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In vitro antibacterial and antibiofilm activity of selected medicinal plants and spices extracts against multidrug resistant *Pseudomonas aeruginosa*

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Abstract
Pseudomonas aeruginosa is a human opportunistic pathogen known to build biofilm and is able to proliferate within the biofilm. Antibacterial substances are of natural origin, and it is thought that their influences on the environment are important and can be used as biological control agents. In the present study, multi-drug resistant and the high yielding biofilm *Pseudomonas aeruginosa* strains have been isolated from the hospital waste. Multi-drug resistance pattern was studied with disc diffusion assay using commercially available antibiotic discs. Ten isolates were multi-drug resistant among eighteen isolates. Modified crystal violet staining method was performed to know the ability of biofilm formation by isolated strains. All of the isolates were able to form biofilm except one. Antibiofilm and Antimicrobial activity of medicinal plants *Centella asiatica* (Thankuni), *Mentha spicata* (Mentha), *Azadirachta indica* (Neem), *Psidium guajava* (Guava) and spices *Syzygium aromaticum* (Cloves), *Cinnamomum zeylanicum* (Cinnamon) extracts were studied with modified crystal violet biofilm assay and Well diffusion assay respectively. Both Methanol and ethyl acetate extract of guava leaf showed lowest antibacterial activity against all selected isolates. MIC values were determined for each extracts and it was ranged from 500 µg mL^{-1} to 2000 µg mL^{-1}. Methanol and ethyl acetate extract of Cinnamon, Neem and Mentha showed high antibiofilm activity suggested that these extracts might act as a potential antibiofilm agent against *Pseudomonas aeruginosa*. Despite of the fact that the extracts were not pure compounds, antimicrobial activity as well as antibiofilm activity were observed. This recommends the potency of these extracts and could be a target for further research to search antibiofilm bioactive compound for therapeutic uses.

Keywords: medicinal plants, spices extracts, multidrug, *Pseudomonas aeruginosa*

1. Introduction
Pseudomonas aeruginosa is a human opportunistic pathogen and it has been emerging as a primary source of nosocomial infections [1], including infections of artificial implants, contact lenses, urinary cathetersacheal tubes [2]. Biofilm is a community of cells attached to either a biotic or abiotic surface enclosed in a complex exopolymeric substance (EPS) [3]. *Pseudomonas* is known to build biofilm and is able to proliferate within the biofilm. Within the biofilm the bacteria are protected from the immune system, from antibiotics and, outside the body, from other adverse environmental factors [4]. The misuse and abuse of antibiotics are recognized to create selective pressure, resulting in the widespread development of resistant bacterial strains [5, 6]. Facing these limitations of antibiotics, there is an increasing need for the discovery and the development of antimicrobial agents that present novel or unexplored properties to efficiently control and manage bacterial infectious diseases [7]. Inhibition of bacterial virulence and/or biofilm formation by targeting nonmicrobicidal mechanisms are examples of increasingly explored antipathogenic approaches [8-11]. According to World Health Organization, to obtain a variety of drugs against many diseases, medicinal plants are one of the potential targets. Among the developed countries, about 80% people use medicinal plants derived traditional medicine. In this post antibiotic era where there is increasing multi drug resistant pathogens, research work on medicinal plants should be carried out to better understand their safety, properties and efficiency [12]. In the recent past, there has been an increased interest in the therapeutic properties of some medicinal plants and natural compounds which have demonstrated for their antibacterial and antibiofilm activities. Present study aimed to isolate multi-drug resistant biofilm forming *Pseudomonas aeruginosa* strains and screening of medicinal plants *Centella asiatica* (Thankuni), *Mentha spicata* (Mentha), *Azadirachta indica* (Neem), *Psidium guajava* (Guava) and spices

Sygizium aromaticum (Cloves), *Cinnamomum zeylanicum* (Cinnamon) extracts for their antibacterial and antibiofilm property against multi-drug resistant *Pseudomonas aeruginosa* strains.

Materials and Methods

1.1. Isolation of *Pseudomonas aeruginosa.* Multidrug resistant clinical isolates of *Pseudomonas aeruginosa* were obtained from the hospital waste of Islamic University, Kushtia, Bangladesh. Isolates were identified as *P. aeruginosa* by Gram staining, its pearlescent appearance on *Pseudomonas* agar followed by morphological tests, staining as well as biochemical tests such as Methyl red, Voges Proskauer, Indole, Catalase and Oxidase.

