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Attenuation of Quorum Sensing, Virulence Factors, and Biofilm Formation in *Pseudomonas aeruginosa* by *Psidium guajava* Leaf Extract

Haitham Qaralleh*

Department of Medical Laboratory Sciences, Mutah University, Mutah, Karak, Jordan

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Abstract

This study was designed to assess the ability of the methanolic extract of *Psidium guajava* leaves to attenuate quorum sensing (QS), biofilm formation, and virulence factors of *Pseudomonas aeruginosa*. Liquid Chromatography - Mass spectrometry (LC-MS) was used to analyze the chemical composition. The effect on *P. aeruginosa* biofilm formation was evaluated using a crystal violet assay, light microscopy, and scanning electron microscopy. The effects on biofilm formation stages, including swarming motility, aggregation ability, surface hydrophobicity, and exopolysaccharide (EPS) production, were also investigated. Besides, the effect of *P. guajava* extract on different *P. aeruginosa* virulence factors, including violacein, pyocyanin, rhamnolipids, protease, and chitinase, was investigated. The mechanism of anti-QS action was evaluated by measuring the effect of the extract on the ability of *P. aeruginosa* to produce acyl-homoserine lactone and the analysis of QS-related gene expression via Real Time – Polymerase Chain Reaction (RT-PCR). LC-MS analysis revealed that quercetin (17.6%), avicularin (17.2%), and kaempferol (15.4%) were the most dominant compounds in the extract. The extract exhibited potent antibiofilm activity at concentrations of 0.37, 0.12, and 0.04 mg/mL, with up to 95% inhibition of biofilm formation. It also significantly interfered with key biofilm development stages, reducing swarming motility by 50% and EPS production by 72.2% at 0.37 mg/mL. The extract suppressed several virulence factors, including pyocyanin production (42.3% inhibition at 0.37 mg/mL), rhamnolipids (59.5% inhibition), and protease activity (49.7% inhibition). Furthermore, the extract reduced the production of acyl-homoserine lactone (AHL) by 29.5% and downregulated QS-related genes, particularly phzA1, indicating its ability to disrupt the QS system. These findings suggest that *P. guajava* methanolic extract holds promise as an anti-infective agent capable of attenuating biofilm formation and virulence factor production in *P. aeruginosa*.

Keywords: *P. guajava, P. aeruginosa*, Anti-infective, Quorum sensing, Biofilm, LC-MS

1. Introduction

Infections caused by *Pseudomonas aeruginosa* are among the most common hospital-acquired infections. *P. aeruginosa* infections can be mild and self-limited, but they may also be systemic, life-threatening infections, especially in those with impaired immune systems such as Acquired Immunodeficiency Syndrome (AIDS) and chemotherapeutic-treated patients (Qin et al. 2022). Moreover, *P. aeruginosa* ranks first in surgical wounds, burns, and diabetic ulcer infections (Bhardwaj et al. 2021). In cystic fibrosis patients, *P. aeruginosa* infection makes matters worse, leading to pulmonary failure and death (Rossi et al. 2021).

Several factors elect *P. aeruginosa* as one of the most common and dangerous pathogenic bacteria. Among these is its ability to resist antibiotics. Equally important is its ability to produce a broad range of virulence factors through an intrinsically interconnected, complex system called the quorum sensing (QS) system. In addition to its high virulence and drug resistance, *P. aeruginosa* tends to live in biofilm communities, making infections difficult to treat (Sahib et al. 2019; Alrawashdeh et al. 2019; Simanek and Paczkowski 2022).

Various approaches have been explored to overcome the challenges of infectious disease treatment, particularly antibiotic resistance. Some scientists are trying to modify the structure of currently used antibiotics, while others are trying to find new antibacterial agents from natural sources. To treat infectious diseases, an attractive approach is now being used to find new anti-infective agents. Anti-infective agents can attenuate virulence factors, biofilm formation, and the QS system of the causative agents (Pulingam et al. 2022; Hasan and Ahmed 2023; Qader and Ganjo 2024). These agents are usually applied at sub-inhibitory concentrations; hence, they exhibit no bacteriostatic or bactericidal activities. Rather, they suppress the QS system and allow the immune system to enhance their effect efficiently (Ma'aitah 2024). In addition, anti-QS agents at sub-inhibitory concentrations exhibited no or minimal toxicity (Haque et al. 2021).

P. aeruginosa, as a community in a biofilm, uses QS systems to achieve maximal effect of their pathogenicity

* Corresponding author. e-mail: haitham@mutah.edu.jo.

and to get through the immune system. The QS system controls the production of various virulence factors in *P. aeruginosa*. First, it starts to produce the chemical signals at a slow rate, which is called autoinducers (AIs) (Brindhadevi et al. 2020). As population density increases, the AI production reaches a threshold, which enables AIs to diffuse into the cytoplasm and complex with specific receptors. This interaction induces gene expression that results in the biosynthesis of virulence factors, which enhances biofilm formation (Vadakkan et al. 2023). It was established that bacteria in biofilms, such as in *P. aeruginosa*, have heightened sensitivity against antibiotics by up to a tenfold factor (Høiby et al. 2010). QS system inhibition could, therefore, reduce the biosynthesis of virulence factors and suppress biofilm formation.

The natural products of plants have been the source of novel biologically active agents (Najmi et al. 2022). Man, since ancient times, has utilized medicinal plants for treatment and relieving the symptoms of diseases. The use of these plants is still growing, specifically in developing countries (Khatib et al. 2021). Scientific testing of some of these traditional plants has provided strong evidence of their effectiveness and safety (Tran et al. 2020). Their traditional use in folk medicine, the successful development of some naturally-based drugs, and their multiple modes of action make them an attractive source for exploring new, promising drugs (Huang et al. 2022) (Bernstein et al. 2021).

Psidium guajava (local name: guava) is a plant tree from the Myetaceae family. Guava fruits are edible as row fruits or as juice. The leaves are known in traditional medicine to relieve respiratory tract symptoms such as flu, cough, and sore throat (Dange et al. 2020). *P. guajava* leaf extract has been found to exhibit antioxidant, antibacterial, anti-inflammatory, anti-allergy, anticough, antiplasmodial, antidiabetic, antinociceptive, antispasmodic, hepatoprotective, and cardioactive effects (Gutiérrez et al. 2008; Anand et al. 2016). However, *P. guajava* leaf extract possesses no or weak antibacterial activity against *P. aeruginosa* (Qaralleh et al. 2020). Thus, this study aimed to evaluate the ability of *P. guajava* methanolic extract to attenuate the quorum sensing system, inhibit biofilm formation, and suppress virulence factors in *P. aeruginosa*. Additionally, we analyzed the extract's chemical composition using LC-MS and examined its mode of action via RT-PCR.

