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The Interference of Soil Bacteria by Quorum Sensing Inhibitory Activity with the Biofilm of Pathogenic *Pseudomonas aeruginosa* Isolates

Samer Raad Abdul Hussain[1], Inam Jasim Lafta[2*]

[1]*Department of Microbiology, College of Veterinary Medicine, University of Diyala, Diyala, Iraq.*
[2]*Department of Microbiology, College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq.*
Address of correspondence:
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Abstract

Pseudomonas aeruginosa's biofilm may have a role in its resistance to antibiotics. By interfering with the quorum sensing (QS) system, biofilm formation in pathogens may be inhibited, thereby reducing resistance to antibiotics. Therefore, new approaches for treating bacterial infections could be offered by applying QS inhibitors (QSIs). Recently, soil microorganisms have been employed as they created compounds with QS inhibition properties. Thus, the current study aimed to examine the ability of facultatively anaerobic soil bacteria to impede the QS of *P. aeruginosa,* thereby interfering with QS-regulated biofilm production. In this research, the soil bacteria were isolated and identified by the Vitek2 system. The soil isolates included five *Proteus mirabilis,* two *Klebsiella pneumoniae* subsp. *pneumoniae,* and one isolate of each *Staphylococcus aureus* and *Escherichia coli.* These isolates were tested for their ability as potential QS inhibitors (QSIs). Then, the whole cell crude aqueous and organic extracts of a *P. mirabilis* soil isolate were made, and their minimum inhibitory concentration (MIC) and sub-MIC were determined against eight clinical *P. aeruginosa* isolates. The clinical bacteria involved four isolated from burn wound infections and the other four from cystic fibrosis patients. The crude aqueous extract was then tested as an anti-biofilm interfering with *P. aeruginosa* using the crystal violet assay and by the use of quantitative real-time polymerase chain reaction (qPCR) targeting the *LasI* gene, the QS regulator. The results showed that the *P. mirabilis* aqueous extract effectively weakened the *P. aeruginosa* biofilm and reduced the expression of the QS regulatory *LasI* gene. To conclude, *P. mirabilis* revealed a promising result in the QS inhibition, and its crude extract successfully rendered the strong biofilm producers *P. aeruginosa* weaker.

Keywords: Soil bacteria, quorum sensing inhibition, *P. aeruginosa,* biofilm.

تداخل جراثيم التربة عن طريق النشاط المثبط لاستشعار النصاب مع الأغشية الحيوية لعزلات الزائفة الزنجارية المسببة للأمراض

سامر رعد عبد الحسين[1] , إنعام جاسم لفته[2*]

[1]فرع الاحياء المجهرية، كلية الطب البيطري، جامعة ديالى، ديالى، العراق

[2]فرع الاحياء المجهرية، كلية الطب البيطري، جامعة بغداد، بغداد، العراق

الخلاصة

*Email: inam.j@covm.uobaghdad.edu.iq

قد يكون للأغشية الحيوية لجرثومة الزائفة الزنجارية دورا في مقاومتها للمضادات الحيوية. من خلال التداخل مع نظام استشعار النصاب، أصبح من الممكن تثبيط تكوين الأغشية الحيوية في المسببات المرضية وبالتالي تقليل مقاومة المضادات الحيوية. لذلك، فإن استخدام مثبطات استشعار النصاب قدم طرقا جديدة لعلاج الالتهابات الجرثومية. في الآونة الأخيرة، استخدمت كائنات حية دقيقة تعيش في التربة لأنها خلقت مركبات ذات خصائص تثبيط استشعار النصاب. لذا هدفت الدراسة الحالية إلى فحص قدرة جراثيم التربة اللاهوائية الاختيارية على إعاقة استشعار النصاب للزائفة الزنجارية، وبالتالي التداخل مع إنتاج الأغشية الحيوية المنظمة بواسطة استشعار النصاب. جرى بهذا البحث عزل جراثيم التربة وتشخيصها باستخدام نظام الفايتك. جرى اختبار عزلات التربة، بضمنها خمسة من المتقلبات الرائعة واثنان من الكلبسيللا الرئوية تحت صنف الرئوية وعزلة من كل من المكورات العنقودية الذهبية وأخرى من الاشريكية القولونية، على قدرتها لتكون مثبطات محتملة لاستشعار النصاب. بعدها، حضر مستخلص مائي خام واخر مستخلص عضوي من جراثيم المتقلبة الرائعة المعزولة من التربة، وجري تحديد التركيز المثبط الأدنى والتركيز تحت المثبط ضد ثمان عزلات سريرية للزائفة الزنجارية. شملت الجراثيم السريرية أربع عزلات من التليف الكيسي وأربع عزلات أخرى من مرضى الحروق. جرى اختبار مستخلص جرثومة المتقلبة الرائعة كمضاد للأغشية الحيوية بإمكانه التداخل مع جراثيم الزائفة الزنجارية المنتجة لتلك الأغشية بالاعتماد على صبغة البنفسج البلوري وباستخدام تفاعل البوليميراز المتسلسل الكمي في الوقت الحقيقي مستهدفًا مورثة تنظم استشعار النصاب. أظهرت النتائج ان المستخلص الخام المائي لجرثومة المتقلبة الرائعة أضعف بشكل فعال الأغشية الحيوية للزائفة الزنجارية وقلل التعبير الجيني للمورثة المنظمة لاستشعار النصاب. مما تقدم تستنتج الدراسة ان جرثومة المتقلبة الرائعة الهوائية ذات أهمية في تثبيط استشعار النصاب، اذ نجح مستخلصها الخام المائي في إضعاف انتاج الأغشية الحيوية من جراثيم الزائفة الزنجارية.

