Journal of Medicinal Plants Studies
www.PlantsJournal.com

ISSN 2320-3862
JMPS 2016; 4(3): 241-247

Received: 21-03-2016
Accepted: 22-04-2016

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In Vitro Antimicrobial Activity of Different Extracts of Long pepper (*Piper longum*) and Water cress (Enhydra fluctuans) against different Pathogenic Bacterial Strains

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Abstract
In this Study to evaluate the anti-microbial activity of Long pepper (*Piper longum*) and watercress (Enhydra fluctuans), the sample plants are collected from four different districts of Bangladesh. Plant parts were extracted by distilled water and organic solvent like ethanol or chloroform or acetone. The antimicrobial properties of the extracts were evaluated on five strains of bacteria e.g. Staphylococcus aureous, Staphylococcus saprophyticus, *E. coli*, Salmonella typhi and shigelladysenteri. Different labeled sterile paper discs are used for sample discs, discs for negative control and standard antibiotic discs. The antimicrobial activity (by measuring inhibition zone diameter) of various extracts were observed. The chloroform and ethanol extracts of both plants have potent against Staphylococcus saprophytias and Staphylococcus aureus with highest zone of inhibition among the tested bacterial strains like 23mm. Similarly ethanol extracts of both plants were more effective against Staphylococcus saprophyticus than any other microorganisms. Acetone extract of long pepper was more effective and produced maximum zone of inhibition of 22mm and watercress of same extract produced 21mm on Staphylococcaceae. Comparatively aqueous extract of Watercress was least effective against Shigella Dysenteriae. The standard antibiotic disc was compared with the plant extract and shown that Tetracycline, Cephradine and Naldixic acid was more potent in producing the zone of inhibition against the same microorganism tested. Ciprofloxacin, Penicillin –G and amoxicillin was less potent in producing the zone of inhibition). Finally different extracts from the both plants shown a strong antibacterial activity with MIC<128 μg/ml, indicating that these plants could be a potent source of antibacterial agent to battle multi drug resistance (MDS) bacterial infection.

Keywords: Long pepper, Water cress, Disc Diffusion Method, Minimum Inhibitory Concentration (MIC), Zone of Inhibition

Introduction
Traditionally used medicinal plants have recently attracted the attention of the pharmaceutical and scientific communities. This has involved the isolation and identification of secondary metabolites produced by plants and their use as active principles in medicinal preparations [1]. Many of the plant secondary metabolites are constitutive, existing in healthy plants in their biologically active forms, but others occur as inactive precursors and are activated in response to tissue damage or pathogen attack [2].

The array of secondary metabolites produced by plants is daunting, with wide ranging chemical, physical and biological activities. These constitute a source of bioactive substances and presently scientific interest has increased due to the search for new drugs of plant origin. A number of plant secondary metabolites (PSM) have been used as anticancer agents. Flavonoid-rich extracts from the mature roots of Scutellaria baicalensis have been shown to exhibit anti-proliferative effects on various cancer lines [3]. Taxol, a diterpene from the Pacific yew has been widely used as a drug for the treatment of ovarian and breast cancer [4]. Limonoids, a group of triterpenes, have been shown to be successful in treatments with in-vitro bioassays on human tumor cell lines, with limonin and isofraxinellone being the most active compounds [5]. A lot of investigation has been conducted for natural antimicrobial agents [6, 7]. According to the World Health Organization (WHO), medicinal plants,