2. Materials and method

2.1. Plant materials

Leaves of *Psidium guajava* were purchased from a traditional herbal market in Jordan. The voucher specimen (MU2023-011) was deposited in the department of biology, Mutah University, Karak, Jordan. The leaves were cleaned to remove dust and any extraneous plant material. The leaves were then processed into a fine powder. The extraction process was performed by soaking 250 g of powdered leaves in 500 mL of methanol and incubating them at room temperature for 24 hours. Then, the solvent was collected after filtration and evaporated using a rotary evaporator. The crude that was produced was collected and stored at 4°C.

2.2. Bacterial species

P. aeruginosa was provided by AlKarak Government Hospital, AlKarak, Jordan. The species was isolated from a urine sample and identified using standard methods, including culture on selective media, analysis of colony morphology, microscopic examination, and biochemical testing. Identification of species level and their antibiotic-resistant profile was made using Biomérieux VITEK®2. It was identified as Beta-Lactamase producing *P. aeruginosa*. *Chromobacterium violaceum* ATCC 12472 was purchased from American Type Culture Collection (ATCC).

Stock solutions of *P. guajava* extract were prepared in 10% DMSO. Bacterial cultures were standardized to 0.5 McFarland turbidity. For the treated samples, the desired final concentrations of the extract were achieved by diluting the stock solution in sterile broth and adding the adjusted bacterial culture. Untreated cultures were prepared using only broth media and the standardized bacterial suspension. In all experiments, a negative control was included, consisting of broth media, 10% DMSO, and the standardized bacterial culture.

2.3. The bacteriostatic effect on P. aeruginosa

The disc diffusion method was performed for preliminary antibacterial activity of methanolic extract *P. guajava* against *P. aeruginosa*. Muller Hinton Agar was prepared and inoculated with 100 µL of bacterial suspension at a concentration of 10^8 CFU/mL. A disc loaded with 1 mg/mL P. guajava methanolic extract was applied on the surface of prepared MHA. The inhibition zone diameter developed after 24h incubation at 37°C was measured in mm (Nigam et al. 2016).

A more precise evaluation of antibacterial activity was conducted using the microdilution method, which can provide a guide to determining the lowest inhibitory concentration (MIC) of *P. guajava* methanolic extract to prevent the complete visible growth of *P. aeruginosa* (Qaralleh et al. 2021). Briefly, several concentrations (4.8, 2.4, 1.2, 0.6, 0.3, and 0.015 mg/mL) of *P. guajava* methanolic extract were prepared in a 96-well plate. Then, 10 µL of bacterial suspension containing 10^4 CFU/mL was added to each well and incubated at 37°C for 24h. Then, the absorbance was measured using ELIZA reader (MCL-2100C, China) at 600 nm.

2.4. Biofilm inhibitory effect

The influence of the extract on *P. aeruginosa* as an anti-biofilm agent was assessed using the crystal violet assay according to (Qaralleh et al. 2020; Qaralleh 2023). A 96-well plate (Polystyrene) was prepared as described before for MIC. Its content was aspirated, washed, and replaced with crystal violet. Then, the dyed plate was incubated at a room temperature for 15 min. The plate contents were removed again, and a decolorization process was initiated by filling the well with 96% ethanol. The plate was incubated at room temperature for 15 min. Using a micropipette, the contents of the wells were transferred to a new 96-well plate, and the absorbance at 570 nm was measured. Untreated culture and culture treated with DMSO (10%) were used as controls. The percentage of inhibition in biofilm formation was calculated in reference to the untreated culture, and the lowest concentration

required to inhibit 50% of the biofilm was reported as MBIC50.

2.5. Effect on the viable cells of the biofilm

A 96-well plate was prepared and incubated as described in the MIC procedure (Gordya et al. 2017). The wells were emptied of their contents and washed adequately to remove free, nonattached cells. Then, 200 µL of a Triphenyltetrazolium chloride (TTC) (Santa Cruz Biotechnology, USA) solution (containing 0.2% glucose and 50 µL of 5 mg/mL TTC) was added to each well and incubated for 6 hours at 37°C with shaking at 150 rpm. The absorbance at 405 nm was measured, and the percentage of reduction in viable cells was calculated in reference to the standard control group.

2.6. Observation of the antibiofilm effect using Light Microscope (LM) and Scanning Electron Microscope (SEM)

A sterile cover slip was placed on the bottom surface of 24-well plates before adding untreated and treated *P. aeruginosa* cultures to the wells. The prepared plates were incubated without shaking for 48h at 37°C. Then, the glass slips were removed, washed, and stained with crystal violet for 1 min. Then, the slips were observed using a LM (NIKON, Japan). In parallel, another set of slips was similarly prepared for SEM observation. However, the slips were incubated in 5% glutaraldehyde for 24h and then dehydrated using a gradual increase in ethanol concentrations in water (10, 30, 50, 70, and 100%). The samples were subjected to critical point drying and finally observed using SEM (Thermo Scientific Phenom Desktop SEM, JU-24112022, Waltham, MA, USA).

2.7. Effect on biofilm stages

2.7.1. Swarming

A jelly-like swarming medium was prepared using 0.5% agar (Sagar et al. 2022). After autoclaving, the tested materials were mixed with the molted agar and poured into petri dishes. The prepared plates were left to solidify, and 1 µL of 24h old *P. aeruginosa* culture was inoculated at the center of the plate using a sterile calibratative loop. The inoculated plates were incubated for 48h, and the swarming area was measured in mm and compared with the typical swarming pattern of the normal untreated cells.

2.7.2. Aggregation

The effect on the aggregation ability of *P. aeruginosa* was evaluated according to (Shanks et al. 2008). In brief, the absorbance of 1 mL of the treated *P. aeruginosa* culture was measured at 600 nm to determine the initial absorbance (iAbs). The culture was then vortexed for 1 min and the post vortixing absorbance was measured again (sAbs). Untreated *P. aeruginosa* was used as a control. The percentage of aggregation for the untreated and treated cultures of *P. aeruginosa* was calculated according to the following formula:

Percentage of aggregation = [(sAbs – iAbs) / sAbs] * 100

2.7.3. Hydrophobicity

The effect on the surface hydrophobicity of *P. aeruginosa* was examined using n-hexadecane (Krishnan et al. 2012). In brief, the absorbance of 1 mL of the treated *P. aeruginosa* culture was measured at 600 nm to determine the initial absorbance (iAbs). This culture was then mixed with 1 mL n-hexadecane (Sigma Aldrich, USA). The mixture was subjected to vortexing (2 min) and left to fractionate. After 15 min., the second absorbance at 600 nm was measured (sAbs). The percentage of hydrophobicity was calculated using the following formula:

Percentage of hydrophobicity = [(sAbs – iAbs) / sAbs] * 100

2.7.4. EPS

Treated and untreated cultures of *P. aeruginosa* were centrifuged. After centrifugation, portions of the supernatant were mixed with cold ethanol in a 1:3 ratios. The solution was incubated at 4°C for 24h. Then, the centrifugation (10000 rpm for 15 min) was made, and to the collected pellets, distilled water (3 mL), 5% cold phenol (1 mL), and 98% sulfuric acid (5 mL) were supplemented. Absorbance at 490 nm (Shimadzu UV-1601, Japan) was reported, and the percentage of EPS was calculated in reference to the standard control group (Razack et al. 2011).