1. Introduction

Pseudomonas aeruginosa is a common non-fermentative bacterium with a high fatality rate of up to 60% and is one of the most frequent causes of nosocomial infections [1]. It causes several inflammations in various body systems and organs because it secretes a range of virulence factors to adapt to unfavorable environments [2]. Lipopolysaccharide (LPS) is one such virulence factor. The endotoxicity of the lipid A in LPS permits tissue injury, attachment, and host receptor recognition [3]. LPS may be connected to the development of biofilms and antibiotic tolerance [4]. The bacterial biofilm may have a role in infections' resistance to antibiotics [5]. The biofilm is an extracellular matrix made up of proteins, polysaccharides, and DNA that bacterial colonies use to create intricate structures and stick to surfaces that are either living or non-living [6]. Bacteria's ability to create biofilms can boost their resistance to antimicrobials by up to 1000 times [7]. Furthermore, the bacterial biofilm's protective structure enables microorganisms to survive under harsh conditions [8]. Therefore, it is desirable to apply methods for regulating biofilm development for therapeutic purposes.

Quorum sensing (QS) is one of the target-regulating mechanisms in the physiological process of bacteria. It alters a variety of phenotypes, including the expression of virulence factors, the creation of biofilms, and the generation of antibiotics [9]. Many genes are controlled by QS systems; *P. aeruginosa* genome contains approximately 10% of the genes under QS systems control [10]. By interfering with the QS system, biofilm formation in pathogens may be inhibited and subsequently disrupted, reducing their resistance to harmful substances such as antibiotics. Therefore, new methods for treating bacterial infections may be offered by the QS system and its related QS inhibitors (QSIs) [11, 12]. A variety of QSIs, such as halogenated furanone C30 and garlic extracts, have been found in macroalgae, plants, fungi, and actinomycetes [12]. Certain soil microorganisms, including the genus *Paenibacillus*, have been discovered to create compounds with QS inhibition properties [13]. The soil Gram-positive *Bacillus* species was found to possess a gene (*aiiA*) that encodes a quorum-quenching enzyme [14]. Furthermore, the filtrate of the soil bacterium *Proteus mirabilis* was tested as a

potential QSI that considerably reduced the ability of the Gram-negative bacteria *P. aeruginosa* to produce biofilms [15]. It is interesting to note that, in contrast to traditional antibiotics, QSIs only help remove bacteria by disrupting biofilm-mediated defenses; they have no effect on the development of pathogens or the selection forces acting against them [11]. Additionally, QSIs may lower the risk of septic shock during infection and diminish the generation of bacterial virulence factors [11]. The primary objective of the present work was to assess the inhibitory impacts of some facultatively anaerobic soil bacteria on the QS and, subsequently, the QS-regulated biofilm of clinical *P. aeruginosa* isolates.

2. Materials and Methods

2.1 Bacterial isolation and identification

2.1.1 Environmental isolates

The environmental samples were collected from the soil of the College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq, during the period from December 2022 to March 2023. These samples were then subjected to bacterial isolation. Briefly, 1 g of each soil sample was suspended in 10 ml of distilled water and mixed well for 5 min. Serial dilutions were made for each suspension from 10^{-1} to 10^{-7}. Then, 0.1 ml was pipetted out from each suspension onto a nutrient agar (TM-MEDIA, India) plate, spread with a glass spreader, and incubated at 37°C for 24 h. Afterward, a single colony was sub-cultured on various enrichment, selective, and differential media, such as blood agar (Hi-Media, India), MacConkey's agar (TM-MEDIA, India), and mannitol salt agar (Hi-Media, India) for isolation of some aerobic bacteria. Later, the soil isolates were identified at the species level using the Vitek-2 assay (bioMerieux, USA).

2.1.2 Clinical isolates

An ethical approval (reference no. 22012 on May 18, 2022) was issued by the Research Ethics Committee of Diyala Health Department, Training and Human Development Center, Research and Knowledge Division, to perform experiments on the human patient samples.

The study involved 8 *P. aeruginosa* isolates acquired from Baqubah General Hospital in Baqubah, Iraq. These bacteria were isolated from 4 cystic fibrosis specimens and 4 burn wound specimens. The isolates were firstly cultivated on Cetrimide agar (Hi-Media, India), and then sub-cultured on Pseudomonas agar base (Hi-Media, India). The Vitek-2 system was used to confirm all *P. aeruginosa* clinical isolates.

