in vitro* study

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ABSTRACT

Enhydra fluctuans Lour. is consumed by various tribes in their ethnomedicinal practices for the treatment of kidney stones and urinary problems. However, no scientific studies were conducted to evaluate its effect on crystal growth. Hence, the present study proposed to investigate the effect of aqueous extract of whole plant of standardized *E. fluctuans* (AEEF) on the growth of calcium phosphate (brushite) crystals. Attempts were also made to evaluate the effect of *in vitro* free radical scavenging and antimicrobial activities of AEEF. *In vitro* studies indicated that AEEF (50, 100, and 200 μg/mL) exhibited an inhibitory role on brushite crystal growth. The average length of the deposited brushite crystals was decreased by citric acid ($1 molL^{-1}$) and AEEF (200, 100, and 50 μg/mL) till day 8. Results showed that AEEF (200 and 100 μg/mL) and citric acid significantly ($P<0.001$) decreased the crystal bed as compared to the control on day 8 of the study. AEEF showed an antimicrobial effect against *Staphylococcus aureus* and exhibited antioxidant activity. In conclusion, the aqueous extract of the whole plant of *Enhydra fluctuans* was found effective in the inhibition of brushite crystals. Further, *in vivo* studies along with molecular studies and bioactivity-guided fractionation are required to strengthen its antilithiatic effect along with identifying the bioactive compounds and the mechanism of action involved therein.

1. Introduction

The accumulation of stones in the kidney and urinary system is known as urolithiasis which has affected about 10 to 12 percent of the world population (Ghodasara et al., 2010). The renal stones may be divided into two subtypes *i.e.* calcareous stones which include calcium oxalate and calcium phosphate and the other subtype type is non-calcareous stones which include uric acid, and struvite (infection stone by protease bacteria), cystine, and uric acid stones (Parmar, 2004). Free radicals/ROS are implicated in several infectious conditions like urolithiasis (Boonla, 2018) and urinary tract infections (Kurutas et al., 2005), etc. They are generated either due to microbial actions or the biochemical reactions in the body. These molecules further aggravate the disease conditions leading to permanent pathologies. The availability of modern medicine in urolithiasis is sparse and hence, due to the scarcity of modern medicine and the occurrence of enormous adverse effects and side effects of the current limited antilithiatic agents like thiazide or alkali-citrate, the research on traditional medicine especially in kidney stones is only the choice which is rising tremendously in the last decade. Previous studies showed the beneficial effects of many herbal drugs on urolithiasis as revealed in various experimental studies (Sharma et al., 2016; Sikarwar et al., 2017). Though traditional and folkloric medicines are highly efficacious, their uses are limited due to a lack of knowledge and scientific evidence for their probable molecular mechanism due to the presence of multi constituents.

Enhydra fluctuans Lour. (Synonym: Helencha) belongs to the family Asteraceae, is a well-known plant is specifically a tropical and subtropical plant and mainly found in India, Bangladesh, Srilanka, Burma, and other southeast Asian countries. Previous studies revealed that *E. fluctuans* possesses antioxidant, analgesic anti-inflammatory, anti-cancer, anti-diarrheal, anthelmintic, antimicrobial, CNS depressant, cytoprotective, hepatoprotective, and thrombolytic activities (Ijaz & Roy, 2014). In ethnomedicinal practices, the decoction of the whole plant of *E. fluctuans* is consumed by various tribes of the Northeast region of India and Bangladesh for the treatment of kidney stones and urinary problems (Lokendrajit et al., 2011; Ahmed et al., 2016, 2017). Recently, the network pharmacology studies predicted the mechanisms of *E. fluctuans* on amelioration of nephrolithiasis (Chattaraj et al., 2023a) while the *in vitro* studies revealed that *E. fluctuans* aqueous extract exhibited an inhibitory effect on calcium oxalate crystallization (Chattaraj et al., 2023b). However, no scientific studies were carried out to delineate its influence on

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calcium phosphate crystals which are also predominantly present in patients with kidney stones. Hence, the present study investigated the effect of an aqueous extract of *E. fluctuans* (AEEF) on *in vitro* growth of calcium phosphate (brushite) crystals. As oxidative stress and microbes play an important role in the pathogenesis of urolithiasis, hence, in the present study attempts were also made to evaluate the effect of *in vitro* free radical scavenging and antimicrobial activities of AEEF.

2. Materials and methods

2.1. Drugs and chemicals

The important chemicals which were used throughout the experimentation are sodium metasilicate (Thermo Fisher Scientific India Pvt. Ltd., India, ≥97%), orthophosphoric acid (S. D. Fine-Chem Ltd., India, 85%), citric acid (Qualikems Fine Chem Pvt. Ltd., India, 99%), calcium chloride dihydrate (Molychem Lab Chemical, India, 98%), Folin-Ciocalteu reagent (Sisco Research Laboratories Pvt. Ltd., India, 2 N), gallic acid (Merck Life Science Pvt. Ltd., India, ≥98.0%), sodium carbonate (HiMedia Laboratories Pvt. Ltd., India, 98.5%), aluminum chloride anhydrous (Finar Limited, India, 98.0%), 1,1-diphenyl-2-picryl-hydrazil (Sisco Research Laboratories Pvt. Ltd., India, 95%). All other chemicals which were used for rest of the experimentation were also of the highest purity grade.

2.2. Collection and processing of the plant material

The plant material was collected in the month of November 2021 from the local market of Durgapur, West Bengal, India. The plant material was identified by a Taxonomist, Dr. R. K. Gupta, Scientist-E, Central National Herbarium, Botanical Survey of India (BSI) and it was identified as *Enhydra fluctuans* Lour. belonging to the family asteraceae. A voucher specimen (BC-01) of the authenticated plant was deposited on dated 12/01/2022 in the herbarium of BSI. The plant material was dried under shade at 25±2 °C and ground into fine powder for further experimentation.

