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Antimicrobial and Cytotoxic Activity of Three Bitter Plants-*Enhydra fluctuans, Andrographis Peniculata* and *Clerodendrum Viscosum.*

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ABSTRACT

Purpose:In this study, three important medicinal plants (*Enhydra fluctuans* Lour, *Clerodendrum viscosum* Vent and *Andrographis peniculata* Wall) of Bangladesh were investigated to analyze their antimicrobial and cytotoxic activities against some pathogenic microorganisms and *Artemia salina* (brine shrimp nauplii). ***Methods:*** The coarse powder material of leaves of each plant was extracted separately with methanol and acetone to yield methanol extracts of leaves of *Enhydra fluctuans* (MLE), *Clerodendrum viscosum* (MLC) and *Andrographis peniculata* (MLA), and acetone extracts of leaves of *Enhydra fluctuans* (ALE), *Clerodendrum viscosum* (ALC) and *Andrographis peniculata* (ALA). The disc diffusion method and the method described by Meyer were used to determine the antimicrobial and cytotoxic activities of each plant extract. ***Results:***Among the test samples, MLE and ALE showed comparatively better antimicrobial activity against a number of bacteria and fungi with inhibition zones in the range of 06-15 mm and according to the intensity of activity, the efficacy against microorganisms were found in the order of *Enhydra fluctuans*>*Andrographi speniculata*>*Clerodendrum viscosum*. In cytotoxicity assay, all samples were found to be active against brine shrimp nauplii (*Artemia salina*) and ALA produced lowest LC_{50} value (7.03 µg/ml). ***Conclusion:*** *Enhydra fluctuans* and *Andrographi speniculata* possesses significant antimicrobial and cytotoxic activities.

Introduction

Bacterial infection is one of the serious global health issues in 21st century.[1] There are several reports of antibiotic resistance of human pathogens to available antibiotics.[2,3] The multiple drug resistance and associated adverse effects of antibiotics on the host including hypersensitivity, immune suppression and allergic reaction are growing and because of this outlook the use of antimicrobial drugs in the future is still uncertain.[4] So there is a need of an alternative source for new antibiotics in the drug development pipeline. Natural products, either as pure compounds or as standardized plant extract provide unlimited opportunities for new drug development because of the unmatched availability of chemical diversity.[5] It is reported that Bangladesh has over 5,000 medicinal plants and uses of these plants for medicinal purposes are remarkable.[6] *Enhydra fluctuans* Lour (Traditional name: Helencha; Family: Compositae), *Clerodendrum viscosum* Vent (Traditional name: Ghetu; Family: Verbenaceae) and *Andrographis peniculata* Wall (Traditional name: Kalomegh; Family: Acanthaceae) are three such medicinally important plants bitter in taste that widely grow in Bangladesh. *Enhydra fluctuans* is nutritious and used in ascites, dropsy, anasarca and snakebite.[7] This plant has been reported to have antioxidative and analgesic activities.[8,9] *Andrographis peniculata* exhibits antihepatotoxic, antimalarial, antihepatitic, antithrombogenic, antiinflammatory, anti-snake venom and antipyretic properties[10-12] and itis also used in the treatment of upper respiratory tract infections.[13] *Clerodendrum viscosum* with antioxidative property is used in the treatment of fever, cough, bronchitis and also applied for herpetic eruptions and as vermifuge and bitter tonic.[14,15] Since there are no reports on antimicrobial and cytotoxic effects of *Enhydra fluctuans*, *Clerodendrum viscosum* and *Andrographis peniculata*, in this study attempts were made to evaluate the antimicrobial and cytotoxic activities of leaves of the above three important species against some pathogenic microorganisms and *Artemia salina* (brine shrimp nauplii).

Materials and Methods

Plant materials

Leaves of *Enhydra fluctuans*, *Clerodendrum viscosum* and *Andrographis peniculata*were collected in the month of October, 2011 from Rajshahi district of Bangladesh and the plant material was taxonomically identified by Professor A.T.M Naderuzzaman,

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Department of Botany, University of Rajshahi. Voucher specimens were deposited under the accession numbers DACB-21401 for *Enhydra fluctuans*, DACB-20710 for *Clerodendrum viscosum* and DACB-18612 for *Andrographis peniculata* at the Bangladesh National Herbarium.