2.2 P. aeruginosa biofilm formation

The capability of the 8 clinical isolates of *P. aeruginosa* to form biofilm was screened via crystal violet assay/microtiter plate method, as stated by Zhang *et al*. [16] with some modifications. Firstly, newly grown colonies of *P. aeruginosa* were inoculated into 5 ml of Brain Heart Infusion (BHI) broth (Hi-Media, India) containing 2% sucrose and incubated for 24 h at 37ºC. The next day, the 96-well microtiter plate containing 180 µl of BHI broth was inoculated with 20 µl of the bacterial suspension (equivalent to 0.5 MacFarland standard that contained 1.5×10^{8} CFU/ml) of each isolate in three replicates and incubated at 37°C for 24 h. To get rid of non-adherent cells, the plate was rinsed three times with normal saline. Then, 200 µl of 99% methanol was added to each well and allowed to sit for 15 min in order to fix the adherent cells. The plate was allowed to dry at room temperature for half an hour. After that, 200 µl of 1% crystal violet was added to each well for 15 min, and then the dye solution was removed by washing the wells with sterile distilled water. Finally, 96% ethanol was used to dissolve the adhering stain. Figure 1 shows the detection of biofilm formation by *P. aeruginosa* (isolates no. 1-8) using the crystal violet assay. The micro-titer plate reader (BIO-TEK, USA) was used to measure the optical density (OD) at 600 nm. The negative control included the use

of BHI broth only. The biofilm formation ability of each isolate was evaluated according to Zhang *et al.* [16], as mentioned below:

Nonbiofilm producer: OD ≤ ODc, weaker biofilm producer: 2 ODc > OD > ODc, moderate producer 4 ODc > OD > 2ODc, strong producer: OD > 4 ODc, cut off value (ODc) = average OD of negative control + (3 *Standard Deviation).

2.3 Preliminary screening for quorum sensing inhibition

According to the study of Sadeq and Lafta [17], all *P. aeruginosa* isolates of this study producing pigments were checked for their ability to screen for QS inhibition (pigment disappearance) using furanone (Tokyo chemical industry, China) as a QSI (positive control). These clinical isolates were then used to screen for the ability of soil bacterial isolates to inhibit their pigment, with the use of furanone as a positive control. A working concentration of 1.7 mg/ml was made from the stock solution of furanone (10 mg/ml), and then different concentrations (20, 22, 25, and 33 µg/ml) of furanone were made. To choose the best conditions that could cause inhibition to the pigment, Pseudomonas agar was prepared and inoculated with an overnight culture of each clinical isolate of *P. aeruginosa.* Then, 4 wells of 6 mm were made per plate, and each was filled with 100 µl of different concentrations of furanone. The same procedure described above was then repeated, but instead of furanone, the wells were filled with 100 µl of an overnight culture of each environmental isolate grown into LB broth (Liofilchem, Italy), incubating the plates at 37°C for 48 h to screen for the ability of the soil isolates as QSIs.

2.4 Preparation of P. mirabilis extracts

Using Dong and Zhang's approach [18] with some modifications, crude aqueous and organic extracts were prepared from *P. mirabilis* isolated from soil.

2.5 Determining MIC and sub-MIC of P. mirabilis extracts

The resazurin broth assay was applied in the current study to establish the MIC and sub-MIC of both crude extracts of *P. mirabilis* **(**the aqueous and organic) based on the procedure described by Ohikhena *et al*. [19]. Serial dilutions (1024, 512, 256, 128, 64, 32, 16, 8, 4, 2, and 1 µg/ml) of the aqueous and organic extracts of *P. mirabilis* were prepared in a microtiter plate. To each well of the microtiter plate, 160 µl of BHI broth was added as a diluent, and 20 µl of each concentration of the extract was added to the wells. A suspension of four *P. aeruginosa* isolates (no. 2, 5, 6, and 8) equivalent to the McFarland standard no. 0.5) was made, and 20 µl of each isolate was added to the wells. The negative control wells contained 180 µl of BHI broth only without the bacteria or the aqueous extract. Afterward, the plate was incubated at 37°C for 24 h. The next day, 20 µl of resazurin dye was applied to each well, and the plate was incubated for a further 2 h. Finally, the MIC and sub-MIC concentrations were estimated, in which MIC represented the lowest concentration of the extract required to prevent the visible growth of *P. aeruginosa*, without changing the color (blue). While the sub-MIC referred to the concentration lower than the MIC value, changing the color from blue to pink.

2.6 Determining the effect of the P. mirabilis aqueous extract on P. aeruginosa

2.6.1 Effects on the biofilm formation

The same protocol applied to the biofilm formation assay was also used to evaluate the anti-biofilm activity of the extract [20]. Briefly, 160 µl of BHI broth and 20 µl of the aqueous crude extract were added to each well of a microtiter plate, and then, 20 µl of *P. aeruginosa* suspension equivalent to MacFarland tube no. 0.5 was added. Three replicates were done for each isolate. The negative control contained 180 µl of BHI broth only without the bacterial suspension or the aqueous extract. Following overnight incubation at 37°C, the medium was

taken away from the wells, which were then rinsed three times with sterile PBS to get rid of any remaining *P. aeruginosa* cells. The plate was then allowed to dry for 15 min at room temperature. After that, 200 µl of crystal violet (0.1%) was added to each well and left to settle for 20 min. The stained wells were rinsed three times with PBS (PH 7.2) and let to dry for 15 min at room temperature to eliminate the unbound dye. Eventually, 200 µl of 95% ethanol was poured into each well, and the OD was measured at 600 nm using the microplate reader.