2.8. Effect on virulence factors

2.8.1. Pyocyanin

Pyocyanin was extracted from treated and untreated cultures of *P. aeruginosa* using chloroform (Hossain et al. 2017). 1 mL of the culture supernatant was mixed with an equal volume of chloroform. Chloroform containing pyocyanin was separated and added to 1.5 mL of 0.2N hydrochloric acid. The absorbance at 520 nm of the developed pink color solution was measured, and the percentage inhibition in pyocyanin production was calculated in reference to the untreated culture.

2.8.2. Rhamnolipids

Rhamnolipids were extracted from treated and untreated cultures of *P. aeruginosa* using diethyl ether (Luo et al. 2017). 1 mL of the culture supernatant was mixed with 3 mL of diethyl ether. Diethyl ether containing rhamnolipids was separated, concentrated using a rotary evaporator, and supplemented with 200 µL of water and 900 µL of 0.18% orcinol (w/v) in 53% (v/v) H_2SO_4. The solution was then heated at 24°C for 30 min and the absorbance at 421 nm was measured. The percentage of inhibition in rhamnolipid production was calculated in reference to the untreated culture.

2.8.3. LasA protease

LasA protease activity was measured using azocasein as a substrate (Andrejko et al. 2013). The reaction was prepared by mixing 1 mL of the culture supernatant with 0.1 mL of azocasein (Sigma Aldrich, USA) and 3 mL of 50 mM phosphate buffer (pH 7.5). The reaction was allowed to react for 1h, at 37°C, and 150 rpm. The reaction was terminated by adding 0.5 mL of trichloroacetic acid (TCA) (Thermo Fischer Scientific, China), followed by centrifugation at 10,000 rpm for 10 minutes. The absorbance of the collected supernatant was measured at 366 nm. The percentage of inhibition in LasA protease activity was calculated in reference to the untreated culture.

2.8.4. Chitinase

Chitinase activity was measured using chitin azure as a substrate (Hossain et al. 2017). The reaction was prepared

by mixing 0.5 mL of the culture supernatant with 4.5 mL of chitin azure (Bioscientific Carbosynth, UK) solution. The reaction was allowed to react for 24h, at 37°C. Then, it was subjected to centrifugation for 10 min and the absorbance of the collected supernatant was measured at 570 nm. The percentage of inhibition in chitinase activity was calculated in reference to the untreated culture.

2.9. Mechanism of quorum sensing

2.9.1. Effect on violacein production

The preliminary evaluation of *P. guajava* methanolic extract on violacein production by *C. violaceum* was made using the well diffusion method. MHA was inoculated with 100 µL of bacterial suspension containing 10^8 CFU/mL. A well of 6 mm was made in MHA and loaded with 100 µL of 1 mg/mL *P. guajava* methanolic extract. The development of the violacein inhibition zone after 24h of incubation at 37°C was measured in mm.

A more precise evaluation of the effect on violacein production was performed quantitatively (Gómez-Gómez et al. 2019). The violacein contents of a 1 mL supernatant from the untreated and treated cultures of *C. violaceum* were extracted using 1 mL DMSO for 30 min. Then, the mixture was centrifuged, and the absorbance at 575 nm for the supernatant was measured. The percentage of violacein inhibition was calculated in reference to the untreated group.

2.9.2. Acyl Homoserein Lactone (AHL) production

AHL was extracted from treated and untreated cultures of *P. aeruginosa* using ethyl acetate (Lee et al. 2017). 2 mL of the culture supernatant was mixed with 3 mL of ethyl acetate. At the end of the incubation (24h and 40°C), 40 µL of the ethyl acetate extract was supplemented with 50 µL from a solution that was prepared by mixing equal volumes of 2 M hydroxyl amine and 3.5 M NaOH, and 90 µL from a solution that was prepared by mixing equal volumes of 10% ferric chloride in 4% HCL and 95% ethanol. Next, absorbance at 520 nm was reported, and the percentage of AHL inhibition was calculated in reference to the standard control group.

2.9.3. Expression of Quorum sensing genes

The total RNA was extracted from the untreated and treated cultures of *P. aeruginosa* using a direct-zoltm RNA miniprep kit (Zymo Research Company, USA). The synthesis of the complementary cDNA was performed using the SensiFASTTM cDNA synthesis kit (Bioline Reagents, UK). The gene expression was prepared using the Bioline SensiFAST™ SYBR® No-ROX Kit (Bioline Reagents, UK), and the tested gene expressions were normalized to the expression of 16S rRNA as a housekeeping gene using the 2ΔCT formula. The primer sequences used in this test are presented in Table 1 (Banerjee et al. 2017).

Table 1. Sequences of primers of *PhazA1, apraA, PelA* and *Psl* genes.

Gene	Primer Sequence 5'-3'		References
16S rRNA	Forward	CAAAACTACTGAGCTAGAGTACG	(Lenz et al. 2008)
	Reverse	TAAGATCTCAAGGATCCCAACGGCT	
PelA	Forward	CCTTCAGCCATCCGTTCTTCT	(Li et al. 2019)
	Reverse	TCGCGTACGAAGTCGACCTT	
PslA	Forward	AAGATCAAGAAACGCGTGGAAT	(Irie et al. 2012)
	Reverse	TGTAGAGGTCGAACCACACCG	
PhazA	Forward	CGAACCACTTCTGGGTCGAGTGC	(Vandeputte et al. 2011)
	Reverse	GGGAATACCGTCACGTTTTATTTGC	
AprA	Forward	GGCAATCCTGGTACCTGATCAA	(Cabrol et al. 2003)
	Reverse	AGCGTCTGGCGCCCGTAGTT	

2.10. Chemical composition

The analysis of the chemical composition of *P. guajava* methanolic extracts was carried out using LC-MS (LC-8030, Shimadzu, Japan). The column used was XDB-C18 (150 mm length × 2.1 mm internal diameter, 3.5 µm film thick), and the mobile phase used was prepared using two solvents: A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). A 50 µL of the sample was injected and eluted using a gradient mobile phase of 5% of A (5 min), 5-100% of B (15 min), and 100% of B (5 min) at a rate of 500 µL/min. 1 µl of the sample was injected using the Shimadzu CBM-20A system controller. Mass spectrometry was performed in positive ion mode using electrospray ionization (ESI-MS), scanning m/z from 100 to 1000. The ESI-MS was operated with a capillary voltage of 125 V, a skimmer voltage of 65 V, and nitrogen gas flow at 10 L/min at 45 psi. The capillary temperature was maintained at 350°C.

