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Original Article

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Antimicrobial and Membrane Stabilization Activity of Plant Extracts Against Pathogenic Bacteria and Fungi

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The use and search for antimicrobial drugs derived from plants have accelerated in recent years. The present study was aimed to determine the antimicrobial, antibiofilm and membrane stabilization activities of plant extracts. Crude extracts of four plants namely, *Mikania scandens* (L.), *Mimosa pudica* (L.), *Murraya paniculata* (L.), and *Syzygium aromaticum* (L.) were tested against eleven human pathogens including four biofilm producing bacterial strains *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Staphylococcus aureus*, and one fungal strain *Candida albicans*. The antimicrobial activities as well as minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts were evaluated using disc diffusion and macro-broth dilution methods respectively. Among the plant extracts, ethanol extract of *S. aromaticum* exhibited the largest zone of inhibition 35 mm in diameter against *Bacillus subtilis* at 500 µg/disc concentrations. The lowest MIC (1000 µg/ml) and MBC (2500 µg/ml) were determined against *P. aeruginosa* with the same extract of *S. aromaticum*. In case of time kill assay, *S. aromaticum* extract showed the lowest optical density (OD_{600}) 0.2 against *E coli* in 3h at MBC concentration. Moreover, the same extract of *S. aromaticum* displayed the strong antibiofilm activity, inhibiting 100% biofilm formation of *E. coli* at 2×MIC concentration. Furthermore, *in vitro* experiment was performed to evaluate membrane stabilization activity of plant extracts and *S. aromaticum* showed 100% activity in stabilizing cell membranes, preventing hemolysis of red blood cells. Therefore, this study provides valuable insight for designing antimicrobial products to efficiently eliminating human infections, and the plant extracts could be the potential antimicrobial agent.

Keywords: Antimicrobial activity, Plant extract, Time Kill Assay, Antibiofilm activity, Membrane Stabilization Activity.

Introduction

The antimicrobial-resistant (AMR) of microorganisms is a global public health problem and showing worrisome resistant mechanism to hydrolyze antibiotics[1-2]. As the antibiotic resistance problem becomes a global perception, there is a continuous need for new drugs and plants have provided a good source of anti-infective agents. Therefore, medicinal plants could play a complementary role against antibiotic resistant pathogenic microorganisms[3].

Mikania scandens (L.) is a twining plant that can be found growing as a weed throughout the country. This plant is a vast source of Vitamins A, B and C. It also contains stigmasterol, â-sitosterol, sesquiterpene, dilactones, mikanolide, dihydromykanolide, deoxymikanolide, and scandenolide[4]. Fresh leaves of *M. scandens* are used to stop and cure hemorrhages from cuts and wounds, and the paste is used for itches and policing wounds[5].

Mimosa pudica (L.) is a creeping perennial flowering plant of the family Fabaceae. This plant is regarded as diuretic, astringent and antispasmodic also used in treating sore gums, convulsions of children as well as a source of blood purifier[6].

Murraya paniculata (L.), commonly known as orange jessamine, is a small, tropical to sub-tropical or shrub with dark green leaves and clusters of white flower under the family Rutaceae. Leaves of this plant contain monoterpene and sesquiteerpene rich oil is used to treat diarrhea, dysentery and diseases of teeth and gum[7-8].

Cloves are the flower buds of a tree *Syzygium aromaticum* (L.) in the family Myrtaceae, commonly used as an aromatic spice, flavoring, or fragrance in consumer products. Clove represents one of the major vegetal sources of phenolic compounds including flavonoids, hidroxibenzoic acids, hidroxicinamic acids and hidroxiphenyl propens. Cloves are using as the sources of antiseptic, relieves pain, controls nausea and vomiting, improving digestion, and antiparasites[9].

Over 60% of microbial illnesses were induced by microbial biofilms, which also account for two-thirds of all bacterial infections in humans[10]. Biofilm is an exopolymeric physical layer on surroundings which resists the pathogens from its unfavorable environment and contributes to recurrent infections likely periodontosis, cystic fibrosis, chronic prostatitis, endocarditis and antibiotic resistance[11]. Pharmacological target for the tackling

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this pathogenic infections, biofilm inhibitory antibiofilm medications should be the subject of intensive study [12]. Various green nonlethal methods for controlling biofilms have been developed recently, but the mode of actions of the innovative antibiofilm drugs are significantly less prone to the emergence of resistance where plant extracts with antibiofilm activity could provide an alternative to control biofilm formation [13].

Furthermore, plant extracts have shown membrane stabilizing activity which refers to their ability to protect and stabilize cell membranes. Plants could be an alternative source of anti-inflammatory drugs. Long-term use of anti-inflammatory medications raises the risk of peptic ulcer disease, acute renal failure, myocardial infection, and other complications. The plant contains many phytoconstituents includes phenolics and flavonoids which are thought to show anti-inflammatory activity. Medicinal plants having anti-inflammatory activity may lead to the identification of a potential therapeutic agent that can be used not only to control inflammation but also to treat a variety of diseases where the inflammation response is increasing the disease process[14].

Materials and Methods

In the present study, crude extracts of fifteen plants (randomly selected) were screened for their antimicrobial activity. Among the fifteen plants, the crude extracts of four plant species viz., *Mikania scandens, Mimosa pudica, Murraya paniculata* and *Syzygium aromaticum* were exhibited good antimicrobial activities, thus, these four plants were finally selected for this study.

Plant materials

Fresh matured leaves of *Mikania scandens* (L.), *Mimosa pudica* (L.), and *Murraya paniculata* (L.) were collected from Chittagong University campus. Flower bud (clove) of *Syzygium aromaticum* (L.) were bought from a grocery store located at Chattogram. The leaves samples were cut into small pieces (1-2cm), washed thoroughly using distilled water, air-dried at room temperature, and pulverized to fine powder mechanically. During this this process, precautions were taken to avoid admixture of different plants.