2.3. Extraction of Enhydra fluctuans

In view of the use of the decoction of the fresh whole plant of *E. fluctuans* in ethnomedicinal practices, the decoction method was used for extracting the aqueous extract from the fresh plant materials. The extraction conditions were established by the preliminary tests. The whole plants (roots, leaves, and stems) of *E. fluctuans* were washed to eliminate soil and other contaminants. The plant materials were cut into small pieces to decrease the particle size. 20 g from it was taken into a beaker containing 400 mL of distilled water and subjected to decoction for 4 h at 100 °C. After 4 h, the extract was filtered by using Whatman filter paper no.1 and the filtrate was dried in a water bath at 100 °C to obtain aqueous extract (AEEF). The percentage yield was calculated and the dried extract was kept in a desiccator for future use.

2.4. Standardization of Enhydra fluctuans

2.4.1. Microscopic evaluation

The leaves and stem of *E. fluctuans* were subjected to microscopic evaluation by using standard methods (Charoensup et al., 2017; Alam & Saqib, 2015). For the preparation of thin transverse sections of the different plant parts such as leaves, stem, and root, fresh plants were collected and washed thoroughly with water. Thin sections from the middle portion of the lamina were cut to obtain a transverse section of the leaves while, thin sections from the stem and roots were cut and placed in water so that they can retain moisture. The thinnest sections were chosen from individual parts and mounted on glass slides with glycerine. The sections were stained with safranin wherever necessary and were covered with the help of coverslips. The sections were observed under a Magnus LX 10 microscope fitted with a camera for the identification of diagnostic characteristics.

2.4.2. Powder microscopy

Powder characterization of the leaves, root and stem of the plant was carried out as per the procedure mentioned in the previous study (Aeri et al., 2019) to identify probable diagnostic characteristics. Leaves, stem, and roots of the plant were separated and dried under shade. Upon drying, the dried parts were powdered and passed through sieve no. 25. The powders from the different parts of the plant were stored in air-tight containers for study. After the preparation of powder, a pinch of powder was taken on a glass slide and mounted with glycerine. The powder sample was covered with cover slip and observed under 10 × 10 magnification for probable identifying characteristics.

2.4.3. Fluorescence analysis

The Fluorescence analysis was done by mixing the powdered crude drug of *E. fluctuans* in various reagents like HCl, H_2SO_4, HNO_3, $FeCl_3$ and picric acid and seen in UV chamber (Make-Mazumdar Enterprises, India; Model-UV CABINET) at 254 nm, 366 nm, and visible light. The method of Chase and Pratt (1949) carried out fluorescence analysis.

2.4.4. Physicochemical parameters

The various physicochemical parameters like ash values, extractive values, and moisture content were determined by standard methods (Khandelwal, 2006).

2.5. Preliminary phytochemical screenings

The preliminary phytochemical screening of AEEF was carried out by various preliminary phytochemical tests (Kokate et al., 2002; Khandelwal, 2006) mentioned in Table 1.

2.6. Quantitative estimation of phytoconstituents

The total phenolic content (TPC) and the total flavonoid content (TFC) of AEEF were estimated by spectrometric methods. Briefly, for the estimation of TPC, 1 ml of Folin-Ciocalteu's reagent, previously diluted (1:20), and 1 ml of AEEF (1000 µg/ml) were mixed. To this mixture, 4 ml of sodium carbonate (75 g/l) and 10 ml of distilled water were added and the resultant solution was allowed to stand for 2 h at 25 °C and further centrifuged at 2000 × g for 5 min. The absorbance of the supernatant was taken at 760 nm. A standard curve was obtained using various concentrations of gallic acid. Results were expressed as mg of gallic acid equivalents per gram of AEEF (Singleton et al., 1999; Dey et al., 2016). TFC of AEEF was estimated in colorimeter (Marinova et al., 2005). AEEF (1 ml), distilled water (4 ml), $NaNO_2$ (5%, 0.3 ml) were mixed properly. 5 min later $AlCl_3$ (10%, 0.3 ml) and NaOH (1 M, 2 ml) were added to the mixture and further diluted up to 10 ml with distilled water. The absorbance resultant solution was taken at 510 nm. A standard curve was obtained using various concentrations of quercetin. Total flavonoid content of the extracts was expressed as mg of quercetin equivalent per gram of AEEF (Marinova et al., 2005).

2.7. In vitro *growth assay of brushite crystals*

This was performed by single diffusion of gel according to the previously described method (Joshi et al., 2005) and some modifications have been included. In this case, a single diffusion gel method was carried out to form calcium hydrogen phosphate dihydrate (CHPD). Citric acid solution with different concentrations was added into the supernatant liquid when those crystals achieved maximum growth to study the inhibitory effect. Glass test tubes of 2.5 cm diameter and 15 cm length were used to grow the crystals. Five millilitres of sodium metasilicate

Table 1
Phytochemical screening of Enhydra fluctuns Lour.