1.2. *Antibiotic sensitivity* Test. Susceptibility of the isolates to commercially used antibiotics was determined by the agar diffusion techniques according to CLSI guideline [13]. Single colony of each isolates were grown in Luria-Bertani (LB) broth separately for 18-24 h and then used to prepare the bacterial inoculums with the turbidity of 0.5 McFarland standard (equal to 1.5×10^8 colony forming units (CFU)/ml). Turbidity of the bacterial suspension was measured at 600 nm. The bacterial inoculum was spread onto Mueller-Hinton agar (MHA) plates (150 mm diameter) using sterile cotton swabs as a lawn culture. Up to Nine commercially used antibiotic disks were placed on the inoculated agar surface. Plates were incubated for 24 h at 37°C prior to determination of results. The diameters of zones of inhibition around the discs were measured in millimeter (mm). Multi-drug resistant (MDR) was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories. The experiment was performed in triplicates.

1.3. Biofilm formation Assay. Biofilm formation assay was carried out by the modified method of crystal violet staining assay in a test tube made with glass [14]. Fresh single colony of the bacterial strain was picked up and grown in LB broth medium for 18-20 h. Bacterial suspensions were added to 5 ml fresh LB broth by maintaining initial absorbance 0.02 at 600 nm and incubated at 37 °C for 36 h in static condition. Only LB broth was also incubated as a negative control. Following 36 h of adhesion and biofilm formation, planktonic cells were removed from the test tube followed by rinsed (twice) with 5 ml distilled water. 5 ml of crystal violet solution (1% w/v) was added to stain the biofilm and incubated at room temperature for 30 min. Excess stains were then washed with 5 ml distilled water twice. The test tubes were then dried in air for 20 to 30 min. The attached dye was solubilized with 95% ethanol and the adherent biofilm was determined by measuring the optical density at 490 nm. LB broth was used as a negative control (background absorbance). All isolates were tested at least three times in triplicate. For interpretation of the biofilm results, the isolates were classified as follow: non-producing, weak, moderate and strong-producing, based on the following optic density (OD) average values: OD(isolate) ≤ OD(control) = non-biofilm producing; OD(control) ≤ OD(isolate) ≤ 2OD(control) = weak producing; 2OD(control) ≤ OD(isolate) ≤ 4OD(control) = moderate producing; 4OD(control) ≤ OD(isolate) = strong producing [15].

1.4. Plants and Spices material. Leaves of plants *Centella asiatica* (Thankuni), *Mentha spicata* (Mentha), *Azadirachta indica* (Neem) and *Psidium guajava* (Guava) were obtained from local region of Kushtia, Bangladesh. Spices *Syzygium aromaticum* (Cloves), and *Cinnamomum zeylanicum* (Cinnamon) were bought from local market of Kushtia, Bangladesh. Plants and spices were identified by prominent botanist Dr. Nilufa Akhter Banu, Department of Biotechnology and Genetic Engineering, Islamic University, Kushtia. The ethnomedicinal data of used plants and spices which are used in this study is given in Table 1.

1.5. Preparation of plants and spices extract. All collected plants and spices material were air dried at room temperature under shade and then ground with a grinder in to fine powders. The powder materials were macerated with methanol and ethyl acetate solvent separately using a ratio of 1 g (plant material) and: 10 ml (solvent) for 72 h. Flasks were agitated daily. Re-extraction was done for a further 24 h. The individual extracts were filtered through what man no. 4 filter paper. The filtrate, each case, was concentrated using a rotary evaporator at 40°C. Stock concentration of 2mg/ml of each dry extract in the excipient Dimethyl sulfoxide (DMSO) were prepared, sterile filtered with 0.22 µm micro filter (Millex ^{R} filter, Carl Roth, Karlsruhe, Germany) and then stored in the dark at 4°C.

1.6. Antibacterial Assay of Extracts. Well Diffusion method [16] was used to test the antibacterial activities of different extracts. Selected multi-drug resistant bacterial isolates were grown in LB broth for 18-20 h and prepared the bacterial inoculums by maintaining turbidity of 0.5 McFarland standard (equal to 1.5×10^8 colony forming units (CFU)/ml). Mueller-Hinton agar (MHA) plates (150 mm diameter) were prepared and the bacterial suspensions were spread over the surface. The wells (9 mm diameter) were made by using cork borer in MHA plates. Each well was loaded with 100 µl of different crude extracts and 100 µl DMSO (without extract) was loaded as negative control. Plates were incubated at 37 ^{0}C for 24 h. The diameters of zones of inhibition around the wells were measured in millimeter (mm). The experiment was performed in triplicates.