2.11. Statistical analysis

The significant difference between the treatment groups and the untreated standard group was assessed using one-way ANOVA and expressed as stars (*, **, or ***) corresponding to the P-values of $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

3. Results

3.1. Antibacterial activity

The inhibition zone observed for the methanolic extract of *P. guajava* against *P. aeruginosa* is 11.67 mm, and the MIC was found to be within the range of 1.1-3.3 mg/mL (Table 2). DMSO, the negative control, showed no antibacterial activity, while the positive control (tetracycline) showed an inhibition zone of 22.5 mm.

Table 2. Diameter of inhibition zone and MIC for the methanolic extract *P. guajava* leaf against *P. aeruginosa*

	Inhibition zones (mm)	MIC (mg/mL)
P. guajava methanolic extract (1 mg/disc)	11.67±0.3	1.1-3.3
Tetracycline (30 µg/disc)	22.5±0.3	-
DMSO	0.0	-

-: not determined

3.2. Antibiofilm

The impact of *P. guajava* extract on the formation of biofilm was investigated using a broad range of concentrations (10-0.0005 mg/mL) (Figure 1). Application of 10, 3.3, 1.1, and 0.37 mg/mL extracts completely (≥ 95%) prevented the formation of *P. aeruginosa* biofilm, while at lower concentrations (0.12–0.0005 mg/mL), the treatment led to a gradual decrease in inhibition. A significant ($P < 0.001$) percent of inhibition equal to 59.5% was reported at a treatment concentration of 0.12 mg/mL, while it dropped significantly to 37.0, 24.5, and 17.5% at 0.04, 0.014, and 0.005 mg/mL. As shown in Figure 1, the MBIC50 can be observed within the range of 0.12 and 0.04 mg/mL.

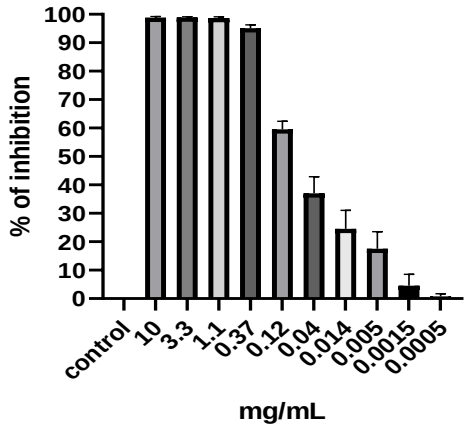


Figure 1. The effect of *P. guajava* methanolic extract on the ability of *P. aeruginosa* to form a biofilm, as indicated by the percentage inhibition. The percentage of inhibition for the untreated (control) culture was considered 0.

As previously mentioned, the MIC of *P. guajava* extract was within the range of 1.1 and 3.3 mg/mL. Consequently, the antibiofilm effect at ≥ 1.1 mg/mL appears to be due to the bacteriostatic action of the extract. Therefore, a TTC assay was performed to examine the effect of the extract on the viable cells within the biofilm matrix. The findings of the TTC assay (Figure 2) provide extra evidence that the inhibition of biofilm formation at concentrations of 10, 3.3, and 1.1 mg/mL is due to the bacteriostatic effect of *P. guajava* methanolic extract, as indicated by the significant reduction in the viable cell to less than 10%. However, the reduction in viable cells was non-significant for treatment concentrations equal to or less than 0.37 mg/mL, indicating that the inhibition of biofilm formation is not due to the bacteriostatic effect but rather to the antibiofilm action of the extract. Therefore, concentrations of 0.37, 0.12, and 0.04 mg/mL, which demonstrated the maximum antibiofilm effect without affecting cell viability, were selected for further investigation.

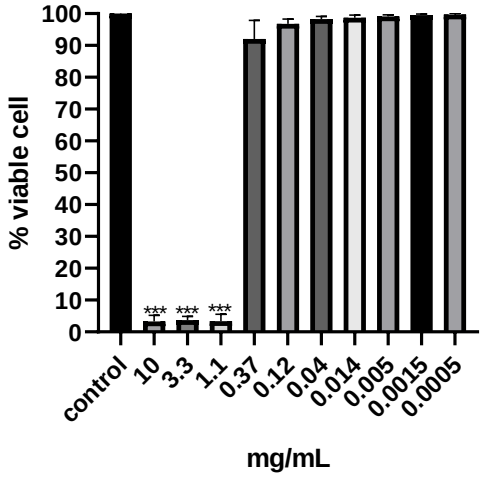


Figure 2. The effect of *P. guajava* methanolic extract on the percentage of viable cell. *, **, or *** corresponding to the P-values of $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively

3.3. Biofilm observation using LM and SEM

To confirm the effect of *P. guajava* methanolic extract on *P. aeruginosa* biofilm, the normal and treated biofilms were prepared and visualized using LM and SEM (Figure 3 and 4). SEM images clearly demonstrate the effect of the *P. guajava* extract on biofilm formation. As shown in the LM and SEM images (Figure 3 and 4), the images of untreated culture showed the ability of *P. aeruginosa* to form a normal biofilm that is composed of multilayered aggregated cells. The ability of the extract to prevent *P. aeruginosa* from building biofilm can be observed in Figure 3B-D, which showed the presence of no aggregated scattered cells. These signs appear to increase clearly as the concentrations of the treatment increase.

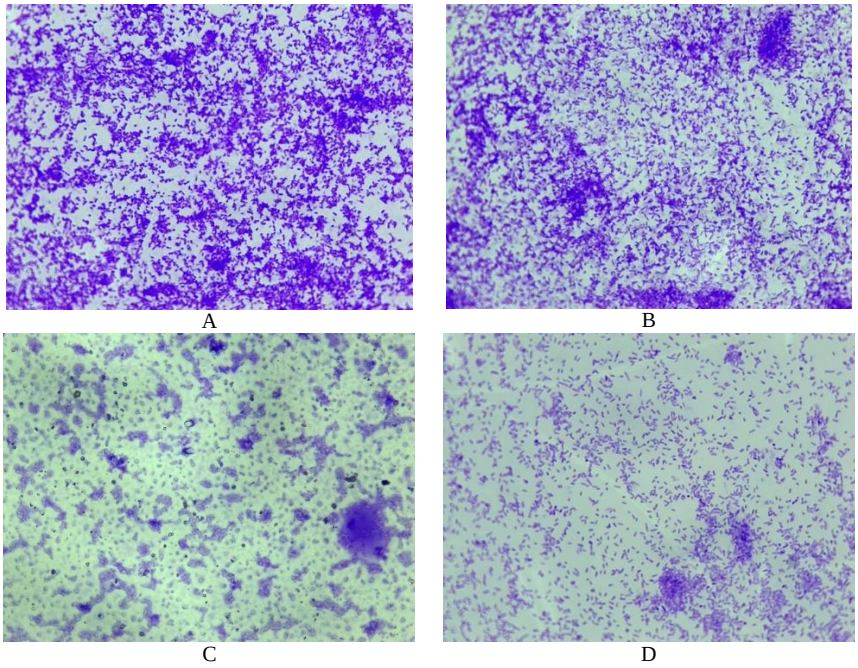


Figure 3. LM images (40X) of *P. aeruginosa* treated with 0 (A), 0.04 (B), 0.12 (C), and 0.37 mg/mL (D) *P. guajava* methanolic extract

