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Original Article

Anti-biofilm activity of plant derived extracts against infectious pathogen-*Pseudomonas aeruginosa* PAO1

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ABSTRACT

Background: Biofilm forming ability of *Pseudomonas aeruginosa* make them vulnerable, because it makes them recalcitrant against various antibiotics. Quorum sensing (QS) is cell density based signaling that helps in bacterial cell–cell communication, which regulated various virulence factors such as pigment and biofilm formation that contribute in the establishment of chronic infections. The interruption of QS is one of the effective approach to control various virulence factors. Present study was intended with the aim to authenticate antibiofilm potential in different solvents based extracts of selected medicinal plant species viz. *Berginia ciliata*, *Clematis grata* and *Clematis viticella* traditionally used by the inhabitants of Himalayan region of Pakistan to treat various pathogenic diseases. *P. aeruginosa* PAO1, an opportunistic pathogen and involves in various life-threatening infections specifically in immune deficient patients was used as a model pathogen.

Methods: Plants were extracted in various organic (ethanol, methanol, acetone, ethyl acetate, hexane, chloroform) as well as in aqueous solvents and their ability to inhibit biofilm was measured. Biofilm of PAO1 was grown in Jensen's medium while growing at 30 °C and crystal violet assay was performed to assess the biofilm inhibiting activity of plant extracts.

Results: Solvents play a vital role in extraction of plant components and it was found that the plants in various solvents exhibit different activity against the PAO1 biofilm. Comparatively, 1% methanolic extract of *B. ciliata* (rhizome with skin), showed more than 80% inhibition of biofilm formation without effecting on the growth of the bacterium. Significant correlation between flavonoids content and antibiofilm activity in methanolic extract revealed the contribution of secondary metabolites in *P. aeruginosa* (PAO1) biofilm inhibition.

Conclusion: Our study revealed that plants under investigation more specifically *B. ciliata* could be a potential candidate for drug discovery to treat *P. aeruginosa* PAO1, induced infectious diseases especially for its biofilm treatment.

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Introduction

Biofilms are complex microbial communities embedded in an extracellular matrix composed of proteins, extracellular DNA (eDNA), lipids, and exo-polysaccharides [1,2]. Biofilm mode of growth of bacteria provide several advantages, one of the most important characteristics of microbial biofilms is that the bacteria stay in microenvironment as long as the conditions are favorable [3]. Mostly in biofilms bacteria account less than 10% of their dry mass, whereas matrix can account over 90%. The matrix produced by the organisms themselves and composed of different types of biopolymers, known as extracellular polymeric substance (EPS) [4]. EPS helps the bacterial cell to live in close proximity and interact, which behave profoundly differ from their planktonic counter parts [5]. These EPS provide an extra covering around the cells providing a shield against various stresses, thus bacteria within the biofilm are recalcitrant to antibiotics, environmental stresses and even escaped host immune response, therefore cause serious problems for industrial as well as in clinical settings [6,7].

Pseudomonas aeruginosa PAO1 is known to be a source of serious life-threatening infections in patients with compromised immune system like AIDS (Acquired Immune Deficiency Syndrome) and cancer, urinary-tract infections (UTI), burn victims, and hospital-acquired pneumonia in patients on respirators; rarely targeting the organisms with intact immune systems. Biofilm forming ability of *P. aeruginosa* makes it more vulnerable as it become resistant to traditionally used antibiotics. *P. aeruginosa* PAO1 biofilms are of significant matter in the pathogenesis of bacterial keratitis, otitis externa, UTI, ventilator-associated pneumonia and burn infections. UTI, catheter infections and formation of dental plaque are the common infections caused by biofilm while cystic fibrosis is documented as the most lethal infection [8]. It is the prominent root cause of mortality (40–60%) in cystic fibrosis patients, where it colonizes the compromised airways epithelia of the lungs, being impossible to eradicate, leading to pulmonary failure and even death. It has been reported that 10–20% of all hospital infections are because of *P. aeruginosa* strain PAO1 [9–12].

Medicinal plants proved to be highly effective to treat various human diseases and are used from thousands of years. Number of plants have been reported having antimicrobial activity and are used for curing the various infectious diseases of human and animals [13]. In the present study various plants were selected based on their previous history of medicinal use i.e. *Bergenia ciliata*, *Clematis grata*, and *Clematis viticella*. The rhizome of *Bergenia ciliate* is used traditionally for curing of many diseases and have antioxidant, anti-inflammatory, anti-diabetic, anti-bacterial, anti-fungal, antiviral, anti-cancer and anti-malarial activities [14]. Similarly, various species of *Clematis* has been used in Europe and Eastern Asia from centuries. It has diuretic, antimalarial, anti-dote in snake bites, anti-dysentery properties. Mostly *Clematis* spp. is used for the treatment of rheumatic pain, fever, eye infections, gonorrheal symptoms, bone illnesses, chronic skin disorders, gout and varicosity [15].