Would be the best source to obtain a variety of drugs and active compounds. Therefore, such plants should be investigated to better understand their properties, safety and efficiency [8]. The indigenous system of medicine namely Ayurvedic, Siddha and Unani has been in existence for several centuries. This system of medicine supports the need of more than 70% of population residing in the rural areas. Besides the demands made by these systems as their raw materials, the demands of medicinal plants made by the modern pharmaceutical industries have also increased manifold [9]. Since a long period of time, plants have been a valuable source of natural products for maintaining human health and infections control because, microbial infections pose a health problem throughout the world, and plants are a possible source of antimicrobial agents [10]. Many of the herbs and spices used by humans to season food yield useful medicinal compounds [11]. Microbial infections pose a health problem throughout the world, and plants are a possible source of antimicrobial agents. Medicinal plants contain active principles which can be used as an alternative to cheap and effective herbal drugs against common bacterial infections [12]. Mainstream medicine is increasingly receptive to the use of antimicrobial and other drugs derived from plants, as traditional antibiotics (products of microorganisms or their synthesized derivatives) become ineffective and as new, particularly viral, diseases remain intractable to this type of drug. Another driving factor for the renewed interest in plant antimicrobials in the past 20 years has been the rapid rate of (plant) species extinction [13]. There is a feeling among natural-products chemists and microbiologists alike that the multitude of potentially useful phytochemical structures which could be synthesized chemically is at risk of being lost irretrievably [14]. Long pepper (*Piper longum*) is cultivated for its fruit, which is usually dried and used as a spice and seasoning. Long pepper has a similar, but hotter, taste to its close relative Piper nigrum. The fruits contain the alkaloid piperine. Water cress (Enhydra fluctuans) is a small genus of marsh herb, distributed in tropical and subtropical regions. The plant is a prostate, spreading, annual herb. This is also an edible semi aquatic herbaceous vegetable plant with notch leaves. The leaves which are slightly bitter are used to treat inflammation, skin diseases, and smallpox [15, 16]. In this study, we aimed to determine the *in vitro* antibacterial properties of extracts from Long pepper (*Piper longum*) and Water cress (Enhydra fluctuans) against some pathogenic bacterial strains.

Materials and Methods

Plant materials and extracts preparation.

Fresh and sound of Long pepper (*Piper longum*) and Water cress (Enhydra fluctuans) were collected from Meherpur, Magura, Faridpur and Kushtia districts. The leaves, stems and roots were taken as sample. The aqueous extraction of the water-soluble ingredients of the plant parts were carried out using the method as described by Asuzu [17]. 15 g of each of the grounded plant part was extracted by successive soaking for 3 days using 35 ml of distilled water for each sample in separate container. The extracts were filtered using Whatman No 1 filter paper, after which the filtrates were concentrated by evaporation at low temperature of 30 °C using water bath. The concentrated extracts were stored in the refrigerator until required. Alternatively for aqueous extraction to be more effective, 10 g of air-dried powder was added to distilled water and boiled on slow heat for 2 hours. It was then filtered through 8 layers of muslin cloth and centrifuged at 5000g for 10 min and supernatant was collected. This procedure was repeated twice. After 6 hours, the supernatant collected at an interval of every 2 hours, was pooled together. Filtrates obtained were then bulked and lyophilised to recover the residues as powder, or in a few cases, as sticky pastes, which were further dried in a desiccator. Aqueous extracts of the plants were employed in this study because the water was likely to extract the same principles as are extracted under in-use conditions. It was then autoclaved at 121 °C temperature and at 15 lbs pressure and stored at 40 °C. These processes of aqueous extraction were applied for all the four test species of plant.

For solvent extraction, 10 g of air-dried powder was taken in 100 ml of organic solvent (absolute ethanol or chloroform or acetone) in a conical flask, plugged with cotton wool and then kept on a rotary shaker at 190-220 rpm for 24 h. After 24 hours the supernatant was collected. Thus the ethanol, chloroform and acetone extracts were prepared separately from the air-dried powder of each of the four plant species. The three organic extract i.e. ethanol extract; acetone extract and chloroform extract were filtered with the aid of a Whatman No 1 filter paper. The extracts were then rotary evaporated to dryness at 40 ^{0}C. The concentrated extracts were stored in the refrigerator until required.

Microbial strains

Five strains of bacteria were used for antibacterial evaluation against the extracts. Among the five strains, *Staphylococcus aureus* and *Staphylococcus saprophyticus* were gram positive and *Escherichia coli*, *Salmonella typhi* and *Shigella dysenteriae* were gram negative. The bacterial strains were maintained on agar slant at 4 ^{o}C in the microbiology laboratory of the Dept. of Applied Nutrition and Food Technology of Islamic University, Kushtia, where the antimicrobial tests were performed.

Culture media

Muller-Hinton Agar and Nutrient Agar Media were used to assay antimicrobial activity and Minimum Inhibitory Concentration (MIC). Mueller-Hinton Agar and Nutrient Agar is considered to be the best for routine susceptibility testing of nonfastidious bacteria.

