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(RESEARCH ARTICLE)

Phytochmical analysis and antibacterial property assessment of helencha (*Enhydra fluctuans*; Family: Asteraceae) extracts

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Abstract

This communication stands for the phytochemical analysis, and antibacterial activity of *Enhydra fluctuans*, alone and in combination with some antibiotics, against gram-negative and gram-positive bacteria. Following disc diffusion, the methanolic extract of *E. fluctuans* (MEE) had antibacterial activity against gram-positive (*B. cereus* and *L. monocytogenes*) and gram-negative (*A. baumannii* and *P. aeruginosa*) clinical as well as standard (*E. coli* ATCC 25922 and *L. monocytogenes* MTCC 657) bacterial strains, displaying ZDI (zone of the diameter of inhibition) values 12 – 24 mm. The aqueous extract of *E. fluctuans* (AEE) showed no activity against *P. aeruginosa*; however, for the other test bacterial isolates AEE had ZDIs of 13 – 21 mm (for gram-negative bacteria) and 10 – 17 mm (for gram-positive bacteria). The MIC (minimum inhibitory concentration) values of MEE, for both gram-negative and gram-positive bacteria, ranged from 2.5 to 10 mg/ml, by agar dilution method. The extracts (AEE and MEE) were screened qualitatively to contain bioactive components: flavonoids, glycosides, steroids, terpenoids, phenols, quinone and saponins, while cardiac glycoside was detected in AEE only. The HPLC chromatogram showed the presence of 14 detectable compounds, within the retention time of 1.53 - 5.69 min, in MEE. The growth inhibitory indices of 0.59 – 1.08, from the antibiotic-MEE combined action, demonstrated synergism against the test bacterial strains. The *E. fluctuans*, alone or in combination with antibiotics, might be useful in combating infections caused by both gram-negative and gram-positive bacteria.

Keywords: Pathogenic bacteria; *Enhydra fluctuans*; Antibacterial activity; Synergism; Phytochemicals.

1. Introduction

Antibiotics are essentially in use in the clinical medicine to reduce the global burden of many of the life-threatening diseases of bacterial infections to humans. However, prolonged use of antibiotics available in the market and their mishandling led to the emergence of antibiotic resistant pathogenic bacteria (ARPB): MDR (multidrug resistant), XDR (extensively drug resistant) and PDR (pan-drug resistant) [1], and their rapid dissemination as well, around the globe. Fighting against the ARPB infections, it is of overriding importance to find new effective antimicrobial agents, mainly the biotherapeutics, from different biological sources including the medicinal plants. The World Health Organization (WHO) considered the (medicinal) plants as the vital source of an array of bioactive components to be used in combating bacterial antibiotic resistances [2]. India holds the capacity of growing enormous number of plants, which have been in use for the remedies of different kinds of human illnesses since ancient. Currently, scientific studies have proved the presence, in different medicinal food plants, of an ample of varieties of 'secondary metabolites' (phenolics, tannins, alkaloids and flavonoids, etc.) possessing antimicrobial properties [3].

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It has been reported that the plant, *Enhydra fluctuans* displayed antioxidant, hepatoprotective, anti-depressant, analgesic and anti-diarrheal activities, and had antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Micrococcus luteus* [4]. Akhe *et al*. [5] developed an HPLC method to analyze and identify the bioactive compounds in *E*. *fluctuans* displaying antibacterial activity. However, no scientific report is available on the issues of bioactive phytochemicals and antibacterial potentialities of *E. fluctuans* from our part of the globe.

The above background prompted us to the analysis of phytochemical components and antibacterial activity of a native of India vegetable plant, *E*. *fluctuans* (known as helencha in Bengali), which grow naturally and enormously in semi aquatic environments of various parts of West Bengal state, India, and are easily available for consumption. Therefore, the current study aims to authenticate the presence of bioactive components in *E*. *fluctuans*, from local niches (Malda, India), and the antibacterial activity of the plant, as well.

2. Material and methods

2.1. Bacterial strains

The clinical bacterial isolates: gram-negative (*Acinetobacter baumannii* and *Pseudomonas aeruginosa*) and gram-positive (*Bacillus cereus* and *Listeria monocytogenes*), and the standard strains of bacteria: *Escherichia coli* ATCC25922 and *Listeria monocytogenes* MTCC657 were utilized in the study. The bacteria were maintained in the laboratory in cystine tryptone agar stabs, at room temperature.