Multi-drug resistant bacteria direct to search for some novel strategies to overcome this problem caused by numerous resistant pathogens. Quorum sensing (QS) mechanism can be targeted approach as an alternative or supplemental method to antibiotics. QS, is a cell density dependent chemical signaling process, mediated by acyl homoserine lactones (AHL) in gram negative bacteria [12,16]. QS regulates number of gene expressions involved in various biological functions such as virulence determinants, bioluminescence, motility, plasmid transfer, secretion of enzymes and toxins, production of bacteriocins, efflux pump and biofilm formation [16]. *P. aeruginosa* which is an opportunistic pathogen, has two well defined QS system i.e. *lasI* and *rhlI*. LasI directs the synthesis of the signal molecule N-(3-oxododecanoyl) homoserine lactone (3O-C_{12}-HSL) while RhlIR synthesizes N-butyryl homoserine lactone (C_4-HSL) [17]. The *las* and *rhl* QS systems in *P. aeruginosa* PAO1 regulate number of genes and gene products that are involved in virulence. Additionally, it was found that the *las* system exerts two levels of control on Rhl, transcriptional and posttranslational [18]. These two QS systems play vital role during the early stages of biofilm formation especially when cells adhered to surface and forming microcolonies. Furthermore, the expression of *lasI* and *rhlI* was maximum when cells located at substratum and their expression decreased with increasing biofilm height [19]. The compounds that interfere with QS system of *P. aeruginosa* PAO1 can help to inhibit its biofilm formation. Therefore, one of the approach to inhibit biofilm formation is to target quorum sensing (QS) regulatory system that ultimately cause the inhibition of biofilm. Plants are the rich resource of number of compounds especially their secondary metabolites have antibacterial, antifungal as well as antibiofilm potential [20]. These compounds have ability to interfere with the QS system by inactivating their receptors in QS signaling pathways [16]. Flavonoids and terpenoids as well as the crude extract obtained from *Senegalia nigrescens* has demonstrated anti-quorum sensing activity [21]. Anti-QS activity of ethanol and ethyl acetate extracts of *Hypericum connatum* Lam. were evaluated against *Chromobacterium violaceum* to inhibit violacein production and was found that the ethanol extract significantly exhibits QS inhibition activity [22,23]. Similarly, in another study it was found the extract of *H. connatum* is capable ti block *las* and *rhl* QS systems of *P. aeruginosa* [24,25]. Similarly, *B. ciliate* and *Clematis* spp., which were also selected in the present study has medicinal value with recorded antibacterial as well as antimicrobial activities and indigenously use for curing many diseases [13]. All these plants are locally use for medication of various diseases but very little is known about their antibiofilm potentcial. Therefore, the present study is focused to screen out these plants for their activity against biofilm of *P. aeruginosa* PAO1.

Materials and methods

Chemicals and reagents

All the chemicals and reagents were purchased from Sigma–Aldrich, USA. Acetone, Chloroform, Ethanol, Ethyl acetate, Hexane, Methanol all are of analytical grade with 99% purity while double distilled water was used for extraction purposes unless otherwise mentioned. Coomassie brilliant blue and acetic acid were used for biofilm assays.

Plant sampling and identification

Various indigenous plant species of Hazara Division were selected based on their previous history of antimicrobial activities. The selected plants were *B. ciliata*, *C. grata* and *C. viticella*. Plant samples were collected from different regions of Hazara Division i.e. Abbottabad (Nathiagali, Thandiyani) and Kohistan, KPK, Pakistan. Each plant was identified by Dr. Arshad Mehmood Abbassi (Department of Environmental Sciences, COMSATS University Islamabad, Abbottabad Campus). Different parts of plants were used for their extraction in various solvents.