Preparation of Subculture

The test organisms were transferred to the nutrient agar slants from the pure cultures with the help of an inoculation loop in aseptic condition using laminar air cabinet. For growth of the test organisms the inoculated slants were incubated at 37 ^{0}C for 18-24 hours in an incubator. These fresh cultures were used for sensitivity test within 2 to 3 days.

Preparation of Inoculums

After incubation at 37 ^{0}C for 18-24 hours, well-isolated colonies were transferred into a sterile screw capped test tube containing sterile distilled water and vortex thoroughly. The bacterial suspension was then compared to 0.5 McFarland standards for turbidity standard. This results in a suspension containing 10^7 to 10^8 CFU/ml. The accuracy of the density of a prepared McFarland standard was checked by using a spectrophotometer with a 1-cm light path; for the 0.5 McFarland standard, the absorbance at a wavelength of 625 nm should be 0.08 to 0.1. If the bacterial suspension does not appear to be the same density as the McFarland 0.5, the turbidity can be reduced by adding sterile saline or increased by adding more bacterial growth.

Inoculation procedure

Within 15 minutes after adjusting the turbidity of the inoculums suspension, a sterile cotton swab was dipped into the suspension. Pressing firmly against the inside wall of the tube just above the fluid level, the swab was rotate to remove excess liquid. Then the swab was streaked over the entire surface of the agar medium three times, rotating the plate approximately 60 degrees after each application to ensure an even distribution of the inoculums. Finally, the swab was streaked all around the edge of the agar surface.

Serial Dilution and Preparation of Sample Solution

All the extracts including aqueous and organic extracts (ethanol extract, acetone extract and chloroform extract) of Long pepper (*Piper longum*) and Water cress (Enhydra fluctuans) were used for serial dilution. First 32 mg of extract were taken in a sterile screw capped test tube containing 2 ml solvent and mixed well immediately with vortex mixture to make the concentration of the solution 16 mg/ml. Then 1 ml of the sample solution having 16 mg/ml extract was transferred to the second sterile test tube and mixed well with another 1 ml solvent to make the volume 2 ml and concentration 8 mg /ml. This process of serial dilution was continued up to five test tubes; finally the concentration was found 16 mg/ml in the first test tube and 1 mg/ml in the last. Then 1024 µl of the solution from the last test tube containing 1 mg/ml or 1 µg/µl was transferred to another sterile test tube and 976 of solvent was added to the test tube to make the final volume 2 ml and concentration 512 µg/ml. The content of the test tube was mixed well with vortex mixture. This process of serial dilution was continued up to ten test tubes; finally the concentration was found 512 µg/ml in the first test tube and 1 µg/ml in the last test tube. The same procedure is followed for each of the extract extracted by different solvent.

Preparation of Discs

Filter paper was punched with the punching machine to prepare discs approximately 6 mm in diameter, which was placed in a screw capped test tube and sterilized in an autoclave machine (15 minutes with 121^0C temperature and 15 lbs inch-2 pressure). The sterilized paper disc was the soaked with sample solution of various concentration. The paper disc was then dried with a dryer. After drying the paper disc was labeled according to different concentration. Finally the labeled paper disc was taken into the sterile air tight vial and stored in refrigerator. It was then ready for use as sample disc. Punched, sterile paper disc (6 mm in diameter) was soaked with each solvent to prepare disc, which would be used as negative control. Standard antibiotic disc were purchased from Mast Diagnostic Centre, England. Following standard antibiotic disc were used as positive control.

Placement of Discs to Inoculated Agar Plates, Diffusion & Incubation

The antimicrobial discs (Sample discs, discs for negative control and standard antibiotic discs) were placed on to the surface of the plates inoculated with the test organism as soon as possible, but no longer than 15 minutes after inoculation. The discs were placed individually with sterile forceps or with a mechanical dispensing apparatus, and then gently press down onto the agar. They were distributed evenly so that they were not closer than 24 mm from center to center. Ordinarily, no more than 12 discs were placed on one 150 mm plate or no more than 5 discs on a 100 mm plate. This prevented overlapping of the zones of inhibition and possible error in measurement. Diffusion of the active compound in the discs begins immediately; therefore, once a disk placed on the agar surface, the disk was not removed or relocated. The plates were refrigerated for an hour or two at 4 ^{0}C to allow pre-diffusion of the extracts from the disk into the seeded agar layer before incubation. After the discs were placed, the plates were inverted and incubate at 37 °C for 18 hours.