Phytoconstituents	Phytochemical Test	Inference	AEEF
Carbohydrates	Molish's test	Formation of violet ring at junction	+
Proteins	Biuret test	Appearance of violet color	+
	Xanthoproteic test	Formation of white precipitate	
Amino acids	Ninhydrine test	Appearance of Purple color	+
Alkaloids	Dragendorff's test	Formation of orange color precipitate	–
	Wagner's Test	Formation of reddish brown precipitate	
Glycosides	Keller-killiani test	Formation of reddish brown color at junction	–
Saponins	Foam Test	Formation of foam	–
Sterols (Phytosterols)	Salkowski test	Appearance of red color in chloroform layer while greenish yellow in acid layer	+
Tannins and Phenolic compounds	Ferric chloride test	Appearance of bluish black color	+
	Lead acetate test	Formation of white precipitate	
Flavonoids	Shinoda Test	Formation of reddish to pink color	+

±=present; -= absent.

solution of specific gravity 1.06 were acidified by adding the perfect required amount (2.7 mL) of orthophosphoric acid to obtain a pH to 5.0 in the mixture, which was subsequently transferred to different test tubes. Just after gelation, 10 mL of 1 M aqueous solution of calcium chloride was poured with proper attention on the set gels. Within two days of pouring the supernatant liquids, the crystals were identified in rapid growth. Elongated platelet-type and star-shaped crystals were found in the gel. The obvious length of growing crystals was measured in different time intervals under the microscope. The plot of growing crystals *versus* time showed that the crystals grew at their maximum length on the 5th day of pouring a supernatant solution to set gel. Next aqueous solution of citric acid as a standard and AEEF (50, 100, and 200 μg/mL) was poured in identical volumes as on the 5th day as calcium chloride solution on the 5th day acquire maximum length of CHPD crystals and their growth was studied up to the 8th day. The photograph of the growing crystals was recorded.

2.8. Assessment of antimicrobial activity

2.8.1. Isolation of UTI-causing bacteria

Bacteria used for the microbial assay was collected from The Mission Hospital are *E. coli, Staphylococcus aureus*, Pseudomonas spp., Klebsiella spp. and *S. typhi* having species numbers U713, 394, P145, 81C, 2313U_748, respectively. Then challenge bacteria were inoculated in nutrient broth.

2.8.2. Disk diffusion method

The antimicrobial activity was evaluated by using the disk diffusion method (Bauer et al., 1966) According to the manufacturer's instructions, a commercially available dehydrated base was used for the preparation of Muella Hinton Agar (MHA). Just after autoclave procedure, it was allowed to cool at 45–50 °C by using a water bath. After that, the freshly prepared and previously cooled medium was poured into the flat bottom glass petridish and standing time for cooling was given and those were stored in the refrigerator if it has to be stored for a further day.

2.8.3. Preparation of antibiotic stock solution

Gentamicin was used as a standard antibiotic. Sterile glass wares were used for the preparation of stock solutions of antibiotics by the help of the formula mentioned below

$$(1000/P) \times V \times C = W$$

Where, P= potency of the antibiotic base, V= required volume in mL, C= final concentration of solution and W= weight of the antimicrobial to be dissolved in V

2.8.4. Preparation of dried filter paper discs

For the preparation of discs, Whatman filter paper no. 1 was used and the diameter was 6 mm each and they were sterilized in a hot air oven by placing them in a petridish. Gentamicin and AEEF (10 mg per disk) were impregnated with respective concentrations by a pipette and dried in a hot air oven. Inoculation of test plates A sterile cotton swab was dipped into the inoculum suspension solution within 15 min after adjusting the turbidity. The swab was rotated for several times and was pressed above the fluid level to get rid of excess inoculums. The swab was streaked all over the sterile agar and thus the dried MHA plate was inoculated. The procedure was repeated for two more times the plate was rotated at 60 each time to determine even distribution of inoculum. Finally, the rim of the agar was swabbed. Before incorporating drug-impregnated disks, the lid was allowed to remain open for 3 to 5 min to absorb moisture. Application of disks to inoculated agar plates as per the method and the impregnated discs of antibiotics and AEEF were dispensed into the surface of the agar plate. Every disk was pressed down to make sure perfect contact with the sterile agar. Then they were placed in an incubator for 35 °C for 15 min.

The incubation was carried out for 16–18 h. Next, the examination was done. By using the zones of measuring scale, the diameter of complete inhibition was determined. Zones were measured to the nearest whole millimeter, a sliding caliper or a ruler was used, that was held on the back of the inverted petridish. a black and non reflecting background was used.

2.9. DPPH (1,1-diphenyl-2-picryl-hydrazil) free radical scavenging activity

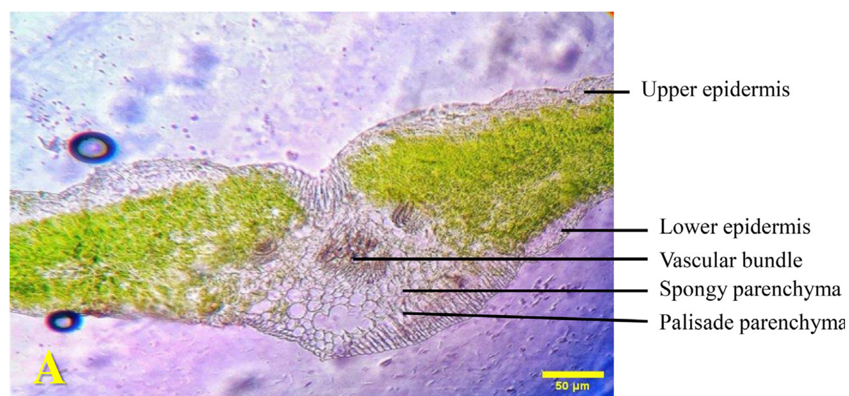
The free radical scavenging activity of AEEF was measured by *in vitro* DPPH method as previously described (Brand-Williams et al., 1995). Briefly, 0.1 mmolL^{-1} solution of DPPH in ethanol was prepared and 3.5 mL of the solution was added to 0.5 mL of different concentrations (200–1000 μg/mL) of AEEF in distilled water. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. The absorbance of the resulting solution was measured at 517 nm. Ascorbic acid was taken as standard antioxidant. The percent DPPH scavenging effect was calculated using the following equation:

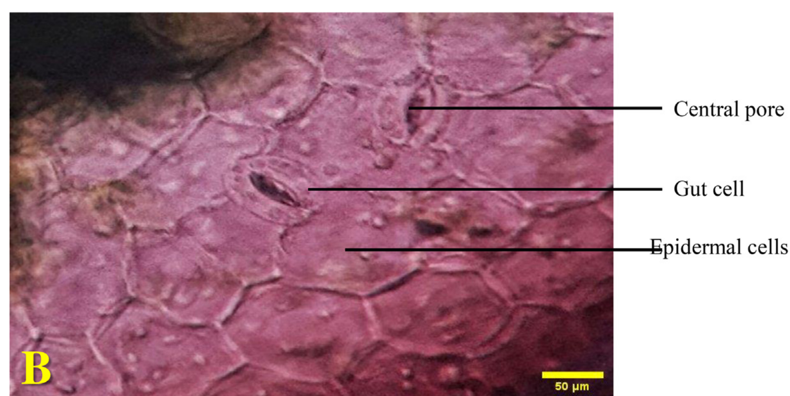
$$\text{DPPH scavenging effect (\%)} = [(A_0 - A_1)/A_0] \times 100$$

Where, A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the test.

2.10. Statistical analysis

The results were analyzed by one way ANOVA, linear regression analysis wherever necessary. The $p<0.05$ was considered significant in all cases.





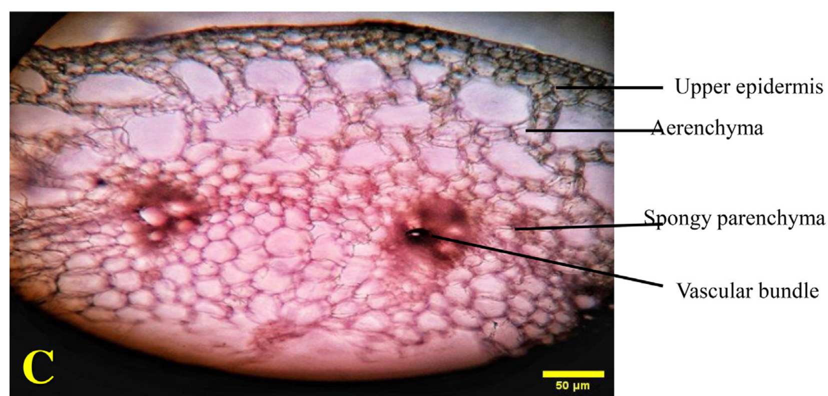


Fig. 1. Transverse sections different parts of *Enhydra fluctuans.*
[*A*=*Leaf, B*=*stem*, C=*Anomocytic stomata*], magnification 400X.

3. Results

3.1. Identification of the plant

The plant material was identified by a taxonomist, R. K. Gupta, Scientist-E & Head of office, Central National Herbarium, Botanical Survey of India. A voucher specimen (BC-01) of the authenticated *Enhydra fluctuans* Lour has been deposited in the herbarium of BSI.

3.2. Microscopy of plant parts

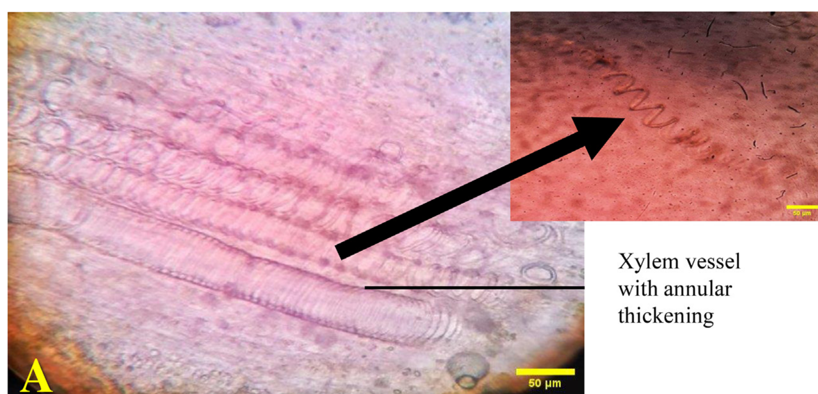
The transverse section of the leaves of the plant showed the presence of the upper and lower epidermis along with the palisade and spongy parenchyma (Fig. 1A). The section from the leaves have also revealed the presence of vascular bundles (Fig. 1A). Peeling of the epidermis from the lamina of the leaves revealed the presence of anomocytic stomata with thickened inner walls of the guard cell (Fig. 1B). The transverse section of the stem had revealed the presence of spongy parenchyma and aerenchyma (Fig. 1C).

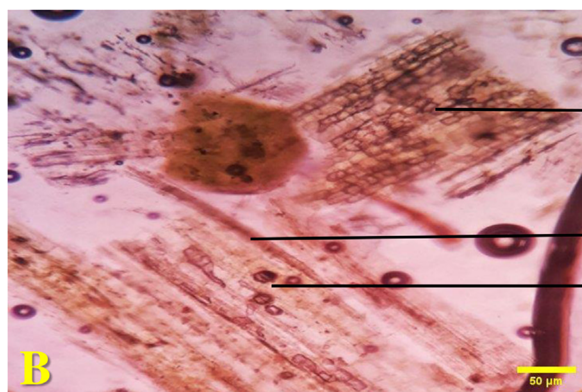
3.3. Powder characteristics

Powder characterization of the leaf of *E. fluctuans* reveal the presence of different diagnostic characteristics which include xylem vessels with annular thickening. Xylem fibres, calcium oxalate crystals and cork cells were also identified by powder microscopy of the stem while powder microscopy of the roots revealed the presence of fibres (Fig. 2).