Preparation of extracts for primary antimicrobial screening

The fine powder of each plant part (leaves and flower buds) was sequentially extracted with polar and non-polar solvents (Petroleum ether, Carbon tetrachloride, Chloroform, Ethyl acetate, and Ethanol) based on solvents polarity index at a ratio of 1:3 (plant material: organic solvent) for 48 hours in a dark vessel with manual application of gentle shaking. The solvent extracts were collected and separated by filtration. These crude extracts were then tested for primary screening of antibacterial activity.

Preparation of Extracts for biological activities

Based on the highest range of zone of inhibitions and activities against pathogenic microorganisms in primary screening, one solvent extract for each plant (Ethyl acetate for *Mikania scandens and Mimosa pudica*, Ethanol for *Murraya paniculata* and Petroleum ether for *Syzygium aromaticum*) were selected for further quantitative extraction process to assay quantitative antimicrobial and other biological activites. The extracts were prepared by amalgamating 200g powder with 500 ml solvents (selected for each plant) in a dark vessel, kept it for five days with manual application of gentle shaking. Then, the extracts were filtered using Whatman filter paper, concentrated at low temperatures (40-50ºC) by a rotary vacuum evaporator, then dried at 40-50ºC. Thus, the dried extract obtained was termed as the crude extract. The dried extract obtained from *Syzygium aromaticum* was essential oil. The crude extract was stored in sealed vials at 4ºC. A measured amount of dried crude extract dissolved in definite volumes of DMSO to give solution (10%, w/v) of known concentration. From this solution desired concentrations were used for further test.

Determination of the Percentage of extracts yield

The crude extract of each sample was weighed and calculated the yield percentage of each sample as follows[15]:

Extract yield % = (W1 / W2) × 100

Here, W1= Net weight of powder in grams after extraction.

And W2= Total weight of wood powder in grams taken for extraction

Treatment of paper disc by extracts

Solutions of known concentration of the crude extracts or essential oil were prepared by dissolving measured amount of the sample in definite volume of solvent. Dried and sterilized filter paper discs (4 mm in diameter) were treated with known concentration (µg dry weight) of extract using micropipette. However, extracts for primary screening, 4mm in diameter paper discs were soaked with respective extract, and these were then tested for primary screening of antimicrobial activities.

Test organisms

Gram negative bacterial strains *Escherichia coli* ATCC25923, *Pseudomonas aeruginosa* ATCC8027, *Salmonella abony* NCTC6017, *Klebsiella pneumoniae* ATCC13883, *Vibrio cholerae* AE14748, *Shigella dysenteriae* AE14612, *Acinetobacter baumannii* ATCC17978 and gram positive bacterial strains *Bacillus cereus* ATCC14579, *Bacillus subtilis* ATCC6633 and *Staphylococcus aureus* ATCC6538, and a human pathogenic fungal strain *Candida albicans* ATCC10231 were used. All of the bacterial and fungal strains used throughout the experiments were taken from stock cultures maintained by the Lab of Department of Microbiology at the University of Chittagong, Bangladesh.

Phytochemical analysis of Extracts

Using established protocols, the extracts were screened for major phytochemicals. Screening of the above medicinal plant extracts

for various phytochemical constituents was carried out by several chemical tests using standard methods[16].

Determination of antimicrobial activity

The antimicrobial activities of the plant extracts were done by disc diffusion method[17]. Luria-Bertani (LB) broth medium per liter contained 10 g tryptone, 5 g of yeast extract and 10 g of NaCl was used for culturing the test microorganisms. Muller-Hinton Agar (MHA) medium (Acid digest casein 17.5g/L, beef extract 20g/L, soluble starch 1.5 g/L and agar 15.0 g/L) was used for antimicrobial assay. All experiments were repeated three times.

Determination of MIC and MBC

Minimum inhibitory concentration (MIC) and minimum bactericidal (MBC) or fungicidal concentration (MFC) of crude extracts were determined by the macro dilution broth technique as described by National Committee for Clinical Laboratory Standards[18]. Luria-Bertaini (LB) broth medium was used for bacterial and fungal cultures. Each tube was inoculated with 1.0 ml of bacterial or fungal (1.6×10^7 CFU/ml) suspension containing 1.0 ml of double strength LB broth with 500 to 5000 ìg/ml plant extract, incubated at 37æ%C for 24h. A control tube was maintained without plant extract. A suspension aliquot of 0.5 ml was placed onto pre-sterilized petri dishes with MHA medium in order to explore the potential MBC of antimicrobial agent in broth culture tubes where the microbial growth was not visible. After that, the plates were incubated for 24h at 37°C in order to observe the microbial growth. MBC were defined as the maximum dilution at which at least 99% of bacteria were inhibited. Testing was performed in triplicate for each extract concentration.