Table 1: Ethnomedicinal data of selected plants and spices for the antibacterial and antibiofilm activity evaluation

Common (local) name	Botanical name (family)	Parts used traditionally	Ethnomedicinal uses	Reported phytochemicals	Ref.
Thankuni	*Centella asiatica* (Apiaceae)	leaf	Wound healing, eczema, burn & scar treatment, treatment of periodontal disease, anti-stress & anti-anxiety medicine	Alkaloids, Flavonoids, Phenols, Saponins, Tannins, Anthraquinone, Steroids, Terpinoids and Cardiac glycosides.	[17]
Cloves	*Syzygium aromaticum* (Myrtaceae)	Flower buds	Antioxidant, anti-septic, local anesthetic, anti-inlammatory, carminative and antiflatulent properties.	Essential oils (acetyl eugenol, beta caryophyllene & vanillin), Tannins (gallotannic acid, methyl salicylate), Flavonoids (eugenin, eugenitin), Triterpenoids and Sesquiterpenes.	[18]
Cinnamon	*Cinnamomum*	Bark	Anti-inflammatory, anti-oxidant,	Essential oil (cinnamaldehyde) Alkaloids,	[19]

	zeylanicum (Lauraceae)		antimicrobial, anti-diabetic and anti-tumor properties.	Flavonoids, Terpenoids and Saponins.	
Mentha (Mint)	*Mentha spicata* (Lamiaceae)	Leaf, Stem & Flower	Fevers, headache, digestive disorders, bronchitis, ulcerative colitis, liver complaints	Flavonoids, Saponins, Cardiac glycoside, Reducing sugars and Steroids	[20]
Neem	*Azadirachta indica* (Meliaceae)	Leaves, flowers, seeds, fruits, roots & bark	Treatment of inflammation, infections, fever, skin diseases and dental disorders	Flavonoid, Alkaloid and Lipids	[21]
Guava	*Psidium guajava* (Myrtaceae)	Leaf	Treatment for infectious diarrhea	Flavonoid, Carotenoids, Anthocyanins, Flavonoids, Alkaloids, Tannins	[22]

1.7. Antibiofilm Assay of Extracts. A modified crystal violet assay was employed to test the effect of plant extract on bio-film formation [14]. Here, 200 µl of different crude extract was added separately with bacterial suspension and incubated at 37 °C for 36 h. Biofilm formation was determined by following the biofilm formation assay.

1.8. Determination of Minimum Inhibitory Concentration (MIC). Antibacterial activities of the extracts were first screened by agar-well diffusion method as described previously [16]. The MIC testing was performed against selected five MDR *Pseudomonas aeruginosa* strains by agar-well diffusion method. After preparing the bacterial inoculums by maintaining 0.5 McFarland standard, it was spreaded on MHA plate. The wells (9 mm diameter) were made by using cork borer in MHA plates. Each well was loaded with 100 µl of different concentration crude extracts ranged from 50 to 2000 µg/ml and 100 µl DMSO (without extract) was loaded as negative control. Plates were incubated at 37 ^{0}C for 24 h. MIC was regarded as lowest concentration that produce a visible zone of inhibition.

1.9. Statistics. Statistical analysis was performed using SPSS version 16.0 software 2007 (SPSS Inc., Chicago, IL). All experiments were performed in triplicates. For antibiofilm activity studies, mean values between extract treated and untreated samples were tested for significance by Student's *t*-test. The significant difference in biofilm reduction by different extracts was compared with the control (strain without the extract was normalized as 100%). The significant level was set at $P<0.5$.

2. Results

2.1. Disk Diffusion Assay. Multidrug resistance pattern of isolates was determined by measuring the diameters of zones of inhibition in millimeter (mm) around the discs by following CLSI guidelines [13]. Among the eighteen isolates, ten were multi-drug resistant. The results obtained from disk diffusion test are illustrated in Table 2.