Measurement of the Zone of Inhibition and Minimum Inhibitory Concentration (MIC)

After 18 hours of incubation, each plate was examined. The plate, which was satisfactorily streaked, and the inoculum was correctly prepared, the resulting zones of inhibition was uniformly circular and there was a confluent lawn of growth. After incubation, the diameter of the zones of complete inhibition (including the diameter of the disk) was measured to the nearest whole millimeter. The measurements were made with a ruler on the undersurface of the plate without opening the lid. The Petri dish was held a few inches above a black, nonreflecting background and illuminated with reflected light [18]. A slight amount of growth may occur within the inhibition zone. In that instance, slight growth (80% inhibition) was ignored and the zone diameter was measured to the margin of heavy growth. The zones of growth inhibition was interpreted and recorded as sensitive, moderately sensitive or resistant to each sample or antibiotics tested. The lowest concentration of the extracts required to inhibit the growth of the organism *in vitro* is MIC and in the present study it was determined following the serial dilution technique according to Reiner [19]. The lowest concentration on which lowest zone of inhibition occurred are the MIC.

Result and Discussion

There is a necessity for the discovery of new ingredients from natural sources, with plants. While several studies are available on the chemical composition, antifungal, antiradical, antioxidant and antibacterial activities of different plant species [20, 21]. However, only a few studies have been carried out with Long pepper and Watercress. Crude plant extracts are traditionally used, in herbal medicine, by the population for the treatment of diseases particularly infectious diseases. Although their efficacy and mechanisms of action have not been tested scientifically in most cases, medicinal preparations from plants often mediate beneficial responses due to their active chemical constituents. The chloroform extract of both Long pepper and Watercress was potent against Staphylococcus saprophyticus, with highest zone of inhibition (22 and 21 mm respectively) among the bacterial strain tested (Table 1).

Table 1: Antimicrobial Activity (diameter of the zone of inhibition) of Chloroform Extract of Long pepper (*Piper longum*) & Water cress (Enhydra flunctuans)

Diameter of the Zone of Inhibition (mm)					
	Staphylococcus aureus	***Staphlococcus saparophyticus***	***Escherichia coli***	**Salmonella typhi**	**Shigella dysenteriae**
MIC*					
Long pepper	16 µg/ml	8 µg/ml	8 µg/ml	8 µg/ml	32 µg/ml
Water cress	4 µg/ml	4 µg/ml	16 µg/ml	8 µg/ml	32 µg/ml
Zone of Inhibition (mm) Long pepper					
16 mg/ml	22 mm	23 mm	20 mm	18 mm	17 mm
64 µg/ml	8 mm	6 mm	4 mm	2 mm	5 mm
Water cress					
16 mg/ml	23 mm	22 mm	17 mm	16 mm	19 mm
128 µg/ml	5 mm	7 mm	7 mm	4 mm	4 mm
Negative Control Disc	-	-	-	-	-

The chloroform extract of *Piper longum* produced maximum zone of inhibition against Staphylococcus saparophyticus (23mm), Staphylococcus aureus (22mm) and Escherichia coli (20mm), similarly ethanol extract was more effective against Staphylococcus saparophyticus (20 mm), Staphylococcus aureus (22 mm) than against any other microorganisms (Table 3). The range of zone of inhibition produced by ethanol extract of *Piper longum* extract against all the test organism was 2 mm to 22 mm. While this range was 2 to 23 mm for chloroform extract. In case of Watercress the chloroform extract had shown maximum zone of inhibition against Staphylococcus aureus (23 mm) and Staphylococcus saparophyticus (22 mm). Acetone extract was more effective and produced maximum zone of inhibition of 18 mm, 17 mm, and 20 mm against *Escherichia coli*, Salmonella typhi and Shigella dysenteriae respectively (Table 2).