2.6.2 Interference with the quorum sensing gene expression

RNA isolation and cDNA synthesis

Firstly, the 8 *P. aeruginosa* isolates were cultured individually in a nutrient broth and each was treated with the aqueous extract of *P. mirabilis* at the sub-MIC. The bacterial cell pellets were then collected in a microcentrifuge tube by spinning the culture for 1 min at 13,000 rpm. The centrifugation process was repeated to achieve adequate cell pellets. Following the manufacturer's instructions, the TRIzol™ RNA extraction kit (Thermo Fisher, US) was used to extract the whole cell RNA from the bacterial cells (pellets), whether or not they had been treated with the aqueous extract. The isolated RNA was kept at -20°C in a freezer until it was coverted into the complementary DNA (cDNA). Afterwards, the cDNA ready to use kit (Bioneer, Korea) was applied to the isolated whole cell RNA of the 8 clinical isolates to perform the cDNA synthesis. The obtained cDNA (20 µl as a total volume) was immediately used as a template for quantitative real-time polymerase chain reaction (qPCR).

qPCR

The qPCR test was performed in this study to evaluate the potential role of the aqueous extract of *P. mirabilis* on the expression of *P. aeruginosa's* target gene (*lasI*). The qPCR reaction mixture was composed of the following: 12.5 µl of 2×qPCR Master mix (Tinzyme, China), 2 µl of cDNA samples (with or without aqueous extract), 1 µl of the forward primer (10 µM), 1 µl of the reverse primer (10 µM) (Table 1), and up to 25 µl of DNase-free distilled water. Then, these components were thoroughly mixed and placed in the qPCR thermal cycler called Exicycler™ (Bioneer, Korea) after setting the qPCR program as follows: initial denaturation at 95°C for 10 min and 1 cycle, then 40 cycles of denaturation (95°C for 20 sec), annealing (1 min at 60°C), and extension (72°C for 60 sec). Melt curve analysis was performed after the qPCR procedure to verify the amplified genes' specificity and rule out the existence of primer dimers or contaminating DNA amplification.

Table 1: The nucleotide sequences of the qPCR primers [21].

Gene	Primer sequence (5' to 3')	Tm (°C)	Product size (bp)
LasI	F: 5'-CCGTAGGGGTGGAGAAGAT-3'	59	110
	R: 5'-ATTGAGTTCGATGCGCAAG-3'	60	
recA	F: 5'- ACTGCCTGGTCATCTTCATC-3'	57.6	104
	R: 5'-CGAGGCGTAGAACTTCAGTG-3'	58.7	

To calculate the data of the qPCR assay, the method of Schmittgen and Livak [22] was used. Comparisons of the Ct values were performed between the target gene (*lasI*) and the reference or housekeeping gene (*recA*). The fold change in gene expression was estimated according to the equations:

$\Delta Ct_{treatment} = Ct_{target} - Ct_{reference}$

$\Delta Ct_{control} = Ct_{target} - Ct_{reference}$

$\Delta\Delta Ct = \Delta Ct_{treatment} - \Delta Ct_{control}$

Fold change in gene expression = $2^{-\Delta\Delta CT}$

3. Results

3.1 Isolation and identification of environmental bacterial isolates

The soil isolates included 5 *P. mirabilis* isolates, 2 *K. pneumoniae* subsp. *pneumoniae,* 1 isolate of each *S. aureus* and *E. coli.*

3.2 Identification of the clinical isolates

The eight *P. aeruginosa* isolates were able to produce the yellowish-greenish pigment on the Cetrimide and Pseudomonas agars (Figure 1). All the isolates were confirmed using the Vitek-2 system (data not shown).

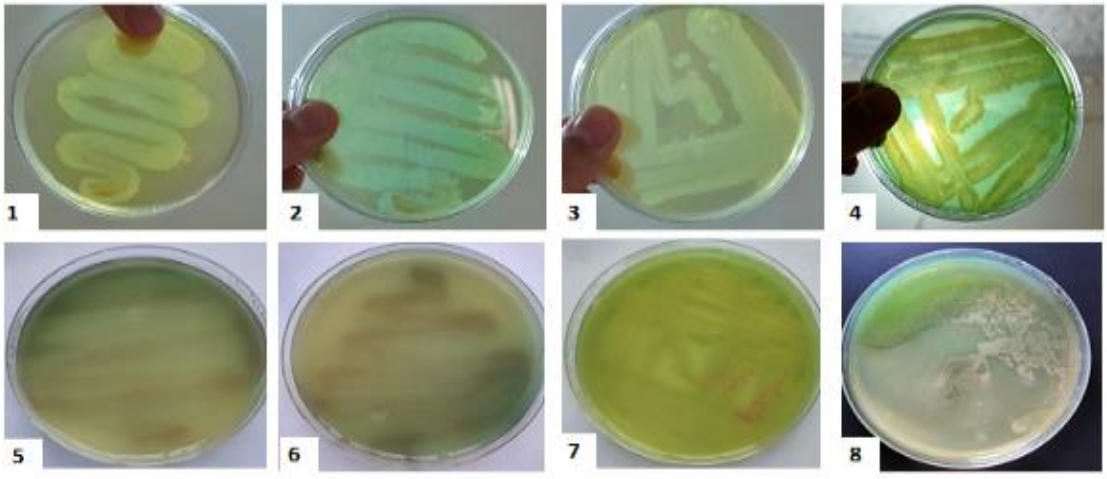

Figure 1: Different isolates of *P. aeruginosa* grown on the selective *Pseudomonas* media. Isolates no. 1, 2, 3, and 4 are obtained from cystic fibrosis patients growing on Cetrimide agar, while isolates no. 5, 6, 7, and 8 are from burn wound infections growing on Pseudomonas agar base.