2.2. Biofilm formation Assay. To know the ability of biofilm formation by isolated *Pseudomonas aeruginosa* strains, biofilm assay was carried out by the modified method of crystal violet staining assay. Status of biofilm production by isolates is shown in Table 2 and Table 3.

Table 2: Antibiogram of *Pseudomonas aeruginosa* isolates

	Zone of inhibition in diameter (mm)[a]									Biofilm
Isolate numbers	**CN 10 µg**	**K 30 µg**	**LEV 5 µg**	**CAZ 30 µg**	**AML 10 µg**	**TE 30 µg**	**E 15 µg**	**F 300 µg**	**RA 5 µg**	**forming capacity**
PS-1	25 (I)	25 (I)	30 (S)	10 (R)	0 (R)	ND	ND	ND	ND	M
PS-2	24 (I)	ND	26 (S)	10 (R)	0 (R)	0 (R)	0 (R)	ND	ND	St
PS-3	22 (R)	22 (R)	26 (S)	ND	0 (R)	0 (R)	0 (R)	ND	ND	St
PS-4	15 (R)	20 (R)	ND	0 (R)	0 (R)	ND	0 (R)	16 (R)	ND	St
PS-5	ND	17 (R)	ND	24 (I)	0 (R)	ND	0 (R)	10 (R)	0 (R)	St
PS-6	20 (R)	ND	ND	13 (R)	0 (R)	ND	20 (R)	ND	0 (R)	M
PS-7	ND	20 (R)	ND	12 (R)	ND	ND	0 (R)	11 (R)	ND	M
PS-8	16 (R)	ND	25 (S)	13 (R)	0 (R)	0 (R)	ND	ND	0 (R)	W
PS-9	15 (R)	ND	ND	10 (R)	ND	ND	0 (R)	0 (R)	0 (R)	St
PS-10	15 (R)	ND	ND	19 (S)	20 (R)	ND	0 (R)	17 (I)	0 (R)	W
PS-11	17 (R)	ND	ND	19 (S)	ND	ND	0 (R)	0 (R)	0 (R)	St
PS-12	ND	ND	ND	23 (S)	13 (R)	ND	0 (R)	0 (R)	13 (R)	W
PS-13	29 (S)	ND	ND	0 (R)	38 (S)	ND	0 (R)	ND	33 (S)	N
PS-14	19 (R)	ND	ND	0 (R)	14 (I)	ND	ND	0 (R)	13 (R)	M
PS-15	10 (R)	ND	ND	12 (R)	0 (R)	ND	ND	0 (R)	0 (R)	St
PS-16	16 (R)	ND	ND	0 (R)	0 (R)	ND	ND	23 (I)	0 (R)	St
PS-17	19 (R)	ND	ND	10 (R)	12 (R)	ND	ND	0 (R)	0 (R)	St
PS-18	19 (R)	ND	ND	0 (R)	30 (S)	ND	ND	0 (R)	0 (R)	St

The zone of inhibition is mean of three replicates for each microbial strain. mm=Millimeter, ND=Not done, R=Resistant, S=Sensitive, I= Intermediate, CN= Gentamycin, K=Kanamycin, E=Erythromycin, LEV= Levofloxacin, CAZ= Ceftazidime, TE= Tetracycline, AML= Amoxycillin, RA= Rifampicin, F=Nitrofurantoin, St=Strong, M=Moderate, and N=Not producing.

Table 3: Status of biofilm production by *Pseudomonas aeruginosa*

	Biofilm Status			
Number of isolates	**Non producing**	**Weak**	**Moderate**	**Strong**
18	1	3	4	10

2.3. Characterization of isolates: To characterize the selected isolates, different staining, morphological characteristics analysis and biochemical tests were performed. Results are shown in Table 4, Table 5 and Table 6.

Table 4: Shape and arrangement of cells of selected isolates

Isolates	Simple Staining	Gram Staining	KOH Test	Shape of Cells
PS 2	Single	Purple (-ve)	Sticky (-ve)	Rod
PS 5	Single	Purple (-ve)	Sticky (-ve)	Rod
PS 9	Single	Purple (-ve)	Sticky (-ve)	Rod
PS 15	Single	Purple (-ve)	Sticky (-ve)	Rod
PS 18	Single	Purple (-ve)	Sticky (-ve)	Rod