Extraction

The collected leaves of each plant were cleaned and shade-dried. The dried leaves were pulverized into a coarse powder by a grinding machine (FFC-15, China). Then half of each plant material was extracted with methanol and remaining part was extracted with acetone at room temperature. Each extract was filtered through filter papers and filtrate was evaporated under reduced pressure at 40^{o}C using a rotary evaporator to have 0.87, 2.2 and 2.4 g methanolextracts of leaves of *Enhydra fluctuans* (MLE), *Clerodendrum viscosum* (MLC) and *Andrographis peniculata* (MLA), respectively whereas 3.5, 2.7 and 4.8g acetone extracts of leaves of *Enhydra fluctuans* (ALE), *Clerodendrum viscosum* (ALC) and *Andrographis peniculata* (ALA), respectively were also obtained.

Antimicrobial assay

Four Gram positive (*Bacillus subtilis* BTCC19, *Bacillus megaterium* BTCC18, *Bacillus cereus* ATCC27853 and *Sarcina lutea* ATCC28106), four Gram negative (*Escherichia coli* ATCC25922, *Shigella sonnei* ATTC8992, *Shigella shiga* ATCC27853 and *Shigella dysenteriae* ATCC14228) pathogenic bacterial strains and five fugal strains (*Aspergillus niger* ATTC235561, *Aspergillus fumigatus* ATTC10231, *Candida albicans* ATTC25889, *Human-3* sp ACCT10558 and *Fusarium sp* ACCT56390) were collected from the Institute of Biological Science (IBSC), University of Rajshahi, Bangladesh. The methanol and acetone extracts of leaves of each plant were tested separately for antibacterial activity by disc diffusion assay method.[16] *Kanamycin* disc (30 µg/disc) and *Nystatin* disc (100 µg/disc) were used as positive antibacterial and antifungal control, respectively. Blank disc impregnated with the respective solvent was used as negative control. The antibacterial activity of each extract was tested against each bacterium at concentrations of 200 µg/disc and 400 µg/disc. For antifungal screening, each extract was tested at concentration of 300 µg/disc and 500 µg/disc. Antibacterial assay plates were incubated at 37±1^{o}C for 24 hrs, whereas antifungal assay plates were incubated at 37±1^{o}C for 48 hrs. Each experiment was carried out in triplicates, and diameter of the zone of inhibition surrounding each disc was recorded.

Cytotoxic assay

The experiment was carried out using the method described by Meyer *et al*.[17] In brief, *Artemia salina* Leach (brine shrimp eggs) was allowed to hatch and mature as nauplii (Larvae) in seawater for 48 hrs at 25^{o}C. Serially diluted test solutions (80 µL in DMSO from a stock solution of 5 mg/mL DMSO) were added to the seawater (5 mL), containing 10 nauplii. After incubation for 24 h at 25^{o}C, the number of survivors was counted. The LC_{50} (50% lethal concentration, µg/ml) was determined from triplicate experiments. Ampicillin trihydrate was used as positive control.

Statistical analysis

The mortality data from cytotoxic experiment were then subjected to probit analysis for the determination of LC_{50} values using the computer software SPSS of 14 version.

Results

Results of antimicrobial test

In antibacterial study, the efficacy of MLE, MLC, MLA, ALE, ALC and ALA to inhibit the growth of four gram (+) positive bacteria and four gram (–) negative bacteria is shown in table 1 and table 2. As shown in table 1 and table 2, MLE and ALE moderately inhibited the growth of *Bacillus cereus*, *Escherchia coli*, *Shigella shiga* and *Shigella sonnei* with the zone of inhibition in the range 06 to 14 mm. ALA and MLA showed activity against *Sarcina lutea*, *Escherichia coli* and *Shigella sonnei* and produced zone of inhibition between 06 to 11 mm (Table 2). MLC and ALC were found inactive against the tested bacteria at concentration of 200 µg/disc, whereas at concentration of 400 µg/disc, it showed mild activity against *Sarcina lutea*, *Escherichia coli* and *Shigella sonnei* exhibiting their zones of inhibition of 06 to 08 mm in diameter (Table 2).