Table 2: Antimicrobial Activity (diameter of the zone of inhibition) of Acetone Extract of Long pepper (*Piper longum*) & Water cress (Enhydra flunctuans)

Diameter of the Zone of Inhibition (mm)					
	Staphylococcus aureus	***Staphlococcus saparophyticus***	***Escherichia coli***	**Salmonella typhi**	**Shigella dysenteriae**
MIC*					
Long pepper	8 µg/ml	8 µg/ml	8 µg/ml	8 µg/ml	32 µg/ml
Water cress	4 µg/ml	4 µg/ml	4 µg/ml	8 µg/ml	16 µg/ml
Zone of Inhibition (mm) Long pepper					
16 mg/ml	21 mm	22 mm	19 mm	18 mm	15 mm
64 µg/ml	5 mm	6 mm	4 mm	3 mm	5 mm
Water cress					
16 mg/ml	22 mm	20 mm	18 mm	17 mm	19 mm
128 µg/ml	5 mm	6 mm	4 mm	4 mm	3 mm
Negative Control Disc					

Comparatively aqueous extract of Watercress was least effective against Shygella dysenteriae, however was equally effective against other microorganism. It has been revealed that all of the four extract of long pepper and Watercress was most effective against Staphylococcus aureus and Staphylococcus saprophyticus, however was effective against the rest of the microorganism tested.

Table 3: Antimicrobial Activity (diameter of the zone of inhibition) of Ethanol Extract of Long pepper (*Piper longum*) & Water cress (Enhydra flunctuans)

Diameter of the Zone of Inhibition (mm)					
	Staphylococcus aureus	***Staphlococcus saparophyticus***	***Escherichia coli***	**Salmonella typhi**	**Shigella dysenteriae**
MIC*					
Long pepper	16 µg/ml	4 µg/ml	8 µg/ml	8 µg/ml	32 µg/ml
Water cress	4 µg/ml	4 µg/ml	16 µg/ml	4 µg/ml	16 µg/ml
Zone of Inhibition (mm) Long pepper					
16 mg/ml	20 mm	22 mm	19 mm	19 mm	18 mm
64 µg/ml	5 mm	5 mm	4 mm	2 mm	5 mm
Water cress					
16 mg/ml	20 mm	21 mm	18 mm	17 mm	20 mm
128 µg/ml	4 mm	7 mm	7 mm	3 mm	3 mm
Negative Control Disc					

The MIC value of different extract of Watercress against Stahhylococcus aureus, Staphyloccus saparophyticus, *Escherichia coli*, Salmonella typhi and Shigella dysenteriae was 4µg/ml. Aqueous extract had shown significant activity

against the test organisms. Aqueous extracts from the two plant species was effective against both of the gram positive microorganism and nearly all gram negative organisms used in this study. The antibacterial activity of aqueous extract of *Piper longum* was surprising. It had shown 24mm of zone of inhibition against Staphylococcus aureus which was maximum produced by all the solvent used in this study.

Table 4: Antimicrobial Activity (diameter of the zone of inhibition) of Aqueous Extract of Long pepper (*Piper longum*) & Water cress (Enhydra flunctuans)