3.4. Fluorescence analysis

The fluorescence analysis revealed the formation of various colours upon exposure of *E. fluctuans* with the various reagents which are depicted in Table 2.





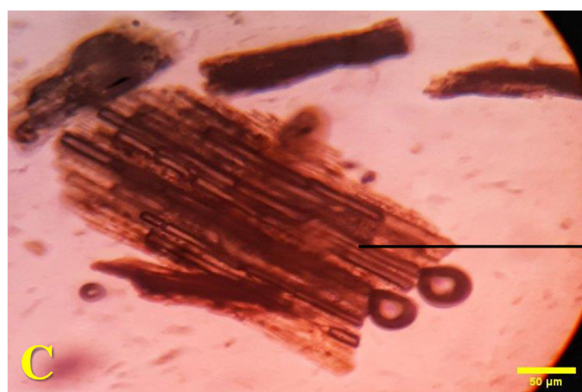


Fig. 2. Powder microscopy of different parts of *Enhydra fluctuans*.
[*A*=*Leaf, B*=*stem*, C=*root*], magnification 400X.

Table 2
Fluorescence analysis of powdered whole plant of *Enhydra fluctuans*.

Sample	Color observed		
	Visible	254 nm	366 nm
Powder	Deep green	Blackish green	Black
Powder + HCl	Yellowish green	Greenish yellow	Bluish black
Powder + H_2SO_4	Greenish brown	Blackish brown	Bluish black
Powder + HNO_3	Deep yellow	Light green	Grayish
Powder + $FeCl_3$	Yellowish green	Greenish yellow	Black
Powder + picric acid	Yellow	Greenish yellow	Bluish black

3.5. Physicochemical evaluation of Enhydra fluctuans

Moisture content, ash values, and extractive values of *E. fluctuans* were depicted in Table 3.

3.6. Preliminary phytochemical screening of Enhydra fluctuns

The AEEF of dark green color and dry sticky consistency was obtained with a percentage yield of 18%. AEEF showed presence of proteins, carbohydrates, saponins, amino acids, flavonoids, sterols, tannins, while alkaloids and glycosides were absent (Table 1).

3.7. Quantitative estimation of phytoconstituents

The TPC and TFC of AEEF were found to be 59.25 mg gallic acid equivalents/g and 472.6 mg quercetin equivalents/g of extract.

3.8. Effect of AEEF on growth assay in single diffusion gel growth method of Brushite crystals

The growth of calcium phosphate (Brushite) crystals was measured as length (thickness of crystal deposition). The crystals acquired maximum length (approximately 1.9 cm) on day 5 after gelatin took place

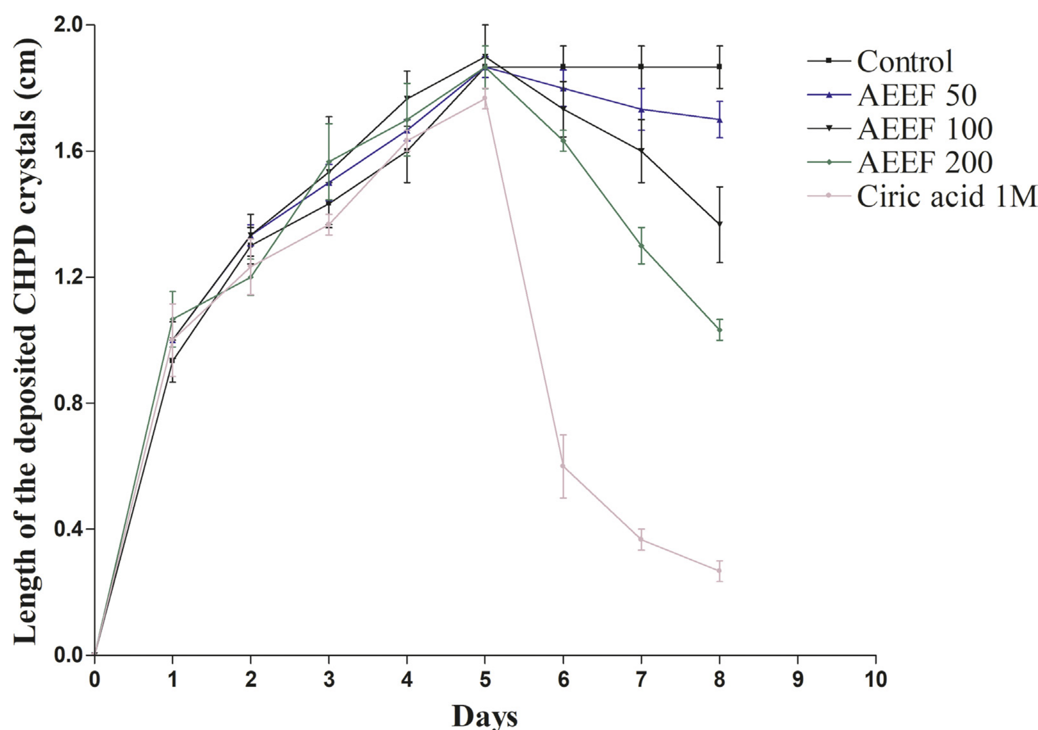


Fig. 3. Effects of citric acid and aqueous extract of *Enhydra fluctuans* on length of deposited Brushite crystals. Results are expresses as mean±SEM.

Table 3
Physicochemical evaluation of *Enhydra fluctuans*.

Standardization parameters	Value (%w/w)
Moisture content	10.667±0.5774
Total ash value	16.667±0.5774
Acid insoluble ash value	11±1
Water soluble ash value	9.333±0.471
Water soluble extractive value	18±1
Methanol soluble extractive value	17±1.73
Ethanol soluble extractive value	2 ± 0
Acetone soluble extractive value	2 ± 0
Chloroform soluble extractive value	1.33±0.577
Petroleum ether soluble extractive value	3 ± 0

Results are expressed as mean ± SD, here $n = 3$.