3.3 Biofilm formation

Almost all of the *P. aeruginosa* isolates included in the study produced a strong biofilm, except for one isolate from a cystic fibrosis patient, which was moderate (Table 2 and Figure 2). Four isolates with the strongest biofilm formation were chosen to be used later. In particular, isolates no. 2, 5, 6, and 8 were selected for testing the anti-biofilm activity because they also revealed multi-drug resistance (data not shown).

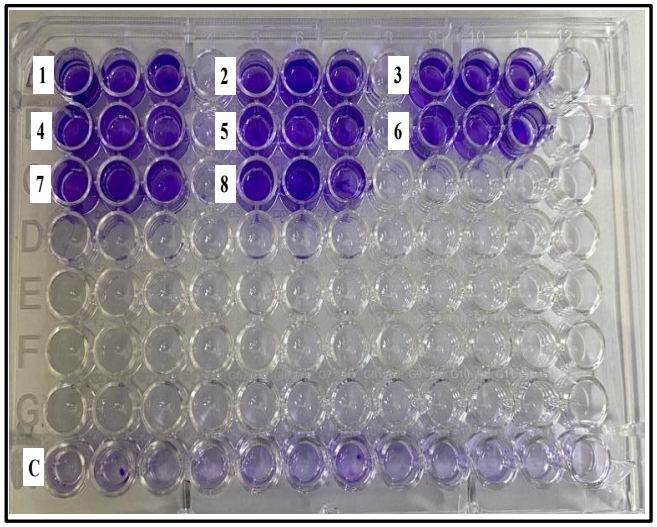

Figure 2: Detection of biofilm formation of *P. aeruginosa* isolates 1-8 compared to the control (C) using the crystal violet assay.

Table 2: Biofilm production by the different isolates of *P. aeruginosa* using the crystal violate assay.

Isolate	OD	Biofilm
1	0.328	Strong
2	0.336	Strong
3	0.284	Strong
4	0.156	Moderate
5	0.339	Strong
6	0.343	Strong
7	0.311	Strong
8	0.453	Strong
-ve control	0.06	-

3.4 Screening for isolates with QS inhibition

Furanone was successfully able to cause a zone of inhibition to *P. aeruginosa* pigment in a concentration-dependent manner, i.e. well no. 4 containing the highest concentration (33 µg/ml) exerted the most prominent effect on the dye disappearance (Figure 3-left). Then, when different *P. aeruginosa* isolates were treated with cultures of the soil bacteria, each of *K. pneumoniae, S. aureus*, and *E. coli* had no effects on the dye of *P. aeruginosa* (data not shown), despite interference with its growth (not within the scope of this study). However, *P. mirabilis* (particularly isolate no. 4) succeeded in interfering with the *P. aeruginosa* stain without reducing its growth (Figure 3 middle and right). In comparison, the other isolates of *P. mirabilis* (isolates no. 2, 3, and 5) did not have significant effects (Figure 3-middle and right).

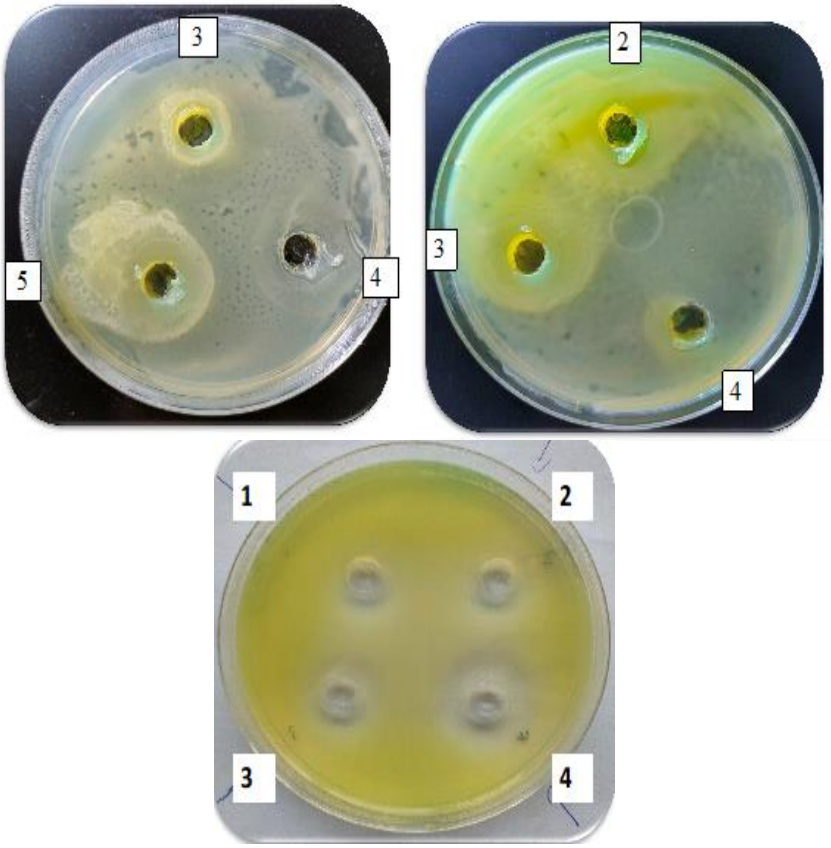

Figure 3: Quorum sensing inhibition (represented by color disappearance) of different isolates of *P. aeruginosa* by:

Left- Furanone (the positive control). Various concentrations of furanone (1- 20 µg/ml, 2- 22 µg/ml, 3- 25 µg/ml, and 4- 33 µg/ml) were tested as QSI against *P. aeruginosa* dye. **Middle-** *Pseudomonas aeruginosa* isolate no. 1 grown on Pseudomonas agar containing three wells filled with culture of *P. mirabilis* isolates no. 2, 3, and 4. **Right-** *Pseudomonas aeruginosa*

isolate no. 6 grown on Pseudomonas agar containing three wells filled with culture of *P. mirabilis* isolates no. 3, 4, and 5 on the corresponding wells of the same numbers.

3.5 Extracts of P. mirabilis

Organic and aqueous extracts were made successfully from a culture of *P. mirabilis*. The aqueous extract tuned into a powder form, with a net weight of 320 mg, while the organic phase was in a paste texture and weighed around 1.3 g.

3.6 MIC and sub-MIC of the *P. mirabilis extract*

Minimum inhibitory concentrations (MICs) as well as sub-MICs of the aqueous extract against the growth of four *P. aeruginosa* isolates (no. 2, 5, 6, and 8) are illustrated in Table 3 and Figure 4. Regarding the organic extract, it is clear from Figure 4 that it had no effects on the growth of the clinical isolates at the MIC and sub-MIC levels; therefore, this extract was omitted.

Table 3: Determining MIC and sub-MIC of aqueous extract of *P. mirabilis* against the growth of *P. aeruginosa* isolates.

P. aeruginosa **Isolate No.**	***P. mirabilis*** **aqueous extract**	
	MIC (µg/ml)	**Sub-MIC (µg/ml)**
2	**32**	**16**
5	**64**	**32**
6	**64**	**32**
8	**32**	**16**

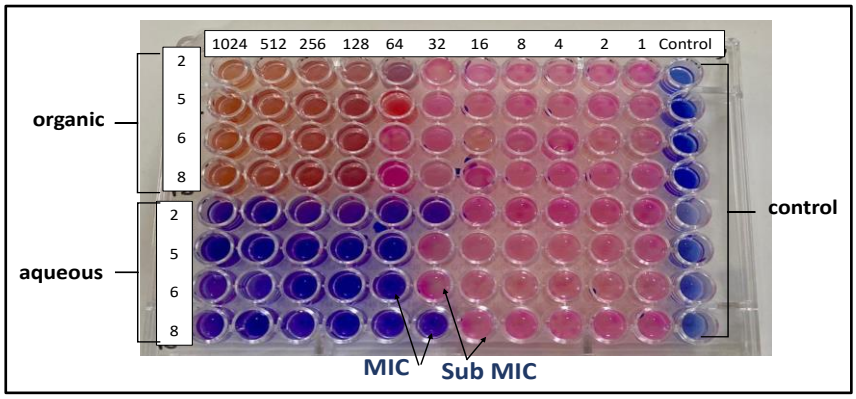

Figure 4: Determining the MIC and sub-MIC of the organic and aqueous extracts of *P. mirabilis* against the *P. aeruginosa* growth (isolates 2, 5, 6, and 8). Two-fold serial dilutions (1-1024 µg/ml) of *P. mirabilis* extract were prepared from a stock (10 mg/ml) in a 96-well microtiter plate, and BHI broth was used as a diluent.

Table 4: The anti-biofilm activity of the aqueous extract of *P. mirabilis* against the biofilm of four isolates of *P. aeruginosa*.

Isolate ID	**OD**	**Biofilm**
2	0.101	weak
5	0.116	weak
6	0.075	weak
8	0.099	weak
-ve control	0.06	

3.7 The anti-biofilm effect of the P. mirabilis aqueous extract

Table 4 and Figure 5 below show the effect of the aqueous extract of *P. mirabilis* as an anti-biofilm that prevents its formation by *P. aeruginosa* isolates already exerted a strong ability to produce the biofilm.