Table 5: Morphological characteristics of selected bacterial isolates

Features	Bacterial Isolates				
	PS 2	PS 5	PS 9	PS 15	PS 18
Shape	Irregular	Irregular	Irregular	Irregular	Irregular
Size	Large	Large	Large	Large	Large
Surface	Smooth	Smooth	Smooth	Smooth	Smooth
Texture	Mucoid	Mucoid	Mucoid	Mucoid	Mucoid
Elevation	Umbonate	Umbonate	Umbonate	Umbonate	Umbonate
Margin	Entire	Entire	Entire	Entire	Entire
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque
Color	Greenish	Greenish	Greenish	Greenish	Greenish
Fluorescence	Positive	Positive	Positive	Positive	Positive
Motility	Positive	Positive	Positive	Positive	Positive
Growth	Profuse	Profuse	Profuse	Profuse	Profuse
Gram reaction	Negative	Negative	Negative	Negative	Negative
Motility	Positive	Positive	Positive	Positive	Positive

Table 6: Biochemical characteristics of selected bacterial isolates

Biochemical tests	Bacterial Isolates				
	PS 2	PS 5	PS 9	PS 15	PS 18
Casein hydrolysis	+ve	+ve	+ve	+ve	+ve
Catalase test	-ve	-ve	-ve	-ve	-ve
Oxidase test	+ve	+ve	+ve	+ve	+ve
Methyl red test	-ve	-ve	-ve	-ve	-ve
Voges-Proskauer test	+ve	+ve	+ve	+ve	+ve
Indole production	-ve	-ve	-ve	-ve	-ve
H_2S test	-ve	-ve	-ve	-ve	-ve
Citrate test	+ve	+ve	+ve	+ve	+ve
Urease test	-ve	-ve	-ve	-ve	-ve
Starch hydrolysis	-ve	-ve	-ve	-ve	-ve
Glucose fermentation	-ve	-ve	-ve	-ve	-ve
Sucrose fermentation	-ve	-ve	-ve	-ve	-ve
Lactose fermentation	-ve	-ve	-ve	-ve	-ve

2.4. Antibacterial activity of plants and spices extracts. Antibacterial activity of medicinal plants Centella asiatica (Thankuni), Mentha spicata (Mentha), *Azadirachta indica* (Neem), *Psidium guajava* (Guava) and spices *Syzygium aromaticum* (Cloves), *Cinnamomum zeylanicum* (Cinnamon) extracts against five multi-drug resistant *Pseudomonas aeruginosa* strains were studied. Results are given in Figure 1 and Figure 2.

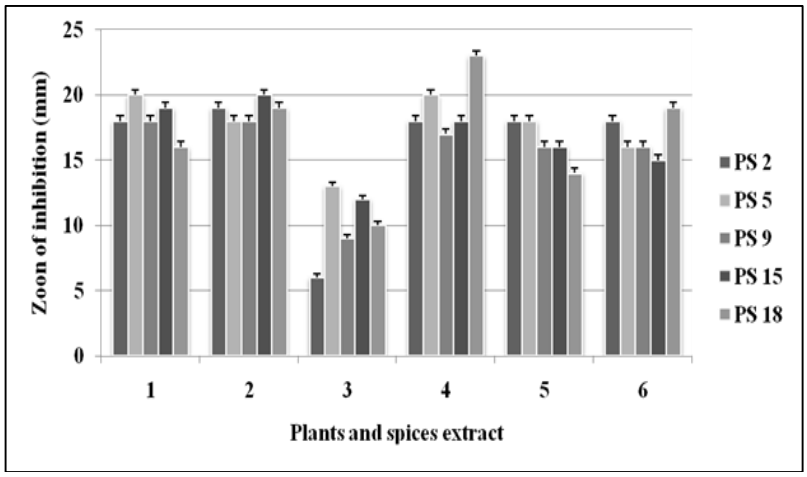

Fig 1: Antibacterial activity of methanol extracts of six medicinal plants against *Pseudomonas aeruginosa* isolates. PS 2, PS 5, PS 9, PS 15, and PS 18 : *Pseudomonas aeruginosa* isolates; 1= Cinnamon extract; 2=Cloves extract; 3=Guava leaf extract; 4=Meant leaf extract; 5=Neem leaf extract, 6=Thankuni leaf extract; Test was repeated three times, Results expressed as mean value with standard deviation