2.2. Plant materials and extract preparation

The whole aerial parts (stem and leaves) of *Enhydra fluctuans* (Bengali name *Helencha*) were collected from local market (Malda town, India) and were brought to the laboratory. The plants were washed repeatedly, cut into pieces and dried under shade for two weeks. The dried plant materials were milled to form fine powder, and were processed to prepare the aqueous extract of *E. fluctuans* (AEE) and methanolic extract of *E. fluctuans* (MEE), as described earlier [6, 7]. For the preparation of MEE, 5 g of sample powder was soaked in 50 ml methanol for 72 h with manual shaking at 2 h intervals, and in order to prepare AEE, 5 g of the powdered *E. fluctuans* was boiled in a conical flask containing 50 ml double distilled water, for 30 min, without pressure. The prepared extracts (following filtration through cheese cloth and thereafter through Whatman No. 1 filter paper): MEE and AEE were stored at 4^0C, for further analysis, within next two weeks of extract preparation. The extracts were prepared afresh according to the need. The concentration of the prepared MEE and AEE was 100 µg/µl.

2.3. Phytochemical analysis

The bioactive components, such as flavonoids, steroids, terpenoids, quinone, phenol, cardiac glycosides, anthraquinone glycosides and saponins, present in the plant extracts have been detected qualitatively, following the protocol mentioned elsewhere [6, 8].

The *Enhydra fluctuans* methanolic extract was subjected to HPLC analysis in the YL 9000 HPLC system with C_{18} column (5 µm; 100Å; 4.6 × 250 mm), as described earlier [7, 8], using the mobile phase that was composed of water and acetonitrile (1:4 ratio), and the detection of the eluting components was done at 254 nm, at 35°C.

2.4. Antibacterial activity of plant extracts

The antibacterial activity of the prepared extracts (AEE and MEE) was determined by agar-well diffusion method [7, 8], in order to measure the zone diameter of the inhibition (ZDI), as well as by agar dilution method [9], in order to assess the minimum inhibitory concentration (MIC), the details of which are described elsewhere. The agar-well diffusion utilized three different concentrations: 2 mg/well, 5 mg/well, 7.5 mg/well, of AEE and MEE, while the extract concentrations ranged 1.3 – 10 mg/ml in agar dilution method, wherein the results were interpreted as described elsewhere [9].

2.5. Combined antibacterial activity of plant extract and antibiotics

The antibacterial activity of plant extract, MEE (5 mg/disc) combined with antibiotics: CX, PI and NA (for *P. aeruginosa*), CF, AM and NA (for *A. baumannii*), CX, PI and CF (for *B. cereus*) and CX, CF and AM (for *L. monocytogenes*), as selected on the basis of their antibiotic susceptibility, was determined following the protocol explained elsewhere. The GII (growth inhibitory index) values calculated were interpreted following the criteria published elsewhere [10, 11], in order to express the nature of the interaction (synergy, additive, or antagonism) between antibiotics and plant extract, MEE.

3. Results and discussion

The antibacterial activities of AEE and MEE, in terms of ZDIs, against gram-positive and gram-negative test bacteria have been represented in Table 1.

Table 1 Antibacterial activity of aqueous extract of *E. fluctuans* (AEE) and methanolic extract of *E. fluctuans* (MEE)

Bacterial strain	ZDI (mm) of AEE (mg/well)			ZDI (mm) of MEE (mg/well)		
	2.0	5.0	7.5	2.0	5.0	7.5
A. baumannii	17	18	21	16	17	20
P. aeruginosa	6	6	6	13	14	18
E. coli ATCC 25922	13	15	19	12	18	20
B. cereus	13	15	17	15	16	18
L. monocytogenes	10	11	12	13	17	21
L. monocytogenes MTCC 657	11	12	13	16	19	24