Extract preparation

The plants were thoroughly washed with tap water to remove soil and dust particles. All plants were then air dried under shade at room temperature for 1–2 weeks. The dried plants were then ground unless they appear in a powder form. For extract preparation, seven different solvents were used which were ethanol, methanol, acetone, ethyl acetate, hexane, chloroform and distilled

water. The powdered sample (5 g each) were added in 20 ml of each solvent and stirred for 24 h at 32 °C. Each sample was vortexed for 5 min and centrifuged at 10,000 rpm to pellet down all plant material, and the supernatant was collected in a clean 100 ml glass reagent bottle. Solvents were added again in the pelleted down sample and remixed it, following the centrifugation and solvents was recollected as above, this step was performed twice for complete extraction of plant samples. The supernatants were pooled together and passed through the Whattman's filter paper no1 to make them free from any plant material [26]. Each extracted sample was concentrated to 5 ml by evaporating the solvent under reduce pressure at temperature of 45 °C by using rotary vacuum evaporator manufacture by Butchi Rotavapor® R-30, Switzerland. The plant extracts were stored at 4 °C until further analysis.

Bacterial strains and growth conditions

P. aeruginosa strain PAO1 was used throughout the experiment for biofilm assays. The strain PAO1 was routinely cultured in Luria–Bertani (LB) media contained 5 g NaCl, 5 g yeast extract and 10 g peptone in a liter. The stock culture of PAO1 was taken from −80 °C and revived on nutrient agar plates while incubating them at 37 °C for 24 h. A freshly obtained bacterial colony was inoculated in LB broth, incubated at 37 °C and 220 rpm in shake flask incubator for 16 h to get a seed culture. The overnight culture of OD 0.8–1 was used for culturing the biofilm [27].

Biofilm inhibition assay

Biofilm of *P. aeruginosa* PAO1 was grown in Jensen's medium (JM) at 30 °C [28]. For culturing the biofilm of PAO1, the freshly obtained seed culture was inoculated in ratio of 1:100 in 96 well micro-titer plate of polypropylene material. The plate was covered and incubated at 37 °C for 24 h to culture the biofilms. Each experiment always has negative control (JM without inoculation), positive control (JM with inoculation 1%) and treatments (JM with inoculation 1% and various concentrations of plant extracts). All experiments were conducted in replicates of three unless otherwise mentioned. Crystal violet assay was performed to assess the biofilm inhibiting activity of plant extracts [29]. For biofilm inhibition assay the medium and planktonic cells were discarded after 24 h of static growth at 37 °C. Each well was rinsed thrice unless and until complete removal of all media and plantonic cells. Adhered cells were stained with 125 μL of crystal violet (0.1%) for 30 min at room temperature. Then the crystal violet (CV) was washed out while using d.H_2O. The CV bound to biofilm was re-solubilized in 30% acetic acid and its absorbance was measured at 550 nm.

Effect of plant extract on bacterial growth

The effect of plant extract on bacterial growth was observed by following the protocol describe [26]. Seed culture was prepared as mentioned above. Three types of controls were used i.e., Control A (LB 99 μL, inoculum 1 μL), Control B (LB 97 μL, solvent 2 μL, inoculum 1 μL), Control C (LB 98 μL, extract 2 μL). Treatments used in this assay contained LB, plant extracts and bacterial inoculums (LB 97 μL, extract 2 μL, inoculum 1 μL). Each treatment and controls were in replicates of three. The experiment was conducted in glass tubes while shaking them for 16 h on shake flask incubator (220 rpm) at 32 °C. The OD was monitored at 600 nm after 16 h of incubation.

Total phenolics content (TPC)

Total phenolic contents were determined by following the Folin-Ciocalteu colorimeteric method described earlier [30] while using the Gallic acid as standard. The standard as well as the extracted samples were blended with distilled water and Folin-Ciocalteu reagent for 6 min and neutralized for 90 min with 7% sodium carbonate solution. After 90 min the absorbance was measured at 760 nm by using the spectrophotometer.

Total flavonoids content (TFC)

Total flavonoids contents were determined by colorimetric method [31]. Catechin was used as standard solution with various concentrations. Sample (1 ml) as well as standards (1 ml) were mixed with 4 ml distilled water and 0.3 ml of 5% $NaNO_3$ (w/v). The mixture was kept for 5 min at room temperature and then 0.3 ml of 10% (w/v) was added. After 6 min 2 ml of NaOH (1 M) was added followed by the addition of distilled water in a volumetric flask. The mixture was shaken well, and the absorbance was measured at 510 nm.

Statistical analysis

Results were verified by applying the statistics on mean data of replicates by performing the ANOVA, DMRT ($P < 0.005$) and correlation between variables. Significant difference between various treatments as well as with that of the control was determined by using the statistical program SPSS as well as graph pad prism (IBM, SPSS).

Results and discussion

Plants were extracted in various solvents as described above, the extracted solvents have shown a significant impact on the inhibition of biofilm. The plant material extracted in alcohols significantly inhibit the biofilm formation as compare to other extracted solvents.