Table 4
Free radical scavenging activity of AEEF.

Treatment	Concentration (μg/ml)	% DPPH inhibition	IC_{50} value
AEEF	200	18.86±0.148	**898.88 μg/ml**
	400	31.55±1.122	
	600	38.29±0.899	
	800	46.19±4.666	
	1000	53.29±1.244	
Ascorbic acid	15	6.17±0.045	**57.21 μg/ml**
	30	31.55±0.682	
	60	50.30±0.045	

Values are mean ± SD, ($n = 3$), IC_{50}= 50% Inhibitory concentration.

and then after the length of the crystals deposited became constant up to day 8. The average length of the deposited CHPD crystals was decreased by citric acid (1molL^{-1}) and AEEF (200, 100, and 50 μg/mL) till day 8. On day 8, the length of deposited crystals in control and citric acid (1molL^{-1}) treated tubes were found to be 1.87 cm and 0.27 cm, respectively while the length of deposited crystals in AEEF (200, 100, and 50 μg/mL) treated tubes were 1.03 cm, 1.37, 1.7, and 0.133, respectively. Results showed that AEEF (200 and 100 μg/mL) and citric acid significantly (P<0.001) decreased the crystal bed as compared to the control on day 8 of the study. Figs. 3 and 4 showed the effects of citric acid and AEEF on the length of deposited CHPD crystals while Fig. 5 showed the inhibitory effect of AEEF on the microscopic length of crystals.

3.9. Assessment of antimicrobial activity

Results revealed that AEEF (10 mg/mL) caused inhibition of the growth of *S. aureus* while it did not cause any effect in the growth of any other bacteria. The diameter of zone of inhibition of AEEF (10 mg/mL) and gentamicin are 23±0.8 and 26±0.82 mm, respectively.

3.10. DPPH scavenging assay

In this study, AEEF in the concentration range of (200–1000 μg/ml) inhibited DPPH radical scavenging as indicated by a concentration-dependent decrease in the purple color of the solution. A similar effect was obtained with the standard antioxidant, Ascorbic acid in the concentration range of 15–60 μg/mL. The linear regression coefficient of AEEF and ascorbic acid were $r^2 = 0.971$ and $r^2 = 0.9250$., respectively, suggested that the DPPH scavenging was concentration dependent. The IC_{50} value of AEEF and ascorbic acid, obtained from regression analysis, were 898.88 and 57.21 μg/mL, respectively. The results were expressed in Table 4.

4. Discussion

In the current scenario, the treatment for urolithiasis has become a severe concern because kidney stones are usually recurrent. Some diuretics like thiazides and alkaline citrate reverse the process of kidney stones, however, their uses are limited due to less efficacy (Sharma et al., 2016). Though some modern techniques like surgery through endoscopy and shock wave therapy are used to take out the stones, however, their

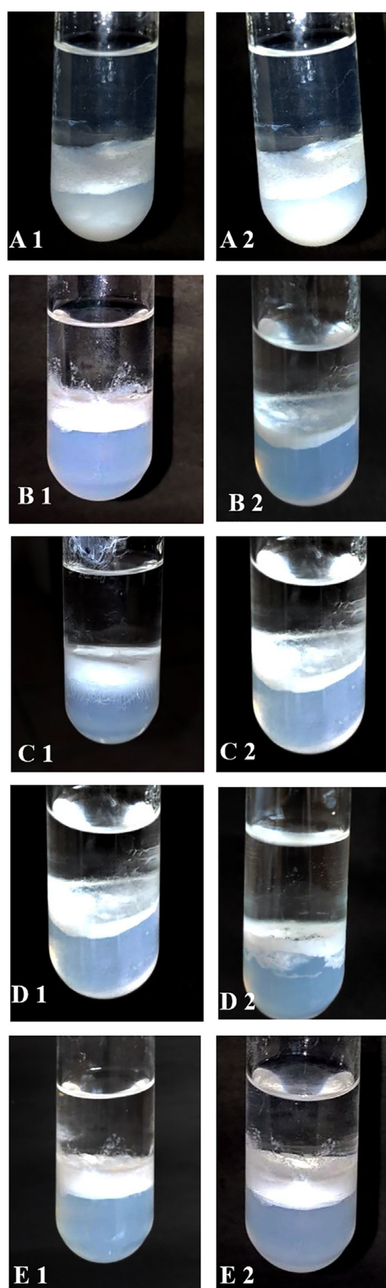


Fig. 4. Effect of citric acid and aqueous extract of *Enhydra fluctuans* on the growth of Brushite crystals.
[A1= *control* (5*th day*), A2= *control* (8*th day*), B1= *before treatment of citric acid* (5*th day*), B2= *after treatment of citric acid* (8*th day*), C1= *before treatment of* ***aqueous extract of Enhydra fluctuans*** 200 μg/mL (5*th day*), C2= *after treatment of* ***aqueous extract of Enhydra fluctuans*** 200 μg/mL (8*th day*), D1= *before treatment of* ***aqueous extract of Enhydra fluctuans*** 100 μg/mL (5*th day*), D2= *after treatment of* ***aqueous extract of Enhydra fluctuans*** 100 μg/mL (8*th day*), E1= *before treatment of* ***aqueous extract of Enhydra fluctuans*** 50 μg/mL (5*th day*), E2= *after treatment of* ***aqueous extract of Enhydra fluctuans*** 50 μg/mL (8*th day*)].

repeated uses may cause the formation of new stones as well as kidney injuries and failure (Kalyan et al., 2009.