ZDI: zone diameter of the inhibition

The AEE extracts had ZDIs of 6 – 21 mm, and the MEE displayed ZDIs in the range from 12 mm to 24 mm. As has been reported by Amin *et al*. [12], both the methanolic and acetonic leaf extracts (400 µg/disc) of *E. fluctuans* showed efficacy against gram-positive (*Bacillus cereus*) and gram-negative (*Escherichia coli*) bacteria with ZDI of 10 mm. The toluene extract of *E. fluctuans* had antibacterial activity against all the test bacteria: *Staphylococcus aureus* (ZDI: 7 – 15 mm), *Escherichia coli* (ZDI: 14 – 20 mm), *Klebsiella pneumoniae* (ZDI: 11 – 18 mm), while the methanolic *E. fluctuans* extracts showed inhibitory action against a single strain of *Staphylococcus aureus* (out of five strains tested), as has been demonstrated by Sarma *et al*. [13]. In the current study, AEE had no zone of inhibition (ZDI: 6 mm) against *P. aeruginosa*, while concentration dependent activity was seen against *A. baumannii* (ZDI: 17 – 21 mm), *B. cereus* (ZDI: 13 – 17 mm) and *L. monocytogenes* (ZDI: 10 – 12 mm) clinical isolates, while the MEE showed antibacterial activity against all gram-negative clinical (*A. baumannii* and *P. aeruginosa*) and standard (*E. coli* ATCC 25922) and all gram-positive clinical (*B. cereus* and *L. monocytogenes*) and standard (*L. monocytogenes* MTCC 657) bacteria tested, displaying ZDIs 12 – 20 mm and 13 – 24 mm, respectively. The concentration dependent antibacterial activity of plant extract against gram-negative (*Pseudomonas aeruginosa, Salmonella typhi, Escherichia coli, Proteus vulgaris, Klebsiella pneumoniae*) as well as gram-positive (*Bacillus cereus, Enterococcus faecalis* and *Staphylococcus aureus*) bacteria, has been reported earlier [14], and that the instant study results, as represented in (Table 1), are in consonance with the findings as reported by Sivaranjani *et al*. [14].

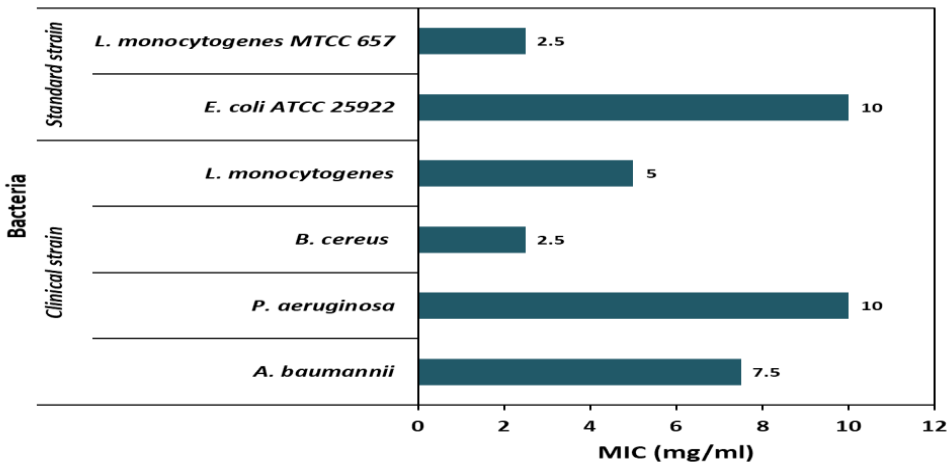

Figure 1 MIC (minimum inhibitory concentration) values of methanolic extract of *E. fluctuans*

The MICs of MEE against the test bacterial strains are represented in Figure 1. The MEE had MICs of 2.5 – 5 mg/ml against gram-positive clinical bacteria, while the values ranged 7.5 – 10 mg/ml for gram-negative clinical bacteria; lower

MIC (2.5 mg/ml) was seen for *L. monocytogenes* MTCC 657 strain compared to the MIC of 10 mg/ml, as displayed against ATCC *E. coli* 25922 (Figure 1); the AEE had MICs >10 mg/ml for all the test bacteria (not shown in the figure). As per the demonstration of Sarma *et al*. [13], the *E. fluctuans* toluene extract MICs for the test clinical bacterial isolates ranged 25 – 50 mg/ml, while the MICs of methanolic *E. fluctuans* extract was 100 mg/ml, for both the gram-negative and gram-positive bacteria. Thus, in the current study, agar-well (in terms of ZDIs) as well as agar dilution (in terms of MICs) techniques achieved excellent antibacterial activity against gram-positive and gram-negative pathogenic bacteria.