Time kill bacterial susceptibility to plant extract

The time-kill bacterial susceptibility to the extracts was investigated followed by the method described by Ajiboye *et al* (2018) [19]. Briefly, bacterial cells were grown overnight in Luria-Bertani (LB) broth medium. Following incubation, bacterial cells were harvested by centrifugation and resuspended in 50 mL fresh LB medium to obtained OD_{600} = 0.1 in 250mL conical flask. Then the cells were grown aerobically in a shaker incubator (200 rpm) at 37°C. At mid-log phase (OD_{600} = 0.5), 15 ml culture was transferred to 3 conical flask. Then crude extract at MBC concentration of test organisms for the test, same concentration of 0.5% dimethyl sulfoxide (DMSO) as a negative control, and an antibiotic (ciprofloxacin 0.50µg/ml) as a positive control were added separately in each flask. Then the mixtures were incubated at 37°C for 3 hours. Following incubation, the absorbance of the culture was determined every 20 min interval until 3 hours incubation time at 600 nm using spectrophotometer. For colony formation, samples of control cultures and cultures treated with extract were removed at intervals 0, 20, 40, 60, 80, 100, 120, 140, 160, and 180 min respectively, and centrifuged to collect the cells as a pellet. The cells were then washed and diluted with 0.9% NaCl, mixed with molten soft LB-agar at 42°C, and poured onto agar plates containing solid LB-agar. Then the plates were incubated at 37°C. Colonies were counted after 24h.

Anti-biofilm activity test

The effect of plant extracts on biofilm formation were evaluated by the crystal violet staining method[20-21] with slightly modified by Christensen *et al* (1985)[22]. Briefly, LB broth medium was used for bacterial culture. 0.5ml of plant extracts (0.5× MIC, 1×MIC, and 2×MIC) was distributed into individual test tubes. Equal volumes of water with DMSO was taken into another tube as a negative control. 0.5ml of bacterial suspension from 24h culture LB broth culture was added to each test tubes yield a final volume of 1ml. Sterile LB broth (1ml) was also added to each tube as an additional nutrient. Then the test tubes were incubated for 3 days at 37°C for cell attachment. After incubation, for removing any loosely attached bacterial cells, the test tubes were washed five times with sterile distilled water. Then the tubes were dried and stained with 1% crystal violet. After 15 minutes the tubes were washed to remove excess stain. Then 2.0 ml of 90% ethanol was added to the test tube to de-stain the tubes. The absorbance of the destained solution was measured using spectrophotometer at 530 nm. Percentage of biofilm inhibition was measured using the following equation:

$$\% \text{ of inhibition} = \frac{100 - (OD_{530}\text{nm of experimental})}{OD_{530}\text{nm of control})} \times 1000$$

Membrane stabilization test

Membrane stabilization test of extracts was done by the hypotonic solution-induced hemolysis method. To study the in vitro anti-inflammatory activity of extracts was investigated using the human red blood cell (HRBC) membrane stabilization process described by Vane and Botting[23].

Fresh whole human blood was collected from six humans and mixed with an equal volume of sterilized Alsever solution*, centrifuged at 3000 rpm for 10 minutes and packed cells were washed three times with isosaline* and prepared 10% v/v suspension with isosaline. Erythrocyte suspension (0.5 ml) mixed with 5ml of hypotonic solution*. Then 25-200 µg/ml of extract in some and standard drug (Diclofenac sodium) in others were added. In control, no extract or drug was added. The mixture was then incubated for 10 min and then centrifuged for 20 min at 3000 rpm and the hemoglobin content was estimated using spectrophotometer at 560 nm after the supernatant was decanted.

$$\% \text{ of hemolysis} = \frac{\text{The optical density of test samples}}{\text{The optical density of the control}} \times 100$$

*Alsever solution: 2% dextrose, 0.8% sodium citrate, 0.05% citric acid and 0.42% sodium chloride in water.

*Isosaline: 0.85% NaCl, pH- 7.2

*Hypotonic solution: 50mM NaCl in 10mM Sodium Phosphate buffer saline, pH- 7.4

Results and Discussion

Fifteen plant samples (randomly selected) collected from Chittagong region, Bangladesh were extracted and screened for

their antibacterial activities. Among them, four plant species viz., *Mikania scandens* (leaf), *Mimosa pudica* (leaf), *Murraya paniculata* (leaf), and Syzygium *aromaticum* (flower bud) extracts exhibited good antimicrobial activity against the microorganisms tested herein.

Yielding percentage of extract

The dry weight yielding percentages of crude extracts obtained from ethyl acetate, ethanol, petroleum ether, carbon tetrachloride and chloroform extracts of the leaves of *Mikania scandens*, *Mimosa pudica*, and *Murraya paniculata* and flower bud of *S. aromaticum* are presented in Figure 1. Where, the ethyl acetate extracts of *S. aromaticum* flower bud (clove) and *M. pudica* leaves exhibited the highest 13.17% and 3.2% yields respectively compared to that of other solvent extracts. However, the highest 22.35% and 23.3% yields were obtained from ethanol extracts of *M. paniculata* and *M. scandens* leaves, respectively (Figure 1).

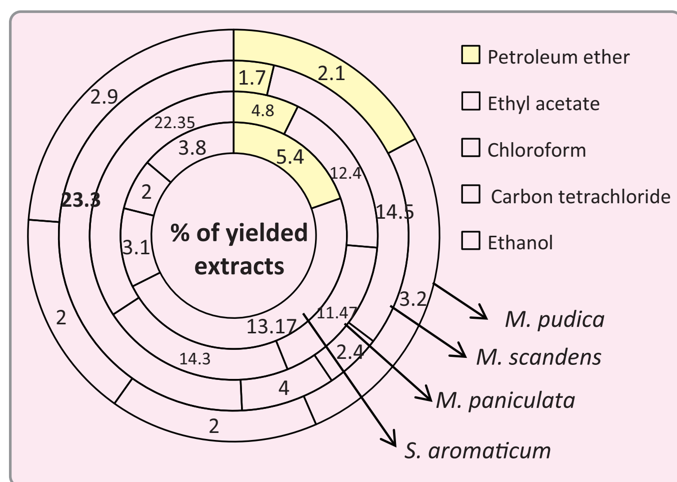

Figure 1. *Percentages of crude extracts (dry weight) yielded from the tested plant extract in different organic solvents (Petroleum ether, ethyl acetate, Chloroform, Carbon tetrachloride and Ethanol).*