Biofilm inhibition by various plants

Two of the selected plants have shown the maximum inhibition activity against the biofilm of PAO1. The methanolic extract of *B. ciliata* (rhizome with skin) and ethanolic extract of *C. grata* (Leaves) efficiently inhibit biofilm formation where 81% and 80% inhibition were recorded respectively (Fig. 1A and B). Additionally, the least activity was observed in hexane extracted plant sample of *C. viticella* (leaves). Although treatments T1 (*B. ciliata* (rhizome with skin) and T3 (*C. grata* (Leaves) as shown in Fig. 1E. Previous studies revealed a strong evidence that the medicinal plants have promising attitude to combat various types of infectious diseases caused by numerous microbial species [32]. Medicinal plants attribute well to cure various diseases due to their antibacterial potential such as *B. ciliate*, *Jasminum officinale*, *Santalum album*, *Oxalis corniculata*, *Artemisia vulgaris*, *Cinnamomum tamala*, and *Ageratina adenophora* [33,34]. Similarly, various ethanolic extracts belong to family Meliaceae, Piperaceae and Sapindaceae interfered with the bacterial QS molecules that inhibit the formation of biofilm [35]. Quorum sensing molecules such as acyl homoserine lactones (AHLs) in *P. aeruginosa*, play important role in release of virulence and toxin is one of the strategy to control its pathogenicity [36–39]. Medicinal plants have numerous phytochemicals like phenolics, flavonoids, quinones, alkaloids, terpenoids, and polystyrenes that play a key role against microbial pathogenicity and proved to be involved in inhibition of QS molecules as well as biofilm [40].

Effect of solvents on biofilm inhibition

Solvents have a principle role in extracting a particular constituent from the plants, therefore the nature of solvent determines