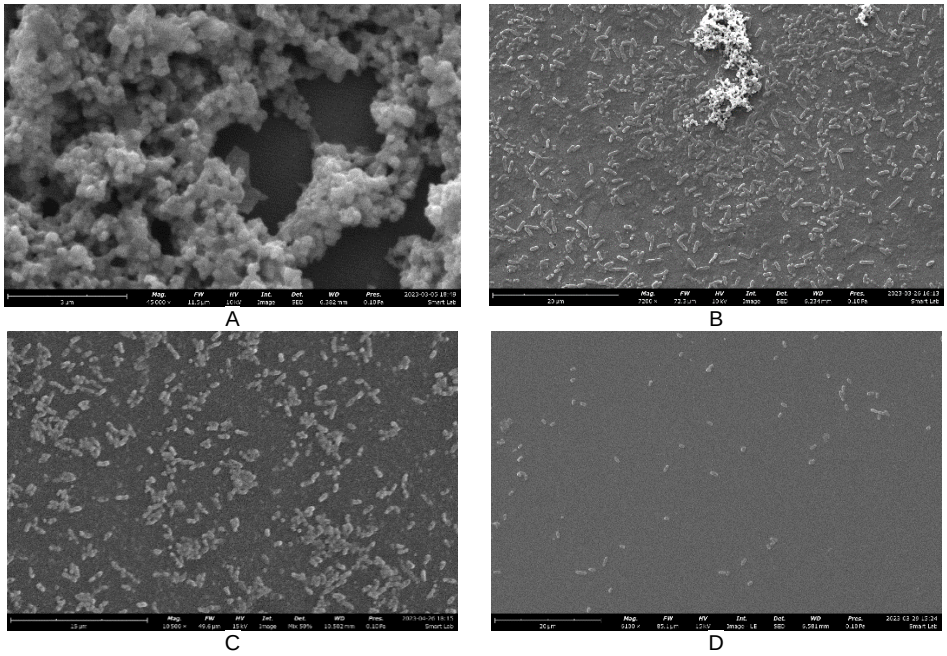


Figure 4. SEM images of *P. aeruginosa* treated with 0 (A), 0.04 (B), 0.12 (C), and 0.37 mg/mL (D) *P. guajava* methanolic extract

3.4. Effect on the biofilm development

The effect of *P. guajava* methanolic extract on swarming motility, aggregation ability, and surface hydrophobicity as significant factors in biofilm development has been evaluated.

3.4.1. Swarming motility

The ability of *P. aeruginosa* to swarm decreased in a dose-dependent manner as a result of the treatment with 0.37, 0.12, and 0.04 mg/mL *P. guajava* extract (Figure 5A). As the concentration of the extract increased, the zone of swarming (mm) decreased. However, the decrease was significant ($p < 0.01$) only at the treatment concentration of 0.37 mg/mL.

3.4.2. Aggregation

The results showed that *P. guajava* methanolic extract displayed a dose-dependent manner of inhibition in the aggregation (Figure 5B). At the treatment concentration of 0.37 mg/mL, the aggregation was significantly ($p < 0.05$) reduced from 28.0% to 10.0%. The aggregation at 0.12 mg/mL was notably reduced to 16.1%, but it was insignificant.

3.4.3. Hydrophobicity

As shown in Figure 5C, the reduction in hydrophobicity was significantly decreased from 62% to 26.3% when the treatment was made using 0.37 mg/mL. In contrast, the reduction due to the treatment with 0.12 or 0.04 mg/mL was insignificant.

3.4.4. EPS

As shown in Figure 5D, EPS production significantly decreased in a dose-dependent manner. At 0.37 and 0.12 mg/mL, EPS production decreased significantly to 72.2 and 88.7%, respectively. At a lower concentration (0.04 mg/mL), the reduction in EPS was insignificant.

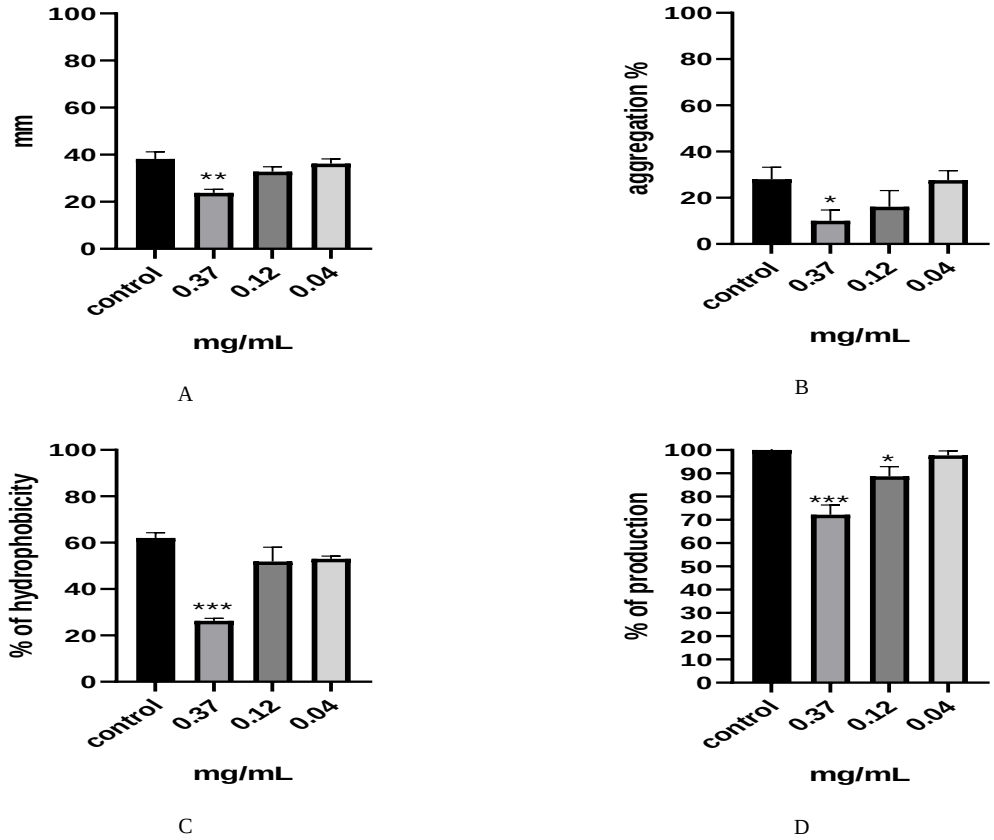


Figure 5. The effect of *P. guajava* methanolic extract on different stages in biofilm formation, including swarming motility (A), aggregation (B), hydrophobicity (C), and EPS production. *, **, or *** corresponding to the P-values of $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively

3.5. Effect on the virulence factors

3.5.1. Pyocyanin

The ability of *P. guajava* methanolic extract to suppress the production of pyocyanin pigment in *P. aeruginosa* was evaluated (Figure 6A). The treatment resulted in significant dose-dependent inhibition of pyocyanin production, with inhibition rates of 42.3% and 24.0% at concentrations of 0.37 and 0.12 mg/mL, respectively. The reduction at 0.04 mg/mL was insignificant.