Primary screening for antimicrobial activity of plant extract

For antimicrobial screening, 4 mm in diameter paper discs were soaked with the crude extracts. Therefore, different (Petroleum ether, ethyl acetate, Chloroform, Carbon tetrachloride and Ethanol) crude extracts of *Mikania scandens, Mimosa pudica, Murraya paniculata* and *Syzygium aromaticum* were screened for their antimicrobial activities and the results are presented in Figure 2. *Mikania scandens* showed highest range of inhibition by ethyl acetate extract against *P. aeruginosa* (22mm) and 16mm in diameter against *S. abony* and *B. cereus*. Carbon tetrachloride extract of *M. scandens* showed activity against all pathogens accept *S. dysenteriae* whereas ethanol, petroleum ether and chloroform extracts exhibited less activity (Figure 2a). However ethyl acetate extracts of *M. pudica* leaf showed a range of inhibition (5-9mm) against *S. aureus, A. baumanni, B. cereus, P. aeruginosa, S. dysenteriae* and *C. albicans* (Figure 2b). However, all extracts of *Syzygium aromaticum* showed antimicrobial activity against all of the tested pathogens except ethanol. The largest zone of inhibition by *S. aromaticum* was 25mm against *B. subtilis* by petroleum ether extract, 23mm against *staphylococcus aureus* by ethyl acetate extract and 21mm against *A. baumanni* by ethanol extract. Moreover, petroleum ether extract showed better activity (23, 17, 16 mm in diameter) against *P. aeruginosa, V. cholerae* & *S. aureus* respectively (Figure 2c). In case of *Murraya paniculata*, Ethanol extract showed better activity than others. It showed activity against eight pathogens where the largest range of zone of inhibition was 10mm in diameter against *C. albicans* (Figure 2d). Similar work was also done by many researchers having similar findings with ours[24-25].

Based on the highest range of zone of inhibitions and antimicrobial activities against a large numbers of pathogenic microorganisms, ethyl acetate extracts of *Mikania scandens and Mimosa pudica*, ethanol extract of *Murraya paniculata* and petroleum ether extract of *Syzygium aromaticum* were selected for further extraction process and other biological tests including quantitative antimicrobial activity, antibiofilm and membrane stabilization assays .

Phytochemical screening

Plant-derived chemicals have been considered as a prolific fount for drug discovery. Extraction of phytochemicals has recently established as a phenomenal subject matter in lead identification for active pharmaceutical moieties. The results of phytochemical study of the plant extracts revealed the presence of saponins in all extracts except *M. pudica* and *S. aromaticum* (Figure 3). Alkaloids and flavonoids were observed in all extracts except *M. scandens.* Betacyanin, carbohydrate found only in *S. aromaticum and M. paniculata* extracts*.* Tannin observed in all extracts except *M. pudica*. Altemimi A, revealed the presence of tannin, saponin, phenols, and phenol, saponin, alkaloid, flavonoid, anthocyanin, and steroid which align with our results[26].

Quantitative antimicrobial activities of plant extracts

For determining of quantitative antimicrobial activities of plant extracts, 500 µg(dry weight)/disc crude extract was used. After primary selection, the ethyl acetate crude extracts of *M. scandens* and *M. pudica* leaves, ethanol extract of *M. paniculata* leaves and petroleum ether extract of *S. aromaticum* flower bud were used for evaluating their antimicrobial activities against all organisms. The results of the antimicrobial activities of the crude extracts at a concentration of 500 µg(dry weight)/disc are presented in Table 1. Therefore, all the plant extracts except *M. pudica* exhibited antimicrobial activities against all the pathogenic organisms tested herein. However, ethyl acetate extract of *M. pudica* did not display antimicrobial activity against *E. coli, K. pneumoniae, S. abony, B. subtilis* and *V. cholerae* at the concentration of 500µg/disc. The largest zone of inhibition exhibited by *M. pudica* against *S. aureus* was 15 mm in diameter. Furthermore, the ethyl acetate extract of *M. scandens* and methanol extract of *M. paniculata* leaves displayed the ranges

Figure 2. *Antimicrobial activity of five crude extracts (Petroleum ether, ethyl acetate, Chloroform, Carbon tetrachloride and Ethanol extracts) of (a) Mikania scandens, (b) Mimosa pudica, (c) Syzygium aromaticum and (d) Murraya paniculata Zone of inhibition in mm in diameter, 4 mm in diameter paper disks were impregnated with different solvents. The highest zone of inhibition of each extract labeled in the figure.*

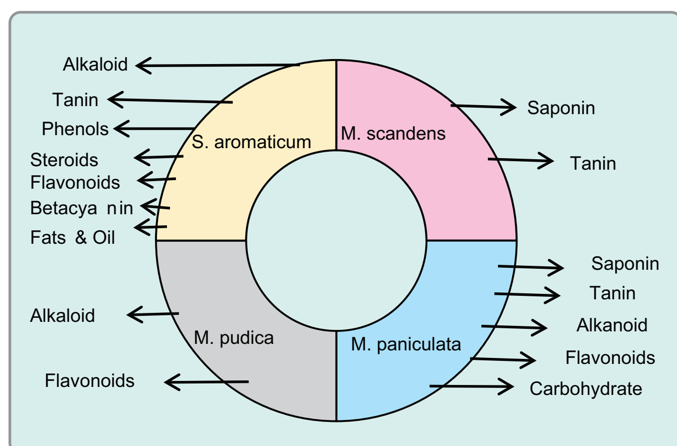

Figure 3. *Phytochemical screening showed the secondary metabolites present in the selected extracts of tested plants.*

of inhibition zones 12-25mm and 5-10 mm in diameters respectively against the organisms at 500 µg/disc extract. In case of *S. aromaticum*, the petroleum ether extract of flower bud (clove) showed good inhibitions against all the organisms ranges from 19-35 mm in dia. Where, the largest zone of inhibition was 35 mm against *B. subtilis* (Table 1). Fatema *et al.* recorded antimicrobial activity of 16 plant extracts showing zone of inhibition 5-22mm where the best antibacterial activity was 22.5 mm against *P. aeruginosa*[27]. According to Sukohar *et al.*, cloves essential oil exhibited antimicrobial activity 19 - 35mm against pathogenic gram-positive and gram negative isolates which is exactly matched with our findings about cloves oil[28].