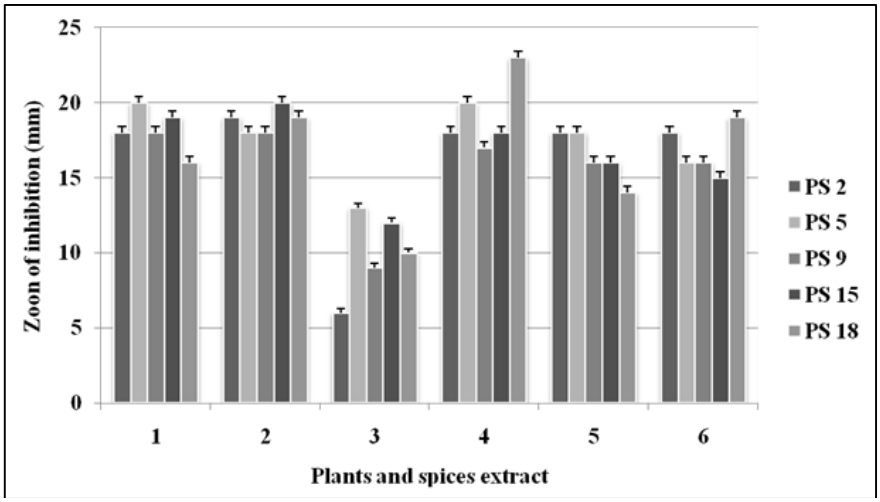

Fig 2: Antibacterial activity of ethyl acetate extracts of six medicinal plants against *Pseudomonas aeruginosa* isolates. PS 2, PS 5, PS 9, PS 15, and PS 18 : *Pseudomonas aeruginosa* isolates; 1= Cinnamon extract; 2=Cloves extract; 3=Guava leaf extract; 4=Meant leaf extract; 5=Neem leaf extract, 6=Thankuni leaf extract; Test was repeated three times, Results expressed as mean value with standard deviation

2.5. Antibiofilm Activity of plants and spices extracts. The anti-biofilm activity of medicinal plants *Centella asiatica* (Thankuni), *Mentha spicata* (Mentha), *Azadirachta indica* (Neem), *Psidium guajava* (Guava) and spices *Syzygium aromaticum* (Cloves), *Cinnamomum zeylanicum* (Cinnamon) extracts against four high biofilm forming multi-drug resistant *Pseudomonas aeruginosa* strains were studied with modified crystal violet biofilm assay. Results are given in Figure 3 and Figure 4.

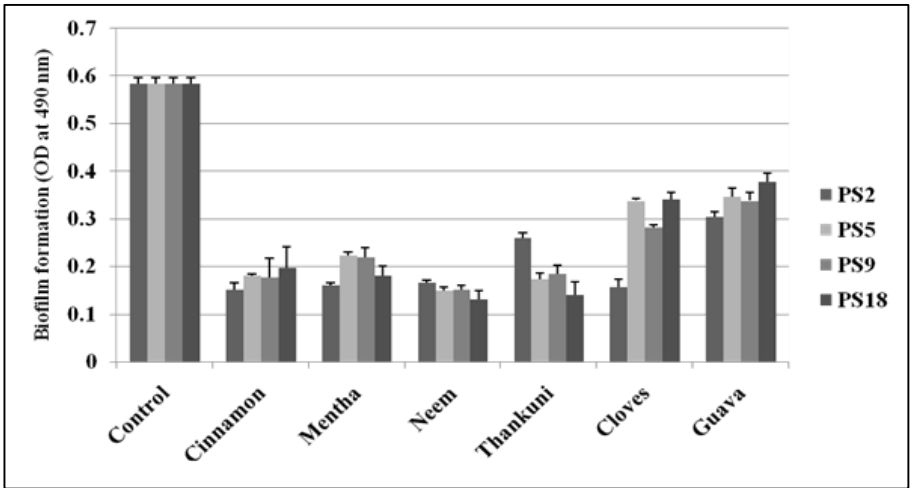

Fig 3: Antibiofilm activity of methanol extracts of six medicinal plants against *Pseudomonas aeruginosa* isolates. PS 2, PS 5, PS 9, PS 15, and PS 18: *Pseudomonas aeruginosa* isolates; Test was repeated three times, Results expressed as mean value with standard deviation