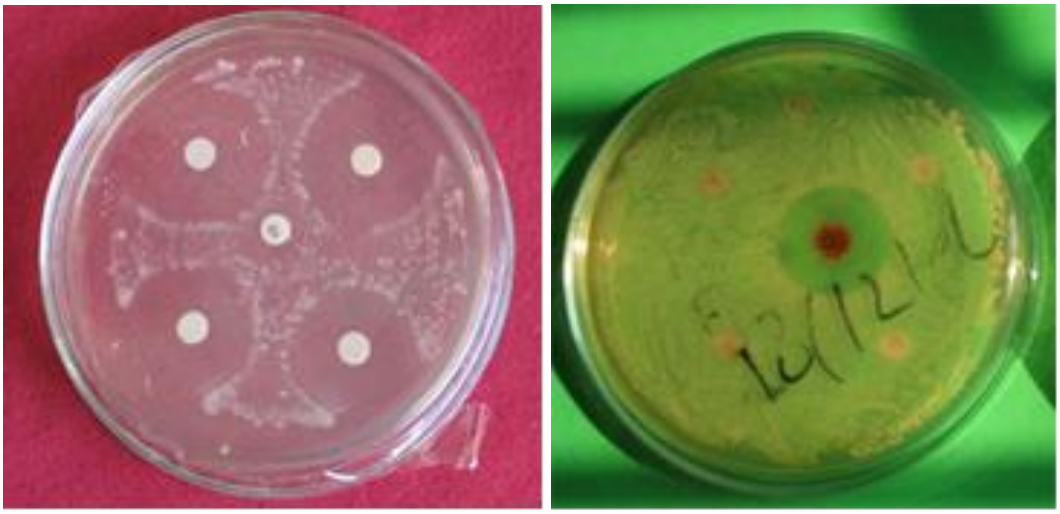
	Diameter of the Zone of Inhibition (mm)				
	Staphylococcus aureus	***Staphlococcus saparophyticus***	***Escherichia coli***	**Salmonella typhi**	**Shigella dysenteriae**
MIC*					
Long pepper	16 µg/ml	64 µg/ml	128 µg/ml	4 µg/ml	16 µg/ml
Water cress	32 µg/ml	256 µg/ml	256 µg/ml	32 µg/ml	4 µg/ml
Zone of Inhibition (mm) Long pepper					
16 mg/ml	24 mm	19 mm	17 mm	20 mm	19 mm
64 µg/ml	3 mm	4 mm	4 mm	7 mm	15 mm
Water cress					
16 mg/ml	17 mm	16 mm	18 mm	19 mm	+
128 µg/ml	4 mm	5 mm	8 mm	5 mm	+
Negative Control Disc	---	---	---	---	---

The standard antibiotic disc was compared with the plant extract and shown that Tetracycline, Cephradine and Naldixic Acid was more potent in producing the zone of inhibition against the same microorganism tested. Ciprofloxacin, Penicillin-G and Amoxicillin was less potent in producing the zone of inhibition (Table 5).

Table 5: Antimicrobial Activity (diameter of the zone of inhibition) of Standard Antibiotic Disc

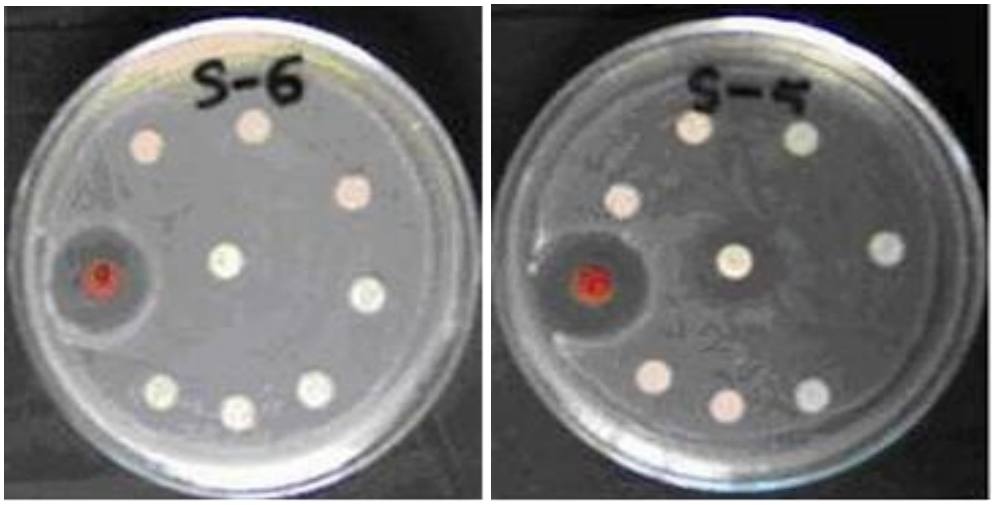
	Diameter of the Zone of Inhibition (mm)				
	Staphylococcus aureus	***Staphlococcus saparophyticus***	***Escherichia coli***	**Salmonella typhi**	**Shigella dysenteriae**
Antibiotic Disc (µg/disc)					
Ciprofloxacin (5)	5 mm	10 mm	+	10 mm	+
Penicillin-G (10)	10 mm	11 mm	+	8 mm	10 mm
Tetracycline (30)	30 mm	29 mm	+	12 mm	12 mm
Amoxycillin (10)	10 mm	11 mm	10 mm	+	+
Cephradine (30)	30 mm	26 mm	24 mm	25 mm	30 mm
Naldixic Acid (30)	30 mm	19 mm	16 mm	14 mm	16 mm