MIC and MBC of the crude extracts

MIC and MBC values of the antimicrobial agents preliminary provides information whether the concentrations are either bacteriostatic or bactericidal. The MIC and MBC of crude extracts were determined by macro dilution broth technique[18] and LB broth medium was used for bacterial and fungal culture.

MIC and MBC values of the crude extracts were determined using the concentrations ranging from 500 to 5000 µg/ml (w/v) with an interval of 500 µg/ml extract. The lowest MIC value was determined

Table 1: *Antimicrobial activity of crude extracts of M. scandens, M. paniculata, M. pudica, and S. aromaticum at 500µg/disc extract against human pathogenic microorganisms.*

Test organisms	Zone of inhibition (mm in diameter) at 500µg/disc extract			
	M. Pudica	*M. scandens*	*M. paniculata*	*S. aromaticum*
Escherichia coli	-	15	10	30
Klebsiella pneumoniae	-	15	7	25
Staphylococcus aureus	15	20	8	25
Salmonella abony	-	21	10	19
Acinetobacter baumannii	6	15	5	23
Bacillus cereus	5	21	8	20
Bacillus subtilis	-	20	5	35
Pseudomonas aeruginosa	10	25	10	33
Vibrio cholera	-	12	10	23
Shigella dysenteriae	10	16	7	20
Candida albicans	10	15	10	20

Note: Bold indicates highest zone of inhibition and (-) means no inhibition

as 1000 µg/ml with *S. aromaticum* extract against *A. baumanni, B. subtilis* and *P. aeruginosa*; therefore, the lowest MBC was 2500 µg/ml against *P. aeruginosa*. The *M. scandens* extract exhibited the lowest MIC 1500 µg/ml against *E. coli, B. cereus, B. subtilis* and *P. aeruginosa* and the lowest MBC 3500 µg/ml against *B. cereus, B. subtilis, V. cholerae* and *C. albicans*. In case of *M. paniculata* extract, the lowest MIC and MBC were recorded as 2000 µg/ml and 4000 µg/ml against *B. subtilis* and *V. cholera* respectively. Similar MIC and MBC values of plant extracts have been reported previously by many researchers[24, 29]. The extract of *M. pudica* did not show any MIC and MBC values up to 2500 µg/ml and 5000 µg/ml respectively (Table 2). However, Singh *et al.* reported the lowest MIC values of *Mimosa pudica* leaf extract against *E. coli and Bacillus subtilis* at the concentrations of 250 mg/ml and 200mg/ml respectively[30], thus, these observations are also in congruent with our work.

The MIC and MBC values of *Mimosa pudica* extracts were undetected up to 2500 µg/ml and 5000 µg/ml extract concentrations respectively. For time-kill bacterial susceptibility, antibiofilm and membrane stabilization activity assays of plant extracts, known concentrations of MIC and MBC are needed, thus, further experiments were done only for *Mikania scandens, Murraya Paniculata* and *Syzygium aromaticum* extracts.

Time-kill Bacterial Susceptibility

Time-kill test is a method for determining the time for bactericidal or fungicidal effect of an antimicrobial agent. This Time-kill Bacterial Susceptibility test is used for obtaining information of dynamic interaction between the antimicrobial agent and the microbial strain. Assays for the rate of *killing bacteria* by the ethyl acetate extracts of *Syzygium aromaticum* flower bud and *Mikania scandens* leaves, and ethanolic extract of *Murraya*

Table 2. *Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Plant extract against human pathogenic bacteria.*

	Name of bacteriaMinimum Inhibitory Concentration (MIC) (µg/ml)							
	M. scandens		*M. paniculata*		*M. pudica*		*S. aromaticum*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Escherichia coli	1500	4000	2500	5000	NF	NF	1500	3500
Klebsiella pneumoniae	2000	5000	NF	NF	NF	NF	1000	3000
Staphylococcus aureus	1500	4000	NF	NF	NF	NF	1000	3000
Salmonella abony	2000	5000	2000	NF	NF	NF	2000	3500
Acinetobacter baumannii	2000	4000	NF	NF	NF	NF	1000	4000
Bacillus cereus	1500	3500	2500	5000	NF	NF	1500	3500
Bacillus subtilis	1500	3500	2500	4000	NF	NF	1000	3500
Pseudomonas aeruginosa	1500	4000	2000	5000	NF	NF	1000	2500
Vibrio cholera	2000	3500	2000	4000	NF	NF	1500	3500
Shigella dysenteriae	2000	4000	NF	NF	NF	NF	1500	3500
Candida albicans	2000	3500	2000	5000	NF	NF	2000	4000

Note: NF= MIC not found up to 2500 µg/ml concentrations, and MBC not found up to 5000 µg/ml concentrations.

paniculata leaves were carried out against a gram negative bacteria *E coli* and a gram positive bacteria *B. cereus* using turbidometric method by measuring absorbance (OD_{600}).