In antifungal activity test, ALE produced zone of inhibition between 07 to 15 mm against *Aspergillus niger*, *Fusarium* sp and *Aspergillus fumigatus*whereas MLE exhibited activity against *Aspergillus niger* and *Fusarium sp* (Table 3). Both MLA and ALA showed moderate activity only against *Human-3* sp and produced inhibition zone ranging from 08 to 14 mm (Table 4). MLC and ALC had no antifungal activity.

Results of cytotoxic assay

All test sample (i.e., MLE, MLC, MLA, ALE, ALC and ALA) showed potent cytotoxicity against brine shrimp nauplii (*Artemia salina*) in comparison with ampicillin trihydrate (LC_{50}: 21.38 µg/ml) (Table 5). Among the samples, ALA (LC_{50}: 7.03 µg/ml) showed the highest toxicity and the mortality was not shown in the negative control experiment.

Discussion

In the continuation of new antimicrobial drug discovery, methanol and acetone extracts of leaves of *Enhydra fluctuans*, *Clerodendrum viscosum* and *Andrographis peniculata* (MLE, MLC, MLA, ALE,ALC and ALA)were investigated, which are being used as a successive medicinal plant in different diseases by folk practitioner in our locality.[7]

Table 1. *In vitro* antibacterial activity of leaves of *Enhydrafluctualns*

Name of microorganism	Zone of inhibition				
	MLE		ALE		Kanamycin
	Dose (µg/disc)				
	200	400	200	400	30
Bacillus subtilis	R	R	R	R	32 ± 1.0
Bacillus megaterium	R	R	R	R	34 ± 1.5
Bacillus cereus	06 ± 0.3	10 ± 1.0	06 ± 0.6	10 ± 1.0	28 ± 1.8
Sarcina lutea	R	R	R	R	35 ± 1.4
Escherichia coli	06 ± 0.6	08 ± 0.3	06 ± 0.3	10 ± 0.6	31 ± 2.1
Shigella sonnei	06 ± 0.6	11 ± 0.5	08 ± 0.6	11 ± 0.3	26 ± 1.2
Shigella shiga	06 ± 0.3	14 ± 0.5	07 ± 0.6	12 ± 1.0	30 ± 1.8
Shigella dysenteriae	R	R	R	R	36 ± 2.0

Data are expressed as mean ± S.E.M (Standard error of mean); MLE: Methanol extract of leaves of Enhydrafluctualns; ALE: Acetone extract of leaves of Enhydrafluctualns; R: Resistance

Table 2. *In vitro* antibacterial activity of leaves of *Clerodendrum viscosum* and *Andrographis peniculata*.

Name of microorganism	Zone of inhibition								
	MLC		ALC		MLA		ALA		Kanamycin
	Dose (µg/disc)								
	200	400	200	400	400	200	400	200	30
Bacillus subtilis	R	R	R	R	R	R	R	R	34 ± 1.3
Bacillus megaterium	R	R	R	R	R	R	R	R	30 ± 1.8
Bacillus cereus	R	R	R	R	R	R	R	R	28 ± 1.5
Sarcina lutea	R	08 ± 0.6	R	07 ± 1.0	06 ± 0.3	11 ± 0.6	06 ± 0.6	09 ± 0.3	30 ± 1.1
Escherichia coli	R	06 ± 0.3	R	06 ± 0.3	06 ± 1.0	08 ± 1.0	06 ± 0.3	10 ± 0.3	32 ± 1.6
Shigella sonnei	R	08 ± 1.8	R	07 ± 1.0	R	08 ± 0.5	08 ± 1.0	11 ± 0.7	38 ± 0.9
Shigella shiga	R	R	R	R	R	R	R	R	26 ± 1.5
Shigella dysenteriae	R	R	R	R	R	R	R	R	31 ± 1.2

Data are expressed as mean ± S.E.M (Standard error of mean); **MLC**: Methanol extract of leaves of *Clerodendrum viscosum*; **ALC**: Acetone extract of leaves of *Clerodendrum viscosum*; **MLA**: Methanol extract of leaves of *Andrographis peniculata*; **ALA**: Acetone extract of leaves of *Andrographis peniculata*; **R**: Resistance