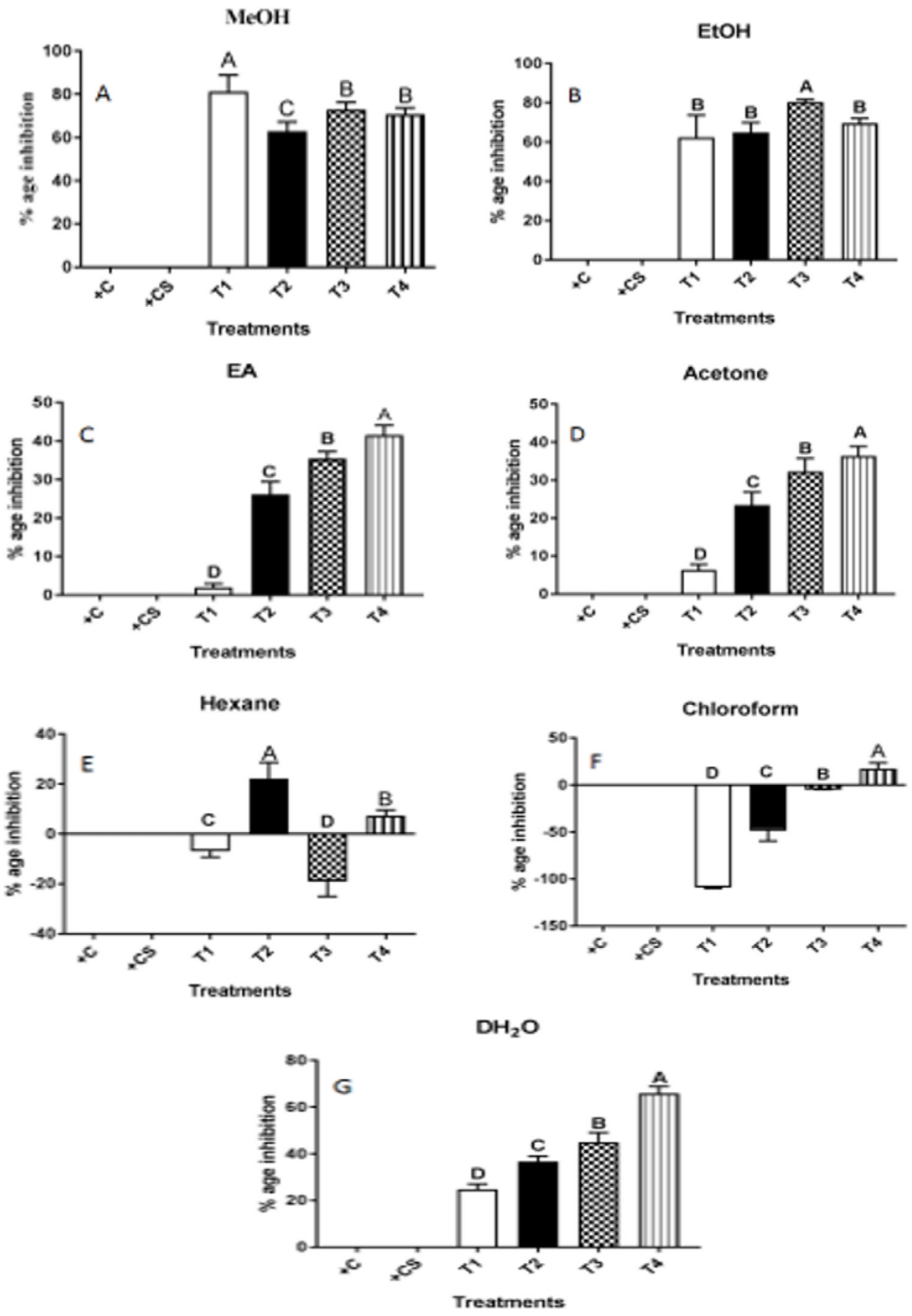

Fig. 1. Plant material extracted in different solvents; T1 (*B. ciliata* with skin), T2 (*B. ciliate* without skin), T3 (*C. grata*) and T4 (*C. viticella*), CS represents the control have respective solvent as treatment, C represents the untreated samples. A–G represents the % age inhibition of biofilm of PAO1 by various plants extracted in different solvents i.e. A (Plants extracted in Methanol), B (Plants extracted in Ethanol), C (Plants extracted in Ethyl Acetate), D (Plants extracted in Acetone), E (Plants extracted in Hexane), F (Plants extracted in Chloroform), G (Plants extracted in Distilled water).

which type of compound will be extracted in it. Due to this reason various solvents were used for the extraction of active compounds from the plants. It has been observed that the solvents played an important role in extracting different constituents of the plant materials [41]. The extraction done in the alcoholic solvents showed very promising results as compare to other solvents as well as the extracts in water (Fig. 2A–D). In previous studies, it has also been proved that due to different nature of phytochemicals their extraction rate is also vary depending upon the nature of chemical solvent [41–43]. The similar types of findings were observed in the present study where the same type of plant exhibit different type of activity against biofilm when extracted in different types of extracts. Same types of results were observed in a previous study where the extracts obtained in different solvents exhibit different types of activity against the pathogens [43,44].

Biofilm inhibition by methanolic extracts

Plant extracts in methanol have shown very promising attitude to inhibit biofilm of *P. aeruginosa* PAO1 irrespective of the type of plant as shown in Fig. 1A. The most significant results were obtained when *B. ciliata* (rhizome with skin) was used against the biofilm, it inhibited more than 80% of the biofilm formation as compared to