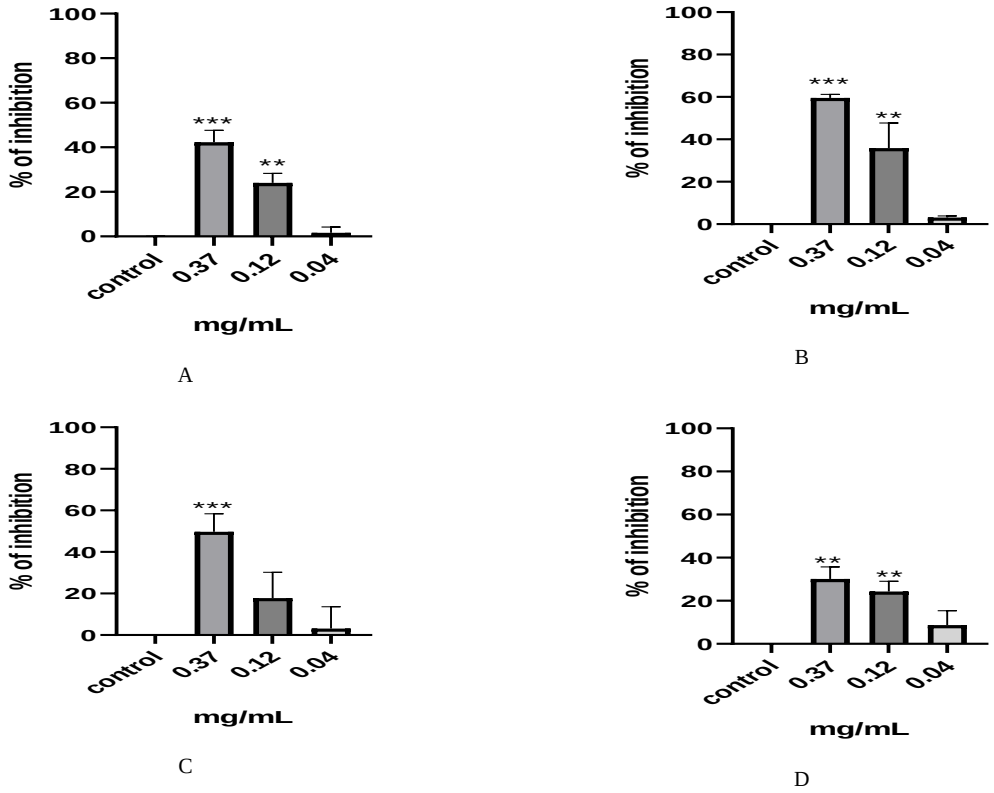


Figure 6. The effect of *P. guajava* methanolic extract on *P. aeruginosa* virulence factors, including pyocyanin (A), rhamnolipids (B), LasA protease (C), and chitinase (D). *, **, or *** corresponding to the P-values of $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively

3.5.2. Rhamnolipids

As shown in Figure 6B, the pattern of the inhibitory effect of *P. guajava* methanolic extract on rhamnolipid production appears to be similar to that of pyocyanin. A dose-dependent inhibitory effect was observed. The percentage of rhamnolipid inhibition was 59.5% and 35.9% at treatment concentrations of 0.37 and 0.12 mg/mL, respectively. No significant reduction was observed due to the treatment with 0.04 mg/mL.

3.5.3. LasA protease

As shown in Figure 6C, the inhibitory effect of *P. guajava* methanolic extract on the activity of LasA protease was displayed in a dose-dependent manner. However, the treatment at 0.37, 0.12, and 0.04 mg/mL led to a decrease in protease activity, as indicated by the percentage inhibition of 49.7, 17.8, and 3.1%, respectively. The statistically significant ($p < 0.001$) reduction was found only at 0.37 mg/mL.

3.5.4. Chitinase

Treating *P. guajava* methanolic extract resulted in a dose-dependent inhibitory effect on the chitinase activity (Figure 6D). Treating *P. aeruginosa* with the extract resulted in a significant ($p < 0.01$) reduction in the chitinase activity, with a percentage inhibition of 30.1 and 24.3% observed at 0.37 and 0.12 mg/mL, respectively. The percentage of inhibition was non-significant at 0.04 mg/mL.

3.6. Mechanism of action

3.6.1. Violacein

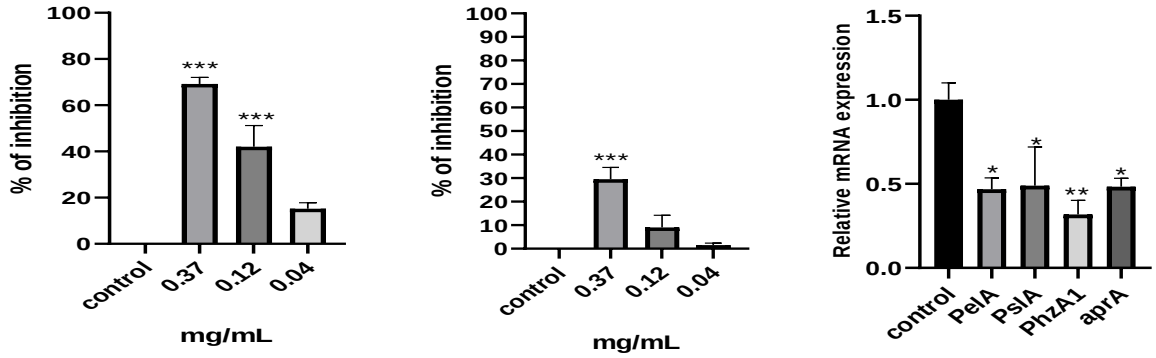
The effect of *P. guajava* methanolic extract on the production of violacein pigment by *C. violacium* was investigated to assess the effect of the extract on the QS system. A preliminary finding using the well diffusion method showed the ability of the extract to inhibit the QS system, as indicated by the development of a 13.5 mm non-pigmented zone around the well. Moreover, the quantitative analysis (Figure 7A) confirmed this finding and observed a significant ($p < 0.001$) reduction in violacein production. A percentage inhibition of 69.2 and 42.0% was observed when the extract was applied at 0.37 and 0.12 mg/mL, respectively.