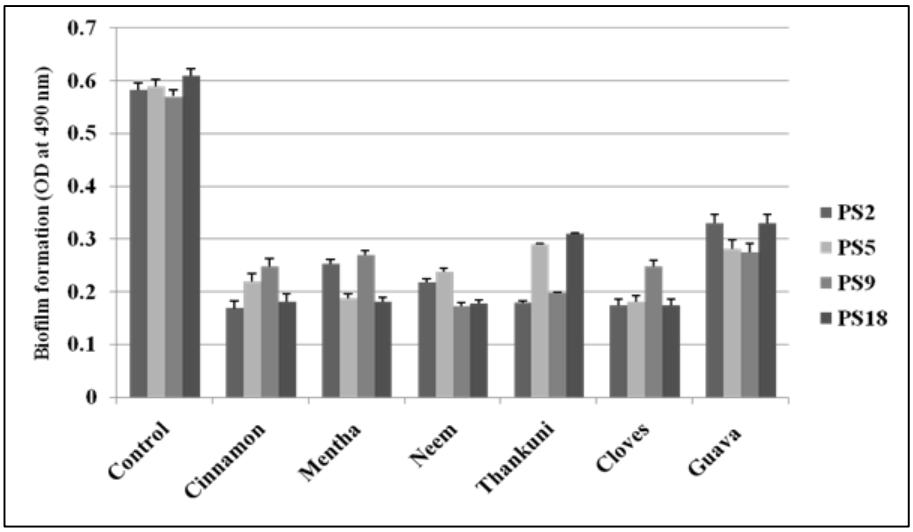

Fig 4: Antibiofilm activity of ethyl acetate extracts of six medicinal plants against *Pseudomonas aeruginosa* isolates. PS 2, PS 5, PS 9, PS 15, and PS 18: *Pseudomonas aeruginosa* isolates; Test was repeated three times, Results expressed as mean value with standard deviation

2.6. *Determination of Minimum Inhibitory Concentration (MIC).* The MIC was determined by agar-well diffusion method and the results are given in Table 4.

Table 7: MIC (µg mL^{-1}) of extracts against selected *Pseudomonas aeruginosa* isolates (PS 2, PS 5, PS 9, PS 15 and PS 18).

Solvent system	Extracts	*Pseudomonas aeruginosa* isolates				
		PS 2	PS 5	PS 9	PS 15	PS 18
Methanol	Cinnamon	500	500	1000	500	500
	Cloves	500	1000	1000	500	500
	Mentha	1000	500	1000	500	500
	Thankuni	1000	1000	1500	1000	1000
	Neem	1000	1500	2000	1500	1000
	Guava	1500	2000	2000	1500	1500
Ethyl acetate	Cinnamon	500	500	500	500	500
	Cloves	1000	500	1000	500	500
	Mentha	500	500	1000	500	500
	Thankuni	1000	1000	1500	1000	1000
	Neem	1500	500	1500	1000	500
	Guava	1000	2000	2000	1500	1500