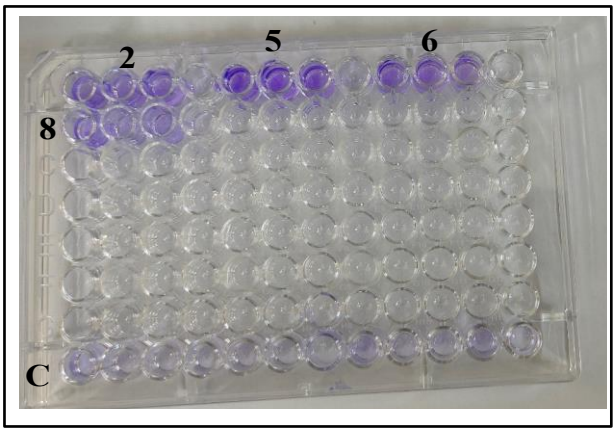

Figure 5: Determining the effect of the *P. mirabilis* aqueous extract as anti-biofilm interfering with the biofilm of *P. aeruginosa* isolates (2, 5, 6, and 8) compared to the negative control wells (C).

3.8 q-PCR

The qPCR assay showed the *recA* and *lasI* expression in *P. aeruginosa,* either treated or not with the aqueous *P. mirabilis* extract (Figure 6). All 8 *P. aeruginosa* isolates showed decreased expression of the QS gene (*LasI*) to varying degrees after treatment with the aqueous extract compared to the negative control (bacteria without treatment with the extract) (Figure 7).

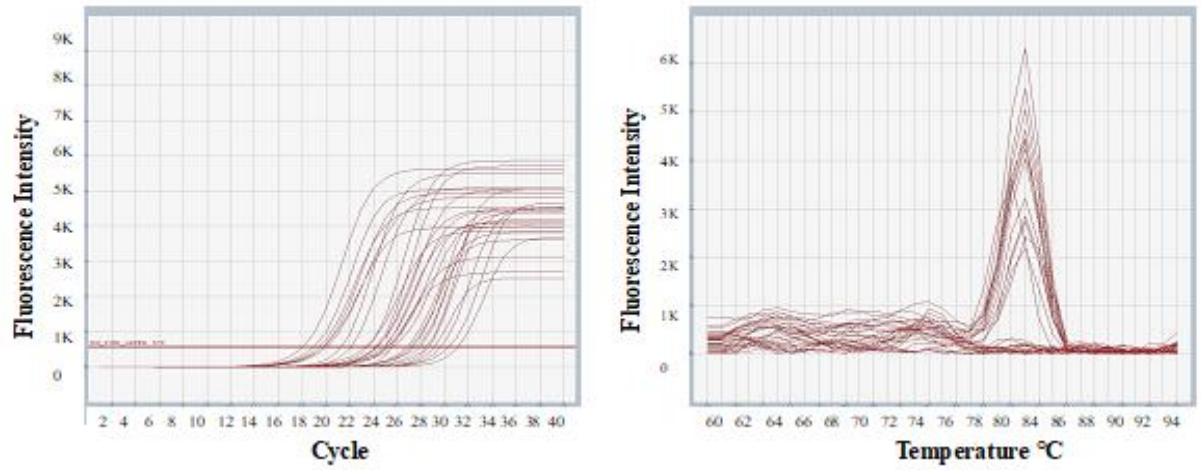

Figure 6: The qPCR approach detects the expression of the *recA* and *lasI* genes in *P. aeruginosa* with or without the aqueous extract. A- Amplification plot of qPCR, B- Melt curve analysis.

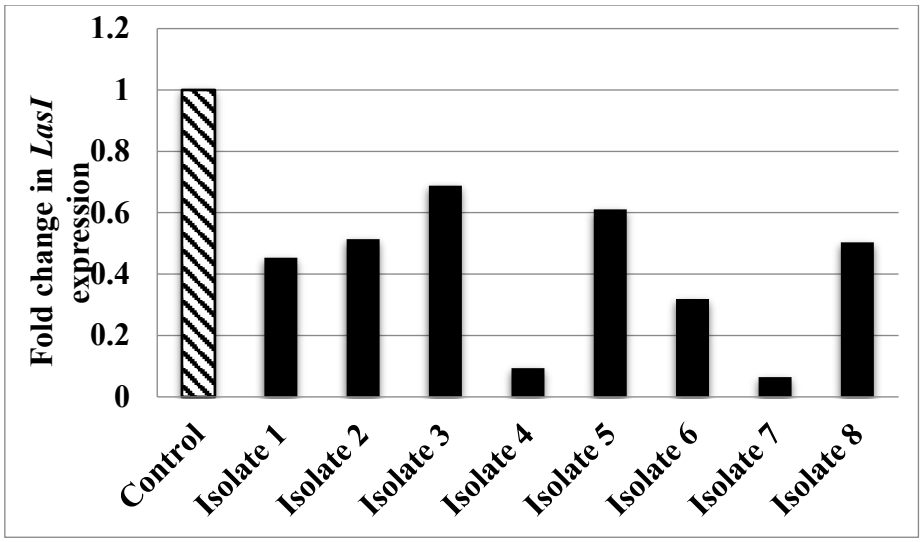

Figure 7: Fold change in the expression of *P. aeruginosa LasI* after treatment with the aqueous extract of *P. mirabilis* compared to the negative control. Black bars (1-8) represent the treated isolates of *P. aeruginosa* versus the white striped bar (negative control).