In the present study, the standardization of the whole plant (root, leaf and stem) of *E. fluctuans* was conducted by estimating the various physicochemical constants like ash and extractive values, loss on drying and pH, fluorescence analysis, microscopic analysis) which have a major role in assessing the quality of herbal raw materials. The rise in ash and altered extractive values may be an indicator of adulteration or wrong handling of crude drugs (Dey et al., 2020). Extractive values determine the nature of chemical constituents present in the plant material. The high yield of water and alcohol extractives and low petroleum ether, chloroform, and ethyl acetate extractives in *E. fluctuans* indicates the abundance of polar constituents in the plant. The results of preliminary phytochemical screening of AEEF showed the presence of polar constituents like carbohydrates, saponins, flavonoids, and phenolics which is correlated with the high water and alcoholic extractive values. It suggests that the aqueous and alcoholic extracts may be the choice for assessing pharmacological effects. In ethnomedicinal practices, the decoction of the fresh whole plant of *E. fluctuans* is consumed by various tribes of the Northeast region of India and Bangladesh for the treatment of kidney stones and urinary problems (Lokendrajit et al., 2011; Ahmed et al., 2016, 2017). The current findings for the selection of the polar extract correlate with the traditional used form of the plant which is used in ethnomedicinal practices. Fluorescence analysis and microscopic analysis are vital techniques that suggest the nature of the phytochemicals present in herbal drugs (Alam & Saqib, 2015). The powder drug analysis was performed when treated with various chemical reagents and observations were made in visible light and UV light of short and long wavelengths. The results of the transverse section of the leaves and stems of *E. fluctuans* showed the presence of palisade, spongy, parenchyma, and aerenchyma cells justifying the plant's ability to survive in an aquatic environment. The section from the leaves also revealed the presence of vascular bundles which was more clearly evident from the results of powder microscopy. The peeling of the epidermis from the lamina of the leaves revealed the presence of an anomocytic stomata with thickened inner walls of the guard cell. The standards estimated in the present study may be useful for the standardization of *E. fluctuans* in future studies to obtain reproducible results.

In view of the preliminary phytochemical analysis and the traditional use of a decoction of *E. fluctuans*, in the present study, the aqueous extract was prepared by a decoction process. In the present study, results indicated that AEEF has an inhibitory role on the brushite crystals growth and also showed diminishing the thickness of the crystal bed which indicates the dissolution of the formed crystals. Further, the microscopic photographs of the deposited crystals indicate a decrease in the microscopic thickness of the crystals as compared to the control. The inhibitory effect of AEEF is comparable to citric acid. The results of the inhibitory role of citric acid are in accordance with the previous finding (Sharma et al., 2016). Hence, the results show that AEEF has a considerable impact on the growth and dissolution of brushite crystals, and further suggest that AEEF may be advantageous to prevent the formation of crystals and their growth.

Though the studies on the exact mechanisms involved in the inhibition of *brushite* crystallization are sparse, however, the various plant extracts were shown to have an inhibitory role in it due to their phytoconstituents (Sharma et al., 2016; Joshi et al., 2005). In previous studies, flavonoids are reported to inhibit crystal formation (Zhong et al., 2012) and deposition (Noorafshan et al., 2013). Saponin-rich fractions from *H. hirsute* are also reported to have an inhibitory role in crystallization (Fouda et al., 2006). The phenolics from various plants are also reported to inhibit the crystallization process (Byahatti et al., 2010; Ahmed et al., 2018; Sharma et al., 2016). In the present study, the results of the preliminary phytochemical screening of AEEF showed the presence of saponins, flavonoids, and phenolics while the results of quantitative estimation revealed the high content of flavonoids and phenolics. Hence, the flavonoids, phenolics and saponins which are present in *E. fluctuans* may act as contributors for the inhibitory action of AEEF in brushite crystal formation.

Bacteria are known to be involved in the development of brushite crystals (Schwaderer & Wolfe, 2017). Antibacterial activity was assessed using disk diffusion assay where effect of different concentration AEEF was tested against the various bacteria which may grow during kidney stones and urinary problems. The effect of the AEEF was compared with standard antibiotics gentamicin. These studies support the observed an-