3. Discussion

Although our understanding of *Pseudomonas aeruginosa* has advanced considerably over the last few years, this bacterium remains a scourge in hospitals, causing virulent and persistent infections despite antibiotic treatment. There is increasing evidence that biofilm-mediated infection facilitates the development of chronic infectious diseases and recurrent infections [23-25]. Relevance in using antibiofilm compounds is based on the restoration of antibiotic effectiveness by facilitating their penetration through compromised biofilm structure. Moreover, a degradation of the biofilm matrix could render infectious bacteria reachable to immune defenses (e.g., polymorphonuclear leukocytes, innate, and specific antibodies) [26-27]. Thus, antibiofilm compounds could be interesting antibiotic adjuvants to prevent or treat chronic infections. In Bangladesh, there are numerous medicinal plants described for treatment of many diseases. The hills and mountains of Bangladesh are covered with more than 500 plant species of which more than 100 are noted for their uses as medicinal herbs [28]. The antimicrobial compounds from plant source have increasing attention in recent years. Although there are many reports available on the antimicrobial properties of plants extracts, there are very few reports are available on the antibiofilm activities of plant extracts. Hence present study aimed to screen medicinal plants *Centella asiatica* (Thankuni), *Mentha spicata* (Mentha), *Azadirachta indica* (Neem), *Psidium guajava* (Guava) and spices *Syzygium aromaticum* (Cloves), *Cinnamomum zeylanicum* (Cinnamon) extracts for their antimicrobial and antibiofilm property against isolated multi-drug resistant *P. aeruginosa* strains. Antibacterial activity of six medicinal plant (*Centella asiatica*, *Syzygium aromaticum*, Cinnamomum zeylanicum, *Mentha spicata*, *Azadirachta indica*, *Psidium guajava*) extracted with different solvents viz methanol, and ethyl acetate were subjected to test against isolated *Pseudomonas* using the test tube and well diffusion method. We confirmed ten isolates as multidrug resistant *P. aeruginosa* strains among the eighteen isolates, results are shown in Table 2. All of the isolates except one were able to form biofilm, results are shown in Table 2. Five high biofilm forming strains were used in further study to screen the presence of antimicrobial and antibiofilm agent in selected plants and spices extract. Selected isolates were characterized through morphological and biochemical tests, results are shown in Table 4-6. Both Methanol and ethyl acetate extract of guava leaf showed highest antibacterial activity against all selected isolates (Figure 1 and Figure 2). Antibiofilm activity assay of methanol extract suggested that Cinnamon, Neem and Mentha extracts are strong antibiofilm agent whereas Thankuni and cloves extracts are moderate and guava leaf extract is a weak antibiofilm agent (Figure 3). Ethyl acetate extracts of Cinnamon, Neem and Mentha showed strong antibiofilm activity (Figure 4). Among the all extracts, Cinnamon spice and Neem plant extract have strong antibiofilm prperty (Figure 3 and Figure 4). There are some reports on plant derive compounds which have antibiofilm activity. Ajoene, an allyl sulfide isolated from garlic (*Allium sativum* L.), has been reported to synergizes with the antibiotic tobramycin in killing biofilm-encapsulated *P. aeruginosa* [29]. Extracts of Ginger (*Zingiber officinale* Rosc.), long used by Indians, Asians, and Arabs to treat numerous ailments [30], inhibit *P. aeruginosa* PA14 biofilm formation [31]. The phenolic compound curcumin, a major constituent of turmeric roots (*Curcuma longa* L.), down regulates virulence factors (pyocyanin, elastase, and protease) in *P. aeruginosa* PAO1 and inhibits adherence of the bacteria to polypropylene surfaces [32]. A recent study revealed that clove extract (*Syzygium aromaticum* (L.) Merr. Et Perry) inhibits QS-controlled gene expression thereby inhibit biofilm formation in *P. aeruginosa* with eugenol as major active constituent [33]. Though Guava leaf extract showed high antibacterial activity but it showed weak antibiofilm activity. On the other hand Neem plant extract showed strong antibiofilm activity and this antibiofilm activity not by antibacterial effect. Neem plant extract might have quorum quenching compound which inhibit QS and thereby inhibit biofilm formation. In this study we observed MIC value 500 µg mL^{-1} in case of Cinnamon extract against 4 isolates among the studied 5 isolates, MIC values were varied from 500 µg mL^{-1} to 2000 µg mL^{-1} for different extracts against different isolates (Table 7). It seems MIC values were quite high, but it can be feasible for crude extract. The observed MIC value for Cinnamon and Cloves are almost similar or lower in range reported by Md. Mahfuzul Haque *et al* [34]. Although certain number of extracts exhibited good antibacterial potency, in contrary to our expectation, a limited potency of some extracts were also observed. Although we have shown the potent *in vitro* activity of few extracts (*e.g.* Cinnamon, Neem and Mentha) against *P. aeruginosa*, we are not certain about if such activity will be translated *in vivo*. Future epidemiological studies are necessary to understand the effectiveness of use of extracts from such medicinal plants in population.

4. Conclusions

Currently, researchers are focused on the therapeutic and pharmacological effects of natural products of plant origin. In conclusion, the findings of this study highlight the antibiofilm and antibacterial activity of some plants and spices extract against multidrug resistant *P. aeruginosa* strains. Complete removal of microbial biofilms still remains a crucial step and a great challenge for clinicians and researchers. Natural antibiofilm agents which are ecologically safe and less hazardous than synthetic compounds are promising target to fight against infectious disease. Our study suggested that Neem, Cinnamon, and Cloves have strong antibacterial and antibiofilm agents. The extracts of these plants and spices should be further analyzed to identify the specific bioactive compounds from them and toxicity studies of the bioactive compounds should also be done to determine the safety indices of the extracts.

5. Conflict of interests

The authors have no conflict of interests.

6. References

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