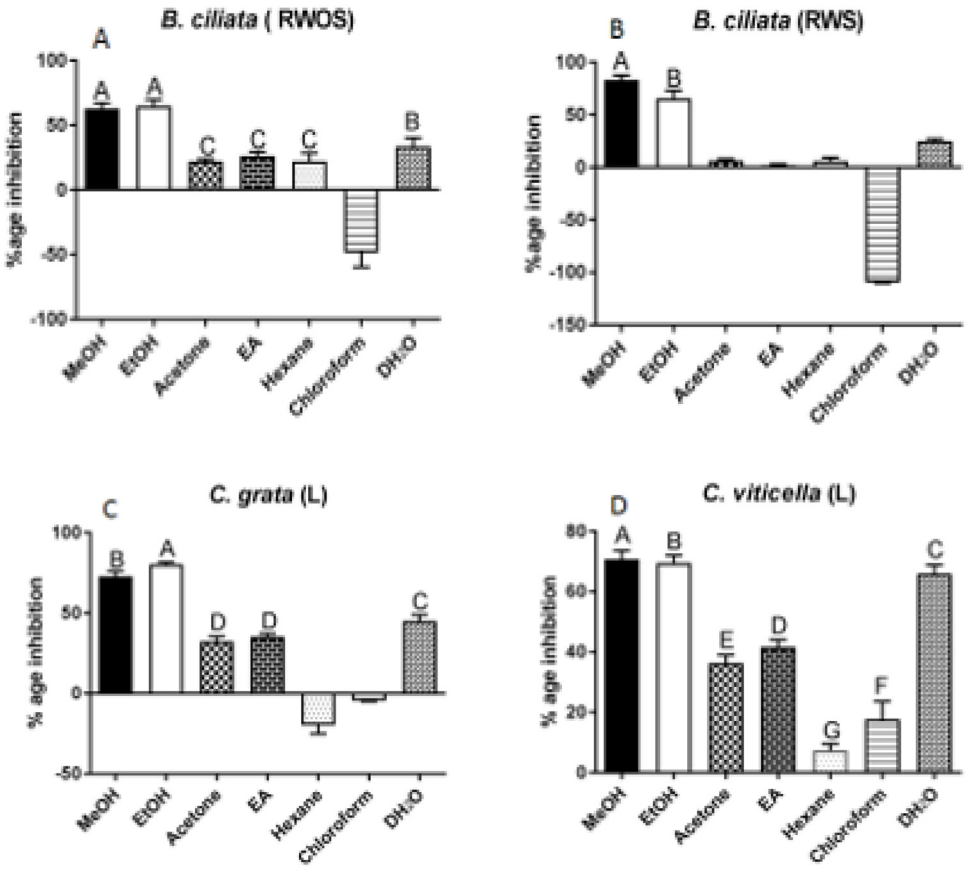

Fig. 2. Comparison of % age inhibition of biofilm of PAO1 by each plant in various solvents, A: % age inhibition of biofilm of PAO1 by *B. cilliata* (rhizome without skin) extracted in various solvents; B: % age inhibition of biofilm of PAO1 by *B. cilliata* (rhizome with skin) extracted in various solvents, C: % age inhibition of biofilm of PAO1 by *C. grata* (leaves) extracted in various solvents, D: % age inhibition of biofilm of PAO1 by *C. viticella* (leaves) extracted in various solvents.

the control as shown in Fig. 1A. The least activity was observed in case of treatment "T2" (*B. ciliata*/rhizome without skin) which inhibited more than 50% of the biofilm formation. In a recent study, *B. ciliate* was used to synthesize the nanoparticles and it was found that the extract as well as the nanoparticles have ability to reduce biofilm formed by *P. aeruginosa*, *Escherichia coli* and *Staphylococcus aureus* [45,46]. Another finding reveals that the methanolic extracts of plants *Betulapendula*, *Equisetum arvense*, *Herniaria glabra*, *Galium odoratum*, *Urtica dioica*, and *Vaccinium vitis-idaea* reduce the virulence factors and significantly reduce biofilm formation [31]. In a recent study, the hydromethanolic extracts of *Eucalyptus globulus* were found to be efficient to reduce the biofilm formation by *S. aureus* [47]. In the present study, the plants extracted in methanol has no effect on the growth of the bacterial strain when used in a concentration of 1%, that clearly indicates that the plant material in methanol adopt some other mechanism to inhibit the formation of biofilm which may be due to interruption in bacterial communication controlled by the quorum sensing molecules and most probably the flavonoids are involve in this inhibition as the biofilm inhibition and the flavonoids in plant samples has positive correlation with each other shown in Table 2.

Biofilm inhibition in ethanol extracts

In case of ethanolic extracts the best activity was obtained when *C. grata* (leaves) were used as anti-biofilm agent, where more than 80% inhibition was recorded as compare to control as shown in Fig. 1B. All the plant extracts in ethanol has shown a significant activity against the PAO1 biofilm as compare to untreated one, as shown in Fig. 1B. The effect of ethanolic extracts is basically on growth of the bacterial strain, as the extracts inhibit bacterial growth that ultimately results in the decrease of biofilm biomass. The ethanolic extracts of medicinal plants including *Beginia* and *Clematis* species have reported to be effective against various microbes as they have antibacterial as well as antifungal activity [14,47]. The ethanolic extracts of eleven plant species i.e. *Melissa officinalis, Mentha piperita, Laurus nobilis, Rhus coriaria, Dianthus coryophyllum, Piper nigrum, Capsicum annum, Juniperus oxycedrus, Erica arborea, Colutea arborescens*, and *Cuminum cyminum* collected from various regions of Turkey have antibacterial and antifungal activity [48]. Here, the ethanolic extracts also have antibacterial activity but not concern with the quorum sensing mechanism of PAO1, hence the antibiofilm activity is just due to its antibacterial activity that ultimately reduces the formation of biofilm of PAO1.

Biofilm inhibition by ethyl acetate extracts

All the extracts obtained in ethyl acetate has shown less than 50% inhibition as compare to control (Fig. 1C). The extract of *B. ciliata* (rhizome with skin) has shown negative impact and enhances the formation of biofilm as shown in Fig. 1C. This is also the clear indication of effect of solvent on extraction of various active compounds from the plants as shown by previous studies [37]. Thus, the compounds that are active in plant are of polar in nature and extracted in ethanolic solvents but not in ethyl acetate, therefore the extracts in ethyl acetate show less activity.