Table 2 Antibiotic-plant extract combined antibacterial activity test results

Bacterial strains	**ZDI (mm)**										
	MEE	**Antibiotic**					**Antibiotic-MEE combination**				
		CX	PI	AM	NA	CF	CX-MEE	PI-MEE	AM-MEE	NA-MEE	CF-MEE
A. baumannii	17	20	21	6	6	18	ND	ND	22	25	25
P. aeruginosa	14	6	6	20	12	20	16	16	ND	16	ND
B. cereus	16	15	23	18	21	15	21	29	ND	ND	23
L. monocytogenes	17	18	24	13	23	12	24	ND	25	ND	25

AM: ampicillin, CF: cefotaxime, CX: cloxacillin, NA: nalidixic acid, PI: piperacillin, MEE: methanolic extract of *E. fluctuans*, ND: not done, ZDI: zone diameter of the inhibition.

Earlier the synergistic interactions between antibiotics and indigenous plant extracts have been reported against clinical isolates of gram-positive and gram-negative bacteria [10, 11]. Herein, the test has been performed on the combination effect between antibiotics (as selected on the basis of the test bacterial susceptibility to antibiotics) and MEE (5 mg/ml) against both gram-positive and gram-negative bacteria, and the results, in terms of ZDIs, are represented in Table 2. The toluene extract of *E. fluctuans*, when combined with oxacillin had increment of ZDI (from 6 mm to 10 – 12 mm), against gram-positive bacteria (*Staphylococcus aureus*) compared to the oxacillin alone, whereas the extract, in combination with ceftazidime, had increased ZDIs, from 6 – 7 mm (ceftazidime alone) to 12 - 15 mm (from the action ceftazidime-*E. fluctuans* extract combination), when tested against gram-negative bacteria [13]. The GIIs from the combined action between MEE and antibiotics are depicted in Table 3. As per the criteria mentioned earlier [10, 11], the GIIs, which ranged 0.59 – 1.08, from the antibiotic-MEE combined action, demonstrated synergism against the bacteria tested in the current study.

Table 3 The nature of interaction and GII values from combined antibacterial activity of antibiotics and plant extract

Bacterial strains	**Combined agents**	**GII**	**Interaction**
P. aeruginosa	CX-MEE	0.80	Synergistic
	PI-MEE	0.80	Synergistic
	NA-MEE	0.60	Synergistic
A. baumannii	CF-MEE	1.08	Synergistic
	AM-MEE	0.95	Synergistic
	NA-MEE	0.71	Synergistic
B. cereus	CX-MEE	0.67	Synergistic
	PI-MEE	0.74	Synergistic
	CF-MEE	0.74	Synergistic
L. monocytogenes	CX-MEE	0.68	Synergistic
	CF-MEE	0.83	Synergistic
	AM-MEE	0.86	Synergistic

AM: ampicillin, CF: cefotaxime, CX: cloxacillin, NA: nalidixic acid, PI: piperacillin, MEE: methanolic extract of *E. fluctuans*, GII: growth inhibitory index.

The formerly published scientific reports showed the presence, in the plant extracts studied, of an array of bioactive phytochemicals accounting the antibacterial activity against antibiotic resistant pathogenic bacteria [7, 15]. In the instant study, both the extracts (AEE and MEE) have been tested qualitatively positive for the presence of flavonoids,

glycosides, steroids, terpenoids, phenols, quinone and saponins, while cardiac glycoside was detected only in the AEE. Akhe *et al*. [5] detected different phytochemicals: flavonoids, tannins, glycoside, steroid, alkaloid and saponin, in ethanol extract of *E. fluctuans* by qualitative analysis. Kuri *et al*. [16] showed the presence of flavonoids, saponins, diterpenes, triterpenes and phenols, in methanolic extracts of *E. fluctuans*, and, similar to the instant study, cardiac glycoside was not detected. Haoya *et al*. [17] reported the presence of steroids, saponins, flavonoids and diterpenes, both in methanolic and ethanolic extracts of *E. fluctuans*, displaying antibacterial activity against *E. coli* (ZDI: 12 mm at 400 µg/disc) with the ethanolic extract. As per the report of Sarma *et al*. [13], the *E. fluctuans* toluene and methanol extracts contained bioactive compounds (saponins, tannins, alkaloids, flavonoids, anthraquinones, cardiac glycosides and steroids) showing a broad spectrum of antibacterial activity. Yadava and Singh [18] isolated a novel bioactive component (from the methanolic extract of the leaves of *Enhydra fluctuans*): isoflavone glycoside, which has been found to exhibit growth inhibitory activity against gram-positive (*Staphylococcus aureus* and *Bacillus cougulans*) and gram-negative bacteria *Escherichia coli*. The preliminary phytochemical screening, as has been demonstrated by Kamal *et al*. [3], of *E. fluctuans* substantiated the existence of alkaloids, saponins, tannins and flavonoids, displaying antibacterial activity against *Escherichia coli, Pseudomonas aeruginosa*, *B. megaterium* and *S. aureus* [3].