Time-kill exhibited the inhibitory effects and *killing* of bacterial pathogens by the MBC concentrations of the *crude extracts and the results are presented in Figure 5 and 6.* The time kill activity showed that exposure of pathogens to the *S. aromaticum, M. scandens* and *M. paniculata* extracts at MBC concentration reduced the absorbance (Figure 5 and 6). A standard antibiotic Ciprofloxacin produced similar alteration in the absorbance of these bacterial cells. In this study it showed that the killing of bacterial cell is time dependent. To determine the time need to kill bacteria, we found that only *S. aromaticum* extract could kill pathogens (*E. coli* and *B. cereus*) in 180 minutes at their MBC concentration (Figure 5a and 6a) whereas, *M. scandens* ((Figure 5b and 6b) and *M. paniculata* (Figure 5c and 6c) extracts decreased the bacterial cell number but didn't kill completely in 180 minutes. Figure 5 and 6 showed that after 3h of incubation, the absorbance of *E.coli* and *B. cereus* by *S. aromaticum* were 0.22 and 0.30 respectively. In case of *M. scandens* extract, the absorbance were 0.3 and 0.39 with *E.coli* and *B. cereus* respectively after 180 minutes. Consequently, *M. paniculata* extract also showed bacterial cell absorbance of 0.4 in 180 min. These results indicate that more time is needed to kill gram positive bacterial cells compared to that of gram negative bacteria. Remarkably, ciprofloxacin had a faster rate of bacterial killing during the first incubation period than plant extracts; however, as the incubation duration progressed, *S. aromaticum* extract's killing rate exceeded compared to that of the antibiotic (Figure 5a and 6a). On the other hand, *M. paniculata* and *M. scandens* extracts exhibited less activity for killing bacterial cells compared to that *S. aromaticum* within 180 minutes (Figure 5 and 6). Similar time-kill susceptibility assay has been reported by Ajiboye *et al.*[19] where they described that the extract lowered the absorbance (0.3) when compared to bacterial cells exposed to only DMSO (0.88), and gram positive bacteria needs more time to kill than gram negative bacteria which is may be due to thick peptidoglycan layer presence in their cell wall. This finding also agreed with our results.

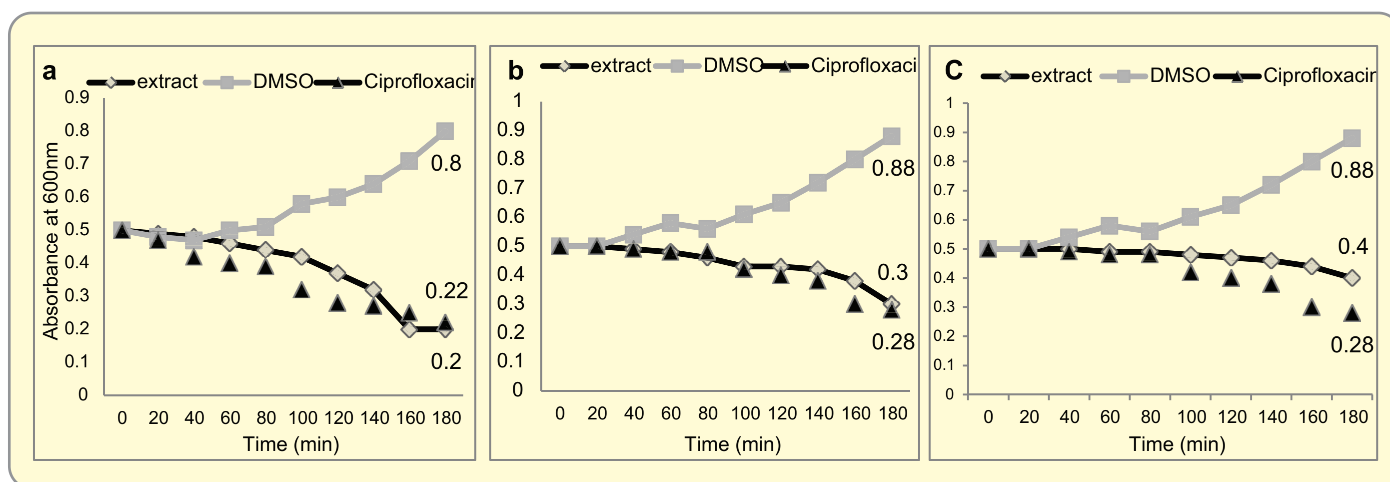

Figure 5: Optical densities of *Escherichia coli* (Gram negative) exposed to extract of (a) *Syzygium aromaticum,*(b) *Mikania scandens* and (c) *Murraya paniculata*