3.6.2. AHL

The results showed that *P. guajava* extract causes a dose-dependent inhibition of AHL production (Figure 7B). The percent of AHL production was reduced significantly ($p < 0.001$) by 29.5% due to the treatment of 0.37 mg/mL extract. The reduction in AHL due to the treatment with the methanolic extract at lower concentrations (0.12 and 0.04 mg/mL) was insignificant.

3.6.3. The effect on PelA, PslA, PhzA1, and aprA genes

The effect of *P. guajava* methanolic extract on the mRNA expression of *PelA*, *PslA*, *PhzA1*, and *aprA* genes was evaluated using RT-PCR (Figure 7C). The results showed that the extract caused a significant reduction in the expression of these genes. Statically, the maximum reduction ($P < 0.01$) was observed in the expression of the *PhzA1* gene.



A B C

Figure 7. The effect of *P. guajava* methanolic extract on the ability of *C. violaceum* to produce violacein pigment (A), *P. aeruginosa* to produce AHL (B), on the mRNA expression of QS dependent genes (C). *, **, or *** corresponding to the P-values of $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

3.7. Chemical composition of P. guajava methanolic extract

The chemical composition of *P. guajava* methanolic extract was analyzed using LC-MS (Table 3). Flavonoids were identified as the most dominant components among a total of 22 compounds. The most dominant content identified was quercetin, representing 17.6% of the total content. Remarkably, the presence of 17.2% of avicularin and 15.4% of kaempferol as dominant components was also reported. These three main contents represent more than 50% of the total contents of the extract. Besides, the LC-MS analysis showed that chlorogenic acid (9.1%), kaempferol-3-glucoside (6.3%), lutein (6.1%), and beta-carotene (5.2%) were present in considerable amounts.

Table 3. Chemical composition of *P. guajava* methanolic leaf extract using LC-MS

	Chemical compound	Classification	%
1	Ellagic acid	Phenolic acid	0.2
2	Leucocyanidin	Leucoanthocyanidin (flavan-3,4-diols)	0.1
3	Querecetin	Flavonoid	17.6
4	Kaempferol-3-glucoside	Glycoside flavonoid	6.3
5	Avicularin	Flavonoid	17.2
6	Ferulic acid	Phenolic acid	7.3
7	Lutein	Carotenoid	6.1
8	Chlorogenic acid	Phenolic acid	9.1
9	Beta-Carotene	Carotenoid	5.2
10	Kaempferol	Flavonoid	15.4
11	Quercetin 3-beta-galactoside	Glycoside flavonoid	0.1
12	Gallic acid	Phenolic acid	2.2
13	Lycopene	Carotenoid	0.1
14	Guaijaverin	Flavonoid	2.2
15	Caffeic acid	Phenolic acid	1.3
16	Phytofluene	Carotenoid	1.3
17	Rubixanthin	Carotenoid	0.1
18	Oleanolic acid	Triterpene	0.1
19	Ursolic acid	Triterpene	0.1
20	Beta-Sitosterol	Triterpene	0.1
21	Uvaol	Triterpene	0.1
22	Protocatechuic acid	Phenolic acid	0.2

4. Discussion

Continuing to use antibiotics directly to kill or inhibit bacteria may become an ineffective therapy due to the ability of many pathogenic bacteria to produce biofilm. Therefore, the use of antibiotics to treat many infectious diseases has become impossible, and this may lead to many health problems that are dangerous to human life (Pulingam et al. 2022). With the diversity of strategies for producing new antibiotics or protocols to control infectious diseases, it is clear that finding a drug that can reduce bacteria's virulence factors is among the best-used strategies currently. This mechanism works by impairing the ability of bacteria to produce virulence and develop biofilms through QS disruption (Haque et al. 2021). Natural products with active ingredients can be excellent candidates to serve as a source for developing anti-QS agents. Thus, *P. guajava* has been selected for this study.

It was demonstrated that *P. guajava* exhibited weak antibacterial action both by using disc diffusion and a microdilution method. Thus, the findings are in agreement with the findings of (Cheruiyot et al. 2009; Qaralleh et al. 2020), which showed that this leaf methanolic extract obtained from the *P. guajava* leaf presents weak antibacterial action or its lack against *P. aeruginosa*.

The plant natural products were earlier reported as antibiofilm agents. *Tilia cordata* and *Nepeta curviflora* polar-essential oils extracts from *Thymbra capitata* were previously reported for their potent antibiofilm activity (Qaralleh 2019; Qaralleh et al. 2020; Qaralleh 2023). In this study, methanolic extract of *P. guajava* showed potent antibiofilm activity at 0.37, 0.12, and 0.04 mg/mL against *P. aeruginosa*. At these concentrations, there was no significant decrease in viable cells, which implicates the antibiofilm activity of the bioactive extract against *P. aeruginosa* as a mode of suppression of biofilm formation rather than through reductions in the number of viable cells. The methanolic extract of *P. guajava* was further confirmed to impede the development of the biofilm of *P. aeruginosa* by both light and scanning electron microscopy.

The transition of bacteria from a planktonic free-living lifestyle to one of living in communities and as a biofilm is an orderly process in a series of successive events. A conditioning film is formed that attracts other bacterial cells to the surface, where they attach and start to accumulate. The cells in the resultant community proliferate, and the biofilm matures. Some of these cells detach from the biofilm to colonize new surfaces during the dispersion phase where they express other biofilms. However, some of these cells in the dispersion phase could reattach themselves to other surfaces to form biofilms (Sauer et al. 2022). In this study, the effect of 0.37 mg/mL of *P. guajava* methanolic extract on different factors that affect biofilm development, including motility, hydrophobicity, aggregation, and EPS production, has been evaluated. Interestingly, *P. guajava* extract causes a significant reduction in these factors.

As shown in the results, the extract has a significant reduction effect on *P. aeruginosa* surface hydrophobicity, aggregation ability, and swarming motility. It is logical to assume that some components of the plant extract have affinity to bind with the adhesion sites, preventing adhesion and aggregation. The reduction in adhesion and aggregation may be due to the inhibition of bacterial motility. Agents that inhibit bacteria from swarming usually prevent the initial stage of biofilm formation (Shrout et al. 2006). In addition, perhaps one of the most important roles for the EPS is to stimulate adhesion and aggregation, and its role extends to maintaining the development of biofilm (Kassinger and van Hoek 2020). The use of the *P. guajava* extract led to the inhibition of these interfering factors, which in turn led to the prevention of the formation of biofilms. It is possible that the plant extract led to this result through several mechanisms.