Abegunde et al. [19] demonstrated phytochemical analysis of aqueous as well as ethanolic extracts of different plants displaying antibacterial activity. Herein, the phyto-components detected in MEE and AEE credibly played role in exhibiting broad spectrum antibacterial activity. The HPLC chromatogram showed the presence of 14 detectable compounds, within the retention time of 1.53 - 5.69 min, in MEE (Figure 2). As reported earlier [7, 8], the HPLC has been found as the essential means of identifying, quantifying, and purifying of bioactive phytochemicals in different indigenous plant extracts. According to Akhe *et al*. [5], the HPLC profiles had four ploy phenolic compounds: catechin hydrate, vanillic acid, *p*-coumaric acid and ellagic acid in the *E. fluctuans* crude extract, whereas catechin hydrate, *p*-coumaric acid, ellagic acid, caffeic acid and kaempferol were identified in the aqueous ethanol fraction, and Gallic acid and kaempferol were detected in the n-hexane fraction. An array of the *E. fluctuans* bioactive components, alone and in combination with antibiotics, plausibly played role in displaying antibacterial activity against gram-positive and gram-negative pathogenic bacteria.

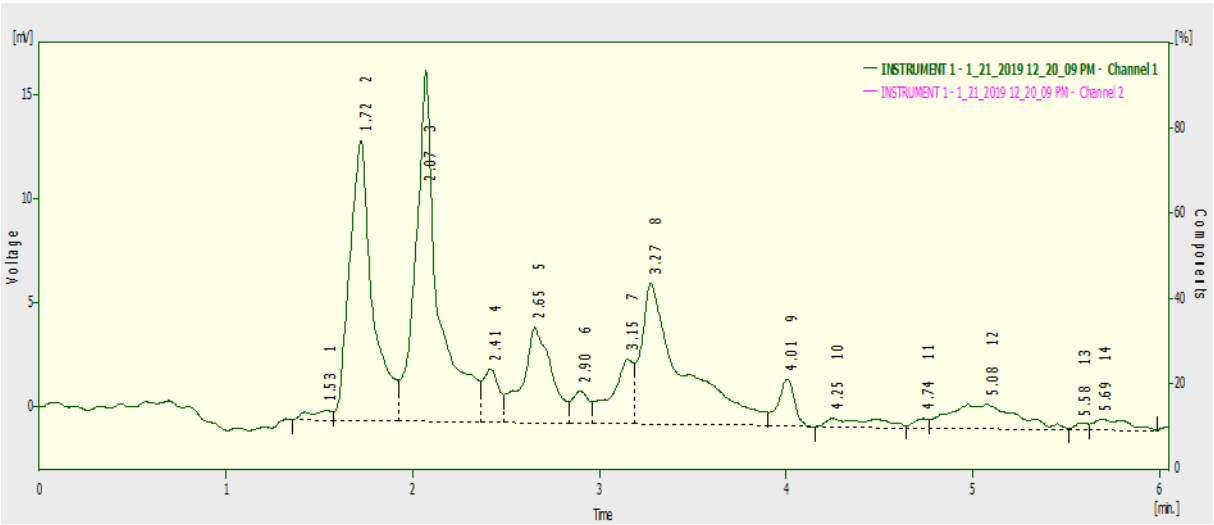

Figure 2 The HPLC chromatogram of methanolic extract of *E. fluctuans*

4. Conclusion

The broad antibacterial spectrum against gram-negative (*Acinetobacter baumannii* and *Pseudomonas aeruginosa*) and gram-positive (*Bacillus cereus* and *Listeria monocytogenes*) pathogenic bacteria of *E. fluctuans* possessing bioactive components might open a new vista in finding the essence of the agent(s) responsible, and thus to prepare the non-antibiotic treatment protocol in tackling the illnesses of bacterial infections to humans. The antibacterial synergy of *E. fluctuans* extract combined with antibiotics, and further pharmacokinetics assay, will aid the process in therapeutic dose determination.

Compliance with ethical standards

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Disclosure of conflict of interest

There is no conflict of interest in publishing the present data of the study.

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