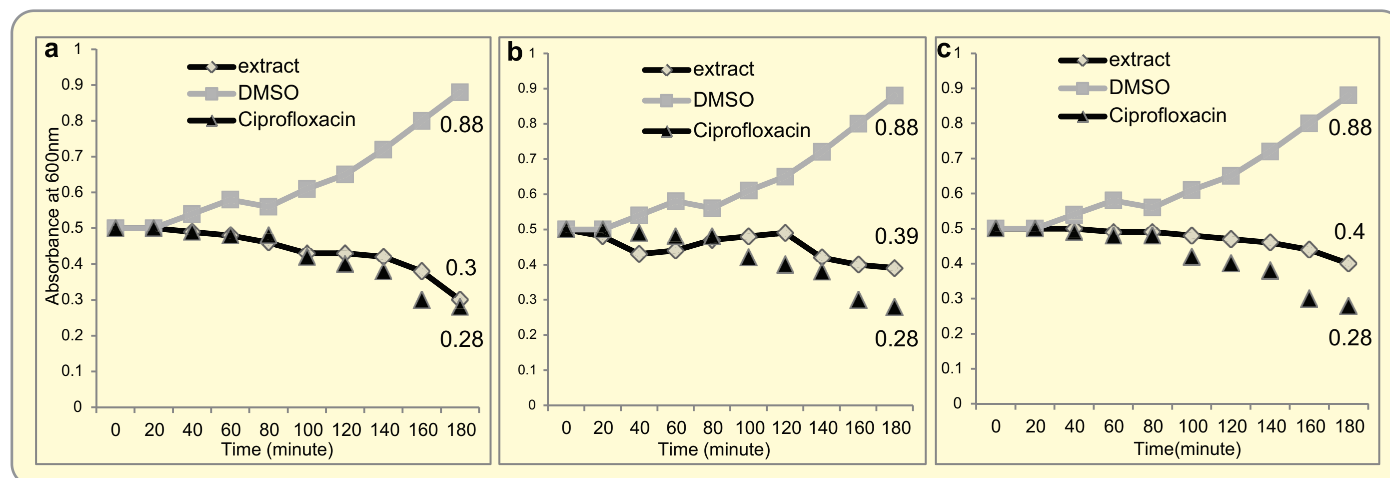

Figure 6: Optical densities of *Bacillus cereus* (Gram positive) exposed to extract of (a) *Syzygium aromaticum,*(b) *Mikania scandens* and (c) *Murraya paniculata*

Antibiofilm activity

For determining the antibiofilm activities of the plant extracts, MIC (1.0×MIC), half MIC (0.5×MIC) and double MIC (2.0×MIC) concentrations of each extract were used. Antibiofilm activities of the ethyl acetate extracts of *Syzygium aromaticum* flower bud and *Mikania scandens* leaves, and ethanolic extract of *Murraya paniculata* leaves were determined *against* four biofilm-producing bacterial pathogens *E. coli, K. pneumoniae, P. aeruginosa* and *S. aureus*, and the results are shown in Figure 7. From Figure 7a, it was appeared that the *M. scandens* extract at the concentration of half MIC inhibited 11-24% biofilm formation of all the four bacterial strains. However, more than 50% inhibition of *E. coli* biofilm formation was attained at the MIC concentration and at double MIC concentration inhibited almost 80% biofilm formation of *E. coli* and *P. aeruginosa* (Figure 7a). The *M. paniculata* extract showed 28-34% inhibition of biofilm at MIC and double MIC concentrations. Though, at 0.5×MIC concentration, it couldn't inhibit the biofilm formation of *K. pneumoniae* and *P. aeruginosa* but showed a little inhibition of *E.coli* (27%) and *B. cereus*(18%) biofilm formation (Figure 7b). Furthermore, *S. aromaticum* extract at concentrations of half of MIC inhibited more than 50% biofilm formation of all the bacterial pathogens except *S. aureus* (Figure 7c). At a concentration of double MIC, *S. aromaticum* inhibited almost 90% and 100% biofilm formation of *K. pneumoniae* and *E. coli* respectively (Figure 7c). Similar antibiofilm activities of other plants extracts were also done by other researchers and they found to inhibit 70-90% biofilm formation [31-32].

Membrane Stabilization Activity

Inflammation is triggered by wounds, infections, or other stimuli (hypotonic solution), which in turn activates lysosomal enzymes or inflammatory cytokines leads to enlarged cell membranes, cause vasodilation, hemoglobin oxidation and increased permeability of blood vessels[23]. Nearly identical to lysosomal membranes, human red blood cell (HRBC) or erythrocyte membranes were used in stabilization assays to determine the anti-inflammatory effects of extracts to get rid of issues likely caused by conventional use of NSAIDs, such as hypertension, stroke, heart failure, acute myocardial infarction, and renal failure[33].

Plant extracts illustrated the dose dependent membrane stabilization at the concentrations of 25-200 ìg/ml (Figure 8). The results were compared with a standard drug (Diclofenac sodium). From the comparison with standard drug it was observed that the concentration of 100 ìg/ml of *S. aromaticum* extract showed maximum activity (100%) (Figure 8b), therefore, the extract of *M. scandens* and *M. paniculata* showed 92% (Figure 8a) and 32% (Figure 8c) activity respectively at 100 µg/ml concentration. Stimulatingly, the standard drug showed less membrane stabilizing activity (57.7%) compared to that of *S. aromaticum and M. scandens* extracts. In case of *M. paniculata* extract, the range of membrane stabilizing activity was 20.5-52.7% at 25 – 200 ìg/ml extracts (Figure 8c). Therefore, our study showed that extracts of *M. scandens* and *S. aromaticum* significantly protect human blood erythrocyte membrane against lysis induced by hypotonic solution. Tantary *et al.*, Umukoro and Ashrobi carried out identical research and figured that the plant extracts they inspected attenuated RBC hemolysis by 70–90%[34,35]. By preventing the expulsion of certain protease and bactericidal enzymes like activated neutrophils lysosomal components, these plant extracts perhaps capable of stabilizing lysosomal membranes or controlling key pro-inflammatory cytokines.

The findings of these studies suggest that plant extracts could be considered as a potential source of natural antimicrobial and anti-inflammatory agents for the development of new drugs and therapeutic agents. However, further research is needed to explore the full potential of this plant and its constituents in the prevention and treatment of various microbial infections and other diseases.