P. guajava extract showed a profound effect on suppressing *P. aeruginosa* virulence factors. This suggests that it may interfere with the QS system. Suppression of the QS system results in decreasing AI production, decreasing virulence factor production, and hence attenuating pathogenicity (Ahmed et al. 2019). Protease and elastase, as secreted virulence factors, are concerned about adhesion and colonization. Furthermore, they act to cause tissue damage and facilitate invasiveness in the tissue host. With the help of the chitinase enzyme, they promote bacterial aggregation and infection. Pyocyanin is produced to function as a chelating agent to extract iron from transferrin (Andrejević et al. 2023). In biofilms, pyocyanin forms complexes with eDNA, increasing surface hydrophobicity and aggregation, thereby maintaining the integrity of the extracellular polymeric substance (EPS) and biofilm stability (Abdelaziz et al. 2023). Rhamnolipids are surfactant factors that mediate motility and biofilm initiation. Reports showed that reducing rhamnolipid production inhibits swarming motility, but when rhamnolipids are supplemented again, their function is restored and they facilitate motility (O'May and Tufenkji 2011). The significant suppression in the production of these factors suggests that *P. guajava* extract exhibits unique anti-QS activity that may disrupt the ability of *P. aeruginosa* to cause disease and impede the progression of the infection.

The sequence of events that make the free-living bacterial cells form a microcolony and reach a threshold level ends in the activation of the QS system and the production of AI. In this study, the results showed that the extract resulted in a significant reduction in AHL production. AHL is a type of AI that is produced due to the activation of the QS system (Ashraf et al. 2018). Therefore, a reduction in AHL may suggest that the extract may suppress the QS system. In *P. aeruginosa*, four QS systems are used: two are AHL-dependent, called the Las and Rhl systems, while the third system, the Pqs system, is quinolone-dependent, and the last one is the Iqs, carbaldehyde-dependent system. However, the Las system works as a master system that regulates all systems; the regulation of these systems is interconnected (Vetrivel et al. 2021). These QS systems regulate virulence factor production (Soukarieh et al. 2018). The Las system controls the production of alkaline protease, Psl EPS, Pel EPS, elastase A, and elastase B through the aprA, psl, pel, lasA, and lasB genes, respectively (Ueda and Wood 2009). All but aprA are also regulated by the Pqs system, which controls the production of rhamnolipids, pyocyanin, and lectin A and B (Lin et al. 2018; Turkina and Vikström 2019).

In the current investigation, the effect of the methanolic extract of *P. guajava* on the mRNA expression of the *psl*, *pel*, *aprA*, and *phzA1* genes was assessed. The production of pel and psl as part of the biofilm matrix is vital in mediating adhesion and organization as well as providing defense. While the *aprA* gene mediates alkaline protease production that denatures complementary and cytokine proteins, the *phzA1* gene controls the secretion of pyocyanin (Mellbye and Schuster 2014) (Pezzoni et al. 2020). When *P. aeruginosa* was treated with 0.37 mg/mL *P. guajava* extract, a significant reduction in *Pel*, *Psl*, *aprA*, and *phzA1* genes was observed. However, the most notable decrease was seen in the expression of the *phzA1* gene. In fact, *Pel*, *Psl*, and *aprA* are Las-dependent genes, while *phzA1* is a pqs-dependent gene. This may suggest that the extract may exert multiple effects through inhibition of the Las and pqs systems, or it may disrupt the interconnection and feedback mechanisms between these QS systems, such as those between the Las system and Rhl, Pqs and Iqs, the Pqs system and Rhl system, and the Iqs system and Pqs system (Pesci et al. 1997).

The inhibitory effect of *P. guajava* extract may be due to one of its active ingredients or a synergism between them. Vasavi and co-authors found that a flavonoid fraction of *P. guajava* significantly reduced biofilm formation, pyocyanin, protease, elastase, violacein production, and swarming motility in relevant strains of *P. aeruginosa* PAO1 and *C. violaceum* at low concentrations ranging from 25 to 400 µg/mL (Vasavi et al. 2014). Quercetin, the most dominant compound in the methanolic extract, has been found to reduce violacein production at 1.2 mg/mL (Vandeputte et al. 2011). At low concentrations (16 µg/mL), quercetin exhibited antibiofilm activity. It inhibited the production of pyocyanin, protease, and elastase and the expression of *lasI*, *lasR*, *rhlI*, and *rhlR* genes in *P. aeruginosa* (Ouyang et al. 2016). Other components, such as kaempferol, have been reported to have anti-QS activity against *E. coli* (Vikram et al. 2010).

In this study, the LC-MS analysis showed that the primary constituents of *P. guajava* methanolic extract are phenolics and flavonoids. The most abundant constituents were quercetin (17.6%), avicularin (17.2%), kaempferol (15.4%), chlorogenic acid (9.1%), kaempferol-3-glucoside (6.3%), lutein (6.1%), and beta-carotene (5.2%). Previous studies showed that the main constituents of *P. guajava* leaves are rutin, naringenin, gallic acid, catechins, epicatechins, kaempferol, quercetin, and guaijaverin (Sampath Kumar et al. 2021). However, the essential oil chemical analysis showed the presence of a significant amount of limonene (42.1%) and caryophyllene (21.3%) (Ogunwande et al. 2003), along with some other compounds, including α-pinene, β-pinene, isopropyl alcohol, menthol, terpenyl acetate, longicyclene, β-bisabolene, and oleanolic acid (Pino et al. 2001; Ogunwande et al. 2003; Begum et al. 2004; Naseer et al. 2018).

5. Conclusion

The methanolic extract of *P. guajava* has demonstrated significant potential in modulating key virulence factors of *P. aeruginosa* by disrupting its QS system. This study highlighted the extract's unique anti-QS properties, evidenced by its ability to selectively inhibit biofilm formation at sub-inhibitory concentrations without significantly affecting bacterial viability. Notably, the extract interfered with several biofilm formation stages, including swarming motility, aggregation, hydrophobicity, and exopolysaccharide (EPS) production, and suppressed important virulence factors such as pyocyanin, rhamnolipids, protease, and chitinase. These findings suggest that *P. guajava* methanolic extract acts through inhibition of the QS-regulated production of acyl-homoserine lactones (AHLs) and a reduction in the expression of QS-related genes (*pel*, *pslA*, *aprA*, and *phzA1*). Given the global rise in antibiotic resistance, the discovery of natural anti-QS agents like *P. guajava* extract provides a promising alternative therapeutic strategy. By targeting virulence mechanisms rather than bacterial viability, this extract reduces the selective pressure for resistance, which is critical in the fight against resistant pathogens. Future studies should focus on isolating the active compounds within the extract and further elucidating their specific mechanisms of action and potential therapeutic application.

6. Disclosure

The author reports no conflict of interests in this work.

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