4. Discussion

Most of the clinical *P. aeruginosa* isolates used in the present study exhibited a clearly identifiable yellowish to green pigment during both initial and subsequent culturing. It is well-known that the majority of *P. aeruginosa* generate one or more extracellular pigments, such as pyomelanin (brown-black), pyocyanin (blue-green), pyoverdine (yellow-green and fluorescent), and pyorubrin (red-brown) [23]. *P. aeruginosa* strains that produce yellow pigment presented a significant challenge because there were no substitute medications to combat these altered strains, which might be linked to the emergence of multi-drug resistance [24]. Moreover, a strong correlation between pyoverdine production and the virulence behavior of a *P. aeruginosa* strain (isolated from cystic fibrosis patients) has been demonstrated in a murine pneumonia model [25]. This indicates the high virulence of the isolates used in the present study. Importantly, all isolates of *P. aeruginosa* under the study were powerful producers of biofilms, except for one isolate from a cystic fibrosis patient, which was moderate. Compared with other studies, Mahmood *et al*. [26] observed that most of *P. aeruginosa* isolated in their research were robust biofilm producers, while a few others were moderate biofilm producers. While Mahdi [27] revealed in his investigation the presence of more weak biofilm isolates than fewer isolates with mild biofilm production. There could be a number of reasons for the variations in biofilm thickness, including variations in the isolates' ability to form a biofilm, variations in the proportion of cells that were able to adhere, or variations in the type and amount of QS signaling molecules (autoinducer) that each isolate produced [28].

Pseudomonas aeruginosa QS system is made up of four interconnected systems that work together to create a sophisticated network of intercellular communications. Through the production of QS signaling molecules, the QS systems control the gene expression of the bacteria's virulence factors and the creation of biofilms in a manner that is dependent on cell density [29]. To combat *P. aeruginosa* biofilms, several novel alternative medications have been created recently, such as bacteriophage treatment, antimicrobial peptides, antimicrobial photodynamic therapy, and QSIs [30]. Research has shown that extracts from some soil microbes have QS inhibitory activity, which significantly impedes the biofilm development by *P. aeruginosa*. Soil contains bacteria, fungi, and viruses in varying amounts depending on soil conditions. The fertility of the soil and the accumulation of organic matter within a short time is dependent on the bacterial amount [31]. In this study, the soil isolates consisted of *P.*

mirabilis, *K. pneumoniae* subsp. *pneumoniae*, *E. coli,* and *S. aureus*. Approximately similar sets of bacterial isolates were detected from the soil in the work of Prashanthi *et al*. [32].

Here, the well-known QSI furanone successfully inhibited *P. aeruginosa* pigment. This is consistent with another study, which found that furanone reduced the pyocyanin and biofilm production by *P. aeruginosa* [33]. Pigments produced by *P. aeruginosa* have been implicated in numerous phenomena, such as virulence factors, antioxidant, QS network, and iron acquisition capabilities [17]. In the current investigation, different isolates of *P. aeruginosa* were used as indicators to screen for QSIs produced by soil bacteria. When different *P. aeruginosa* isolates were treated with cultures of the soil bacteria; each of *K. pneumoniae, S. aureus*, and *E. coli* had no effects on the *P. aeruginosa* stain, despite interference with its growth. Nevertheless, the filtrate of *P. mirabilis* isolate no. 4, in particular, succeeded in interfering with the *P. aeruginosa* dye without reducing its growth. In comparison, the other isolates of *P. mirabilis* did not have strong effects, suggesting that the QS inhibitory activity could be strain-specific. Despite being well-known as opportunistic pathogens, *Proteus* species exhibit many beneficial aspects of their life in natural settings. *Proteus* species frequently exhibit remarkable metabolic traits that enable them to adapt to a variety of circumstances and find remote niches in natural settings where they may be identified as autochthons [34]. In contaminated environments, *Proteus* species may function as efficient and specialized rhizobacteria that promote plant development or as bio-remediators of hydrocarbons, pesticides, herbicides, aromatic chemicals, azo dyes, and heavy metals [34].

Interestingly, in this study, the crude aqueous extract of *P. mirabilis* weakened the strong biofilm produced by four *P. aeruginosa* isolates. There are limited investigations on the effect of *P. mirabilis* extract on the biofilm production of different bacterial species. One such study is that of Yu *et al.* [15], who reported *P. mirabilis* as a potential QSIs producer. In that study, concentrations ranging from 0.1 to 1.1% of *P. mirabilis* filtrates inhibited biofilm formation by multiple pathogens, for instance, *P. aeruginosa,* by approximately 59%. The current study suggests an inhibition of biofilm-controlling QS genes. This was verified here at the molecular level, where qPCR showed reduced expression to the QS regulatory gene *LasI* of the eight *P. aeruginosa* isolates, which were treated with sub-MIC of the aqueous extract of *P. mirabilis,* relative to the clinical isolates without treatment.

5. Conclusion

To conclude, the crude aqueous extract of the bacterium isolated from soil (*P. mirabilis*) showed potent effects by inhibiting the biofilm production by interfering with the QS signals of the pathogenic *P. aeruginosa* clinical isolates both phenotypically and genotypically leading to weakening of their biofilm.

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Conflicts of interest

There are no conflicts of interest.

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Author Contributions

I.J.L. put the conception and design of the study, wrote the manuscript, and analyzed the data. Samer Raad Abdul Hussain performed all the experiments and wrote the first draft of the manuscript. All authors have read and approved the final manuscript.

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