Table 3. *In vitro* antifungal activity of leaves of *Enhydrafluctualns*

Name of microorganism	Zone of inhibition				
	MLE		ALE		Nystatin
	Dose (µg/disc)				
	300	500	300	500	100
Aspergillus niger	07 ± 0.6	13 ± 0.3	07 ± 1.0	15 ± 0.6	25 ± 1.1
Aspergillus fumigatus	R	R	R	13 ± 0.5	30 ± 1.8
***Human-3*sp**	R	R	R	R	28 ± 1.0
Fusarium sp	06 ± 0.6	09 ± 0.3	06 ± 1.0	10 ± 1.0	31 ± 1.5

Data are expressed as mean ± S.E.M (Standard error of mean); MLE: Methanol extract of leaves of Enhydra fluctualns; ALE: Acetone extract of leaves of Enhydra fluctualns; R: Resistance

Table 4. *In vitro* antifungal activity of leaves of *Clerodendrum viscosum* and *Andrographis peniculata*.

Name of microorganism	Zone of inhibition								
	MLC		ALC		MLA		ALA		Nystatin
	Dose (µg/disc)								
	300	500	300	500	300	500	300	500	100
Aspergillus niger	R	R	R	R	R	R	R	R	25 ± 1.1
Aspergillus fumigatus	R	R	R	R	R	R	R	R	30 ± 1.8
***Human-3* sp**	R	R	R	R	07 ± 1.8	07 ± 1.8	07 ± 1.8	07 ± 1.8	28 ± 1.0
Fusarium sp	R	R	R	R	R	R	R	R	31 ± 1.5

Data are expressed as mean ± S.E.M (Standard error of mean); **MLC**: Methanol extract of leaves of *Clerodendrum viscosum*; **ALC**: Acetone extract of leaves of *Clerodendrum viscosum*; **MLA**: Methanol extract of leaves of*Andrographis peniculata*; **ALA**: Acetone extract of leaves of *Andrographis peniculata*; **R**: Resistance

Table 5. Cytotoxicity of the leaves of Enhydra fluctuans, Clerodendrum viscosum and Andrographis peniculata against brine shrimp nauplii.

Sample	LC_{50} (µg/mL)
Amphicillin trihydrate	21.38 ± 0.29
MLE	13.33 ± 0.47
ALE	11.37 ± 0.19
MLC	10.58 ± 0.76
ALC	13.68 ± 0.30
MLA	10.81 ± 0.84
ALA	7.03 ± 0.53

Data are expressed as mean ± S.E.M (Standard error of mean)

In the present investigation, mild to moderate antimicribial activity of these crude extracts were found against the test microorganisms at the concentrations of 200 and 400 µg/disc. Among the samples, the methanol (MLE) and acetone (ALE) extracts of leaves of *Enhydra fluctuans* showed better efficacy against both gram positive (+) and gram negative (-) bacteria as well as three pathogenic fungi. In literature, it has been found that the presence of terpenoids, flavonoids, steroids and glycosides like chemicals in crude extract plays an important role for producing antimicrobial activity.[18-20] The presence of phytoconstitutents like alkaloids, steroids, flavonoids, terpenoids, glycosides etc, in the leaves of *Enhydra fluctuans*, *Clerodendrum viscosum* and *Andrographis peniculata* has been previously confirmed by several studies[7] and these phytoconstitutents possibly might have contributed to the anti-bacterial activity of the three plants.

In the Brine Shrimp lethality study, all extracts obtained from the leaves of the three plants were tested for their cytotoxicity against brine shrimp nauplii and showed positive results indicating that these are biologically active. Several studies have shown that brine shrimp bioassay has been an excellent method to screen the cytotoxic property of medicinal plants and for the isolation of a great variety of biologically active compounds.[21] Finally the overall results of this study act a scientific basis to investigate the relationship between the phytochemicals of the three plant extracts and the biological activities that may explore the actual antimicrobial and cytotoxic chemical constituents present in the crude extracts. Our future studies to isolate these active phytochemicals and determine their activities against microorganisms, are in progress.

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Conflict of interest

The authors have declared that there is no conflict of interest.

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