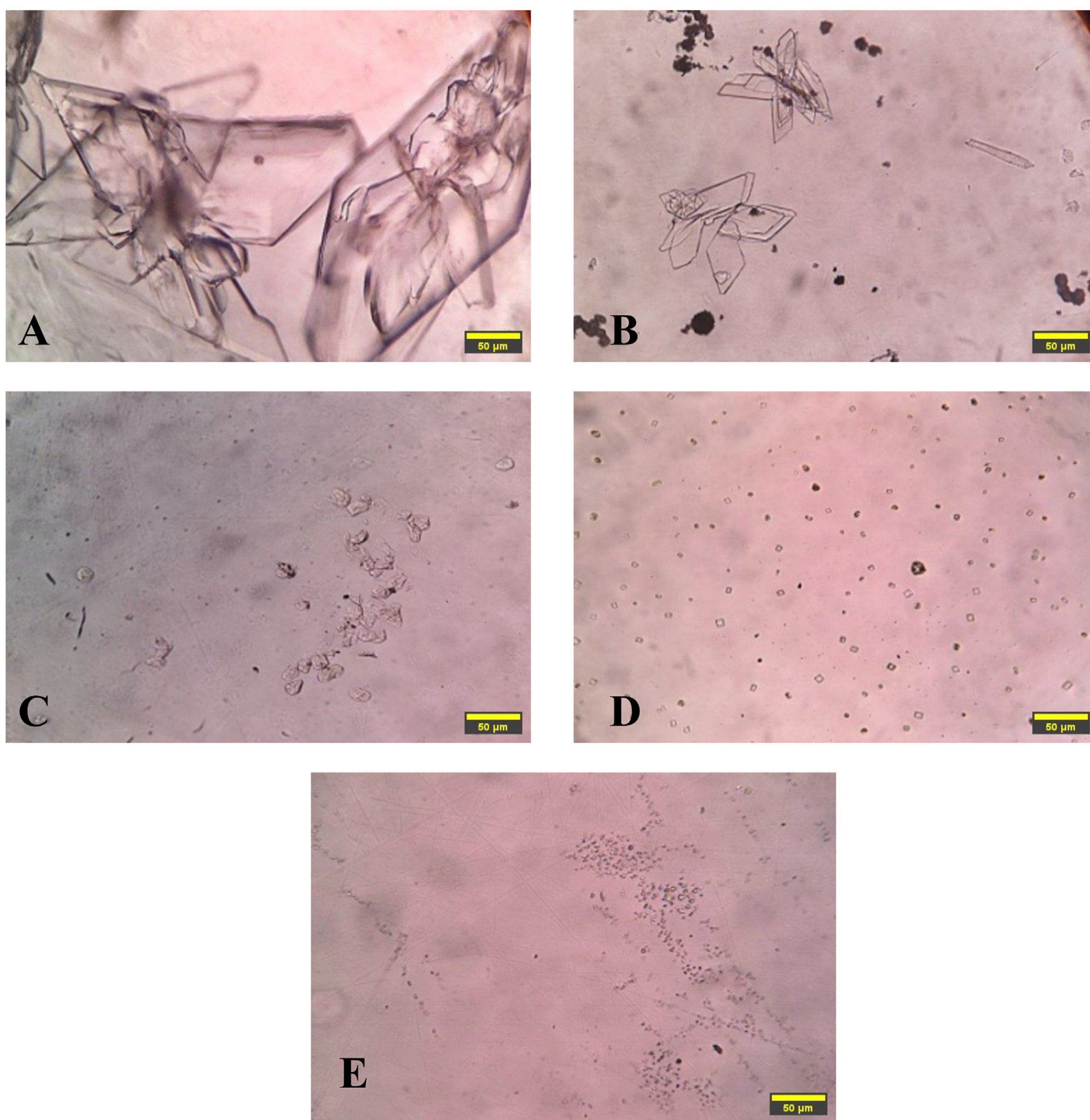


Fig. 5. Effect of Citric acid and AEEF on the size of crystals.
[A= C*ontrol, B=* ***aqueous extract of Enhydra fluctuans*** (50 μg/mL), C= ***aqueous extract of Enhydra fluctuans*** (100 μg/mL), *D=* ***aqueous extract of Enhydra fluctuans*** (200 μg/mL) *and* E= C*itric acid*], magnification 400X.

tibacterial action of AEEF. The antimicrobial activity of the AEEF may be attributed to its flavonoid and phenolic contents. The various bioactive phytoconstituents like flavonoids, phenolics and tannins have been demonstrated to show antimicrobial potential. Thus, the antibacterial activity of the AEEF may be attributed to the presence of these constituents. Hence, the antibacterial property of AEEF may be helpful for preventing infection during urolithiasis and urinary diseases.

The pathology behind calcification during nephrolithiasis is elicited by free radicals (Khan, 2014). Hence, the free radical scavenging activity of AEEF was assessed in the present study. The antioxidant potential of antioxidants is generally attributed to various mechanisms, among which are the prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and, radical scavenging (Kumar et al., 2012). In the present study, the antioxidant activity of the extract was evaluated by studying its DPPH free radical scavenging. DPPH is stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Gulcin et al., 2003). The DPPH scavenging effect was compared with ascorbic acid as the standard antioxidant. The IC_{50} value of both AEEF and ascorbic acid suggests that AEEF is fifteen times less potent than that of ascorbic acid. As AEEF is a crude aqueous extract, it is obvious that very strong% DPPH scavenging is not expected as that of ascorbic acid. However, this indicates the free radical scavenging potential of the AEEF. The antioxidant potential of AEEF may be attributed to the presence of flavonoids and phenolics as revealed in phytochemical screening and quantitative estimation of phytoconstituents of AEEF. Polyphenolic compounds, like flavonoids and phenolic acids, commonly found in plants have been re-

ported to have multiple biological effects, including antioxidant activity (Brown & Rice-Evans, 1998; Kahkonen et al., 1999) and supports the present findings. The antioxidant property of AEEF may be beneficial for the reversal of oxidative damage in patients of kidney stones.

The present findings validate the ethnomedicinal use of the decoction of the whole plant of *E. fluctans* in the treatment of kidney stones and urinary problems. In order to substantiate its *in-vitro* effect, further *in-vivo* studies in experimental animals and the identification of bioactive compounds of *E. flctuans* need to be carried out in future.

5. Conclusion

In conclusion, the aqueous extract of the whole plant of *E. fluctuans* Lour. exhibited an inhibitory effect on the growth of brushite crystals. Further, *in vivo* studies along with molecular studies and bioactivity-guided fractionation are required to strengthen its antilithiatic effect along with identifying the bioactive compounds and the mechanism of action involved therein.

Authors' contribution

Yadu Nandan Dey contributed to the generation of concept, literature search, drafting of manuscript and supervision of the research work. Bornika Chattaraj and Arijit Nandi contributed significantly to the *in vitro* studies and drafting of the manuscript. Anwesha Das contributed significantly to the literature review and preparation of the manuscript. Arun Baidya Sourav Mahata and Aritra Chowdhury assisted in the *in vitro* studies. Soumya Mitra contributed in the microscopic analysis of plant parts while Sumon Roy and Subrata Chakraborty contributed in the antimicrobial assay. All authors of this manuscript have read and approved the manuscript for submission.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Data Availability

No data was used for the research described in the article.

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