Plants might be a potent source of natural remedy and for Plants have been a valuable source of natural products for upholding human health as, microbial infections posture a health problem throughout the world, and plants are conceivable source of antimicrobial agents [22]. World Health Organization (WHO) also foresees, medicinal plants could be the best source to gain a variety of drugs and active compounds. Therefore, it is necessary to investigate such plants to better recognize their properties, safety and efficiency [23]. There are a number of methods, as agar diffusion, cup method, paper disc, to evaluate the antimicrobial properties and disc diffusion is a methods that is easy to perform and minimum tools and equipment's are required. In addition a number of factors influence on the accuracy of the methods and if the factors could be controlled, the antimicrobial properties would be evaluated effectively [24].



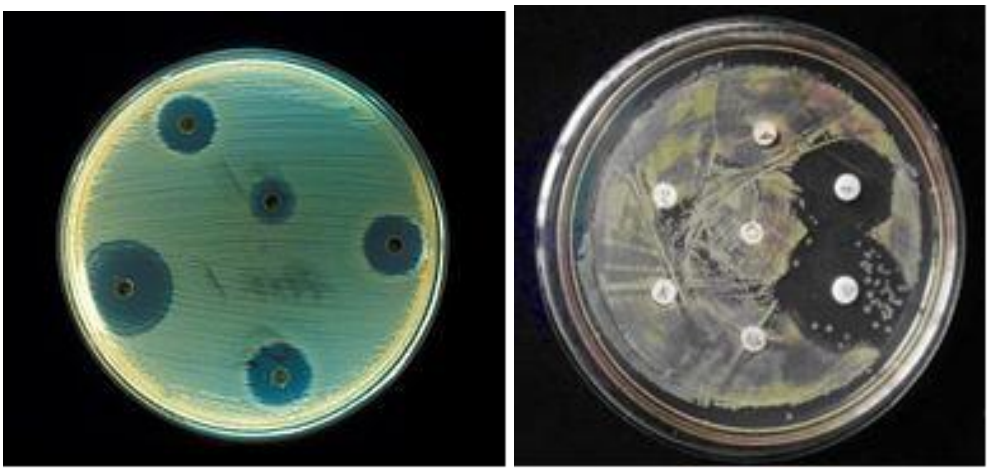
1 (A) **1(B)**

Fig 1: Zone of inhibition produced by chloroform extract of Enhydra fluctuans (A) and *Piper longum* (B) against *Staphylococcus aureus*.



2 (A) **2(B)**

Fig 2: Zone of inhibition produced by acetone extract of Enhydra fluctuans against Salmonela typhi (A) Enhydra fluctuans against Shigella Dysenteriae (B)



3 (A) **3(B)**

Fig 3: Zone of inhibition produced by aqueous extract of *Piper longum against Staphylococcus saparophyticus* (A) and *Escherichia coli Shigella dysenteriae* (B)

In our studies, we have tried to control and maintain all of the factors, like thickness and uniformity of the media and temperature that affects the outcome of the investigation. However, our experiment have shown the potent antimicrobial activity, which was performed *in vitro* against few bacterial stain, we are not convinced about if such activity will be interpreted in vivo. We hope, further studies including epidemiological and clinical trial studies are necessary to comprehend the effect of use of extracts from such medicinal plants in population.

Conclusion

Although our study have shown the effective *in vitro* activity of plant extracts for certain bacterial strains, in vivo and epidemiological studies are might help to understand the effective use of extracts from such medicinal plants in population. In this study we evaluated the antibacterial activity of two commonly used traditional medicinal plants from Bangladesh. Different extracts from both plants shown a strong antibacterial activity with MIC<128 μg/ml, indicating that these plants could be a potent source of antibacterial agent to battle multi drug resistance (MDR) bacterial infections. These plants can also be used to discover bioactive natural products in the form of antimicrobial phytochemicals that might help for the development of new pharmaceutical drugs.

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