Biofilm inhibition in acetone extracts

Plant extracts in acetone are not as efficient as obtained in alcoholic solvents and one of the extracted plant in acetone has

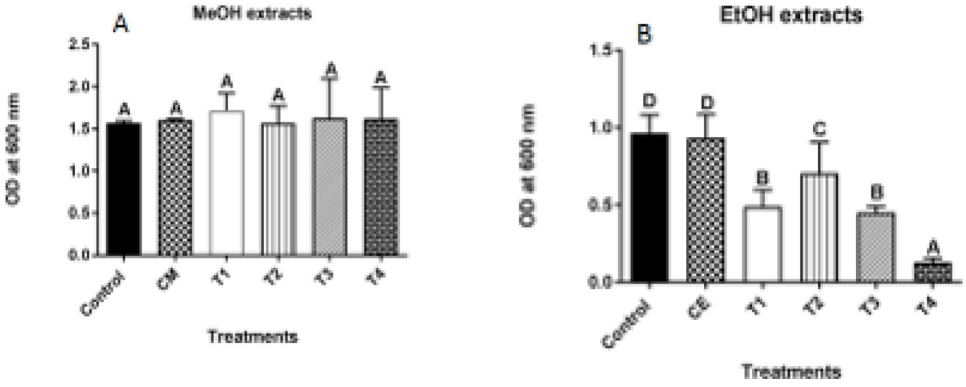

Fig. 3. A: Effect of 1% methanolic solvents on the growth of PAO1; B: effect of 1% ethanolic solvents on the growth of PAO1.

negative impact that enhances the biofilm formation as compare to the untreated one (Fig. 1D). The maximum biofilm inhibition was observed in *C. viticella* (leaves) but it was still less than 40%. In case of *B. ciliata* (rhizome with skin), it significantly increases the formation of biofilm as compare to control and about 15% increase in biofilm biomass was observed as shown in Fig. 1D. This clearly indicates that the active compounds in most of the selected plant species are not extracted in acetone and hence the activity against the PAO1 biofilm has not been observed. The methanolic extracts of same plants have highest activity which indicates that the compounds that has been extracted in methanol are highly polar and that solvent is most compatible to extract the biofilm active compounds from these plants as compare to less polar solvents. Previously, it has also been concluded by various researchers that the solvents have very much prominent effect on the extraction of various types of phytochemicals from the plants which is determined by the nature of the compound [44].

Biofilm inhibition in hexane extracts

Plant extracts in hexane has shown very less activity against the biofilm as compare to the other solvents (Fig. 1E). The maximum activity observed was in case of *B. ciliate* (rhizome without skin) which was only 22%. It indicates that the hexane is not a suitable solvent for extraction of the selected plant materials, one of the recent study also favor the present findings where they conclude that the polar solvents demonstrated the highest number of bioactive compounds such as polyphenols, antioxidants etc. [49].

Biofilm inhibition by chloroform extracts

The least activity was observed in plants extracted in chloroform, most of the plants extracts in chloroform significantly enhanced the biofilm biomass as compare to control (untreated) shown in Fig. 1F, only a single extract of *Clematic viticella* (Leaves) has inhibited 17% of biofilm formation while all others have significantly increased the biofilm formation. The negative effect i.e. the increase in biomass of biofilm was observed in most of the plants extracted in chloroform, clearly indicates that in chloroform some other compounds have been extracted that instead of inhibition facilitate the formation of PAO1 biofilm as shown in Fig. 1F.

Biofilm inhibition assay in distilled water extracts

All the aqueous extracts have significantly reduced the biofilm biomass as compare to the untreated samples. The most significant activity was observed in case of *C. viticella* (leaves) where more than 65% inhibition of PAO1 biofilm was recorded as shown in Fig. 1G.

Effect of extracts on growth

The plant extracts obtained in methanol as well as in ethanol has shown a very promising attitude against the biofilm formation of PAO1. Therefore, their same percentage was used to determine their effect on PAO1 growth. It has been found that the extracts in ethanol significantly decrease the growth of bacterium which was shown in Fig. 3A and B. The methanolic extracts have no effect on the growth of bacterium as shown in Fig. 3A, this indicates that the extracts in methanol have some other mechanism that inhibit the formation of biofilm other than killing of bacterial cells.