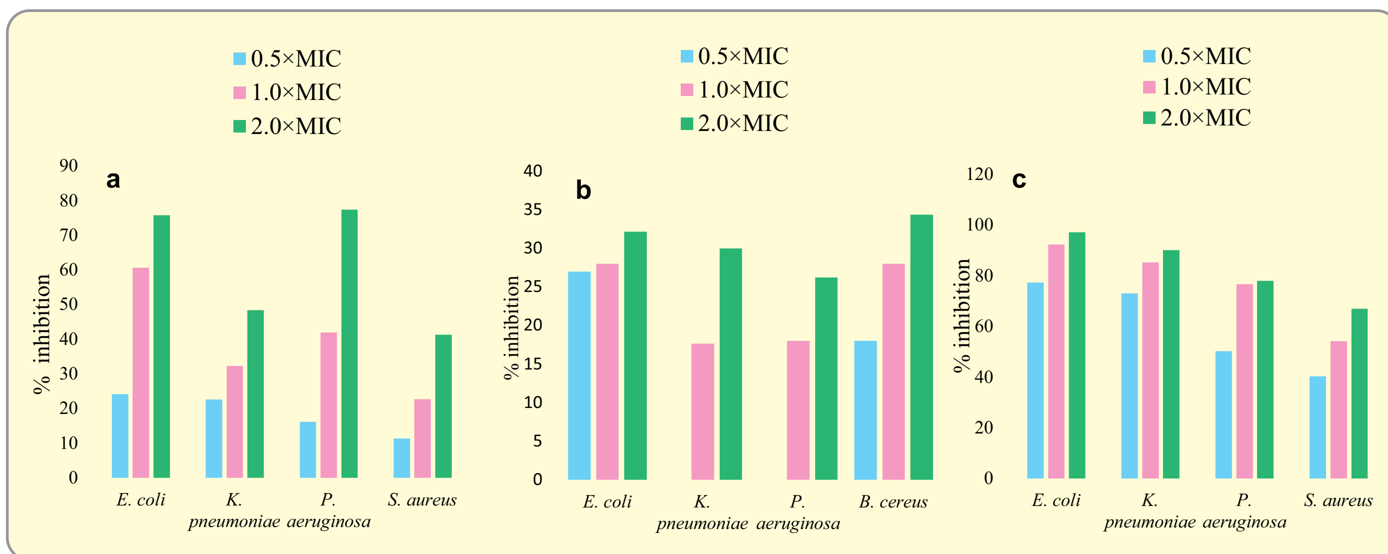

Figure 7. *Antibiofilm activity of plant extracts a) M. scandens b) M. paniculata and c) S. aromaticum against Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa and Staphylococcus aureus.*

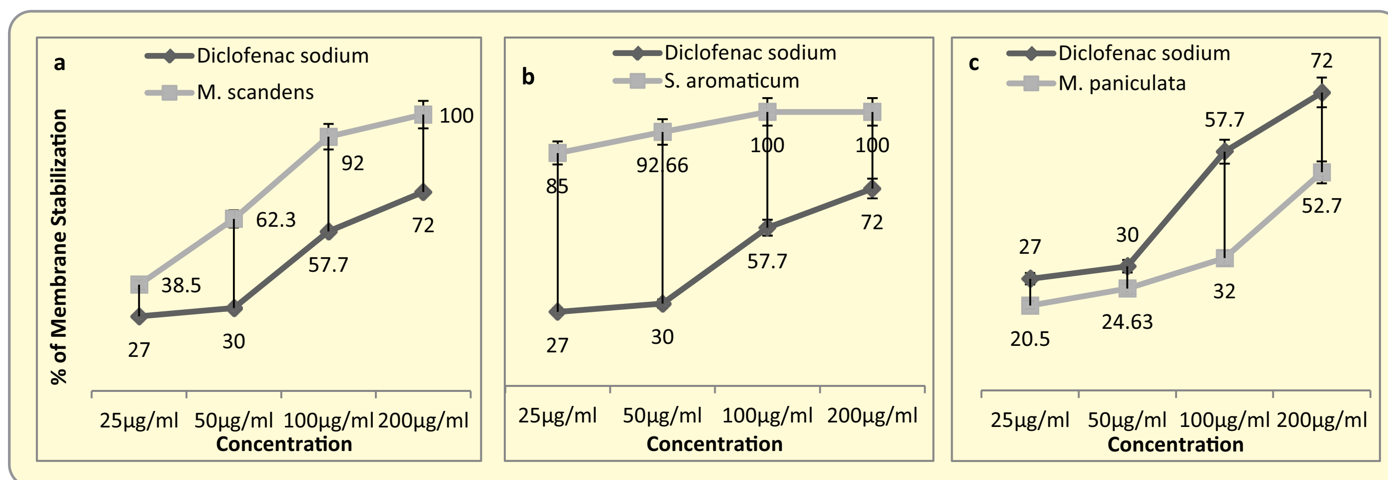

Figure 8. *Effect of (a) M. scandens, (b) S. aromaticum and (c) M. paniculata extracts on RBC membrane stabilization and comparison of activity with standard drug (Diclofenac Sodium) at different concentration of 25 – 200 ìg/ml.*

Conclusion

The study is to validate the application of plant extracts as the antimicrobial as well as antibiofilm and membrane stabilizing substance in conventional folk medicine in Bangladesh. In our investigation, it is clear that the extracts of *M. scandens* and *S. aromaticum* could be the alternative source of antimicrobial drugs against several pathogenic strains including biofilm producing microorganisms. It is additionally assumed that the antimicrobial effect may be associated with flavonoids, tannins, saponin, and steroids which also provoke anti-inflammatory or membrane stabilizing activity. The extracts also showed a higher antibiofilm and membrane stabilizing activities which indicating that it is possible to isolate novel antibacterial agents from plant extract, capable of eradicating antibiotic resistant biofilm producing bacteria as well as can be used as complementary of NSAID drugs. Therefore, further investigation is required to isolate and identify active constituents or bioactive natural products which could represent a noteworthy alternative to commercial antibiotics and drugs helping treatment of human antibiotic resistant infections.

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