Qaralleh, H., 2024
J. Basic Appl. Res biomed 10(1): 80-88

10.51152/jbarbiomed.v10i1.245

JBAAR
Journal of Basic and Applied Research in Biomedicine
ISSN 2413-7014

Original Article

Limonene as a Multi-Target Antibiofilm and Quorum Sensing Inhibitor Against *Pseudomonas aeruginosa*

Haitham Qaralleh

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

*Corresponding Author: haitham@mutah.edu.jo

Received: 29-11-2024
Revised: 22-12-2024
Published: 23-12-2024

Keywords:
Limonene,
Antibiofilm,
Anti-quorum sensing,
Anti-infective,
Pseudomonas aeruginosa

Abstract: *Pseudomonas aeruginosa*, a common causative pathogen of nosocomial infections, has recently evolved multidrug resistance, creating an urgent demand to develop alternate antimicrobials targeting virulence systems. In this study, we elucidate the inhibitory effects of limonene, a monoterpene abundantly present in citrus essential oils, on biofilm formation and quorum sensing systems of *P. aeruginosa*. The determination of limonene antibacterial activity determined the MIC to be 0.63 mg/mL. Limonene showed strong antibiofilm activity, inhibiting by 97.18% at MIC, while at sub-MIC (0.078 and 0.039 mg/mL), it also demonstrated good efficacy. Mechanistic analyses revealed that limonene inhibits biofilm formation via the modulation of bacterial aggregation, surface hydrophobicity, swarming motility, and extracellular polymeric substance (EPS) release. Under sub-MIC levels, limonene