Phenolics and biofilm

As the methanolic and ethanolic extracts inhibit biofilm formation, therefore phytochemical analysis was performed to find the preliminary knowledge about the active compounds contained by these extracts Table 1. Total phenolics in methanolic extracts has negative correlation with biofilm as shown in Table 2, although the extracts shown to be very effective against the biofilm that indicates that the inhibition is not due to phenolic contents of the plants but there exist some other compounds or mechanism that is involve in this inhibition. The phenolics in ethanol as well in hexane has some positive correlation with biofilm inhibition although it is not very strong. This might be due to few phenolics which are extracted in ethanol but not in methanol that shows activity against the biofilm as it was recorded by previous reports where the solvents have their critical role in the extraction of various antioxidants including plant phenols [44,50]. In hexane although the correlation seems to be positive, but the inhibition recorded in hexane extracts are either negative or very less as shown in Fig. 1E, which indicate that these extracts have no significant role in the inhibition of biofilms.

Flavonoids and biofilm

The correlation studies depict that the flavonoids have very strong relation with anti-biofilm activity when the plant were extracted in methanol as shown in Table 2, while no correlation was found in case of extracts of same plants obtained in ethanol as well in hexane. Flavonoids are the group of natural product exhibit broad range of medicinal applications found in various parts of plants [52]. Previous studies reveal that the number of flavonoids play vital role in the inhibition of biofilm by combating the quorum sensing molecules [9]. Flavonoids are observed to be very effective against the biofilm formed by various microbial species [52–54]. Catechin one of the flavonoid isolated from *Combretum albiflorum* bark has potential to reduce the quorum sensing controlled virulence factors in PAO1 including the biofilm [54,55]. Similar findings were also been reported, where numerous types of flavonoids exhibit

Table 1
Measured levels of total phenolics and flavonoids contents.

Plant species	TPC (mg GAE/100 g DW)			TFC (mg QE/100 g DW)		
	ME	EE	HE	ME	EE	HE
Bergenia ciliate (rhizome with skin)	0.794 ± 0.01	0.896 ± 0.05	0.367 ± 0.03	1.253 ± 0.07	1.253 ± 0.00	1.600 ± 0.03
Bergenia ciliate (rhizome without skin)	1.313 ± 0.01	0.444 ± 0.05	0.526 ± 0.02	0.742 ± 0.01	0.742 ± 0.01	1.792 ± 0.02
Clematis grata	1.085 ± 0.04	0.817 ± 0.02	0.500 ± 0.01	1.346 ± 0.04	1.346 ± 0.02	2.155 ± 0.04
Clematis viticella	0.548 ± 0.04	0.578 ± 0.01	0.501 ± 0.02	1.187 ± 0.05	1.187 ± 0.02	2.215 ± 0.01

Table 2
Correlations coefficient matrixes of polyphenolics and anti-biofilm activity.

Variables	TPC (ME)	TPC (EE)	TPC (HE)	TFC (ME)	TFC (EE)	TFC (HE)	BF (ME)	BF (EE)	BF (HE)
TPC (ME)	1.000								
TPC (EE)	−0.283	1.000							
TPC (HE)	0.390	−0.766	1.000						
TFC (ME)	−0.572	0.842	−0.454	1.000					
TFC (EE)	−0.786	−0.229	−0.231	−0.049	1.000				
TFC (HE)	−0.279	−0.202	0.656	0.329	−0.008	1.000			
BF (ME)	−0.527	0.914	−0.932	**0.746**	0.156	−0.356	1.000		
BF (EE)	0.054	0.395	0.285	0.644	−0.604	0.685	0.048	1.000	
BF (HE)	0.212	−0.898	0.410	−0.921	0.420	−0.203	−0.680	−0.757	1.000

Bold value indicate strong association between tested variables.

potential to reduce the virulence factors including production of pyocyanin and elastase as well biofilm inhibition without effecting the growth of PAO1 [51,56,57], which was also similar in our findings where methanolic extracts have no effect on growth but have potential to inhibit biofilm as shown in Fig. 3A. Flavonoids act via antagonism of the autoinducer binding receptors, LasR and RhIR that inhibit quorum sensing which ultimately results in the inhibition of biofilm [58].

Conclusion

Antibiofilm activity was studied in *P. aeruginosa* PAO1, which is an opportunistic pathogen, particularly in immune deficient patients. Among three tested plant species, methanolic extract of *B. ciliata* (1%, concentration) exhibited more than 80% inhibition of biofilm formation without any adverse effect on the growth of bacterium. In addition, strong positive association among antibiofilm activity in methanolic extract and total flavonoids indicating the key role of phytochemicals in *P. aeruginosa* biofilm inhibition. Therefore, methanolic extract of *B. ciliata* could be a potential candidate for drug discovery, particularly to treat immune deficient patients.

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Competing interests

None declared.

Ethical approval

Not required.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jiph.2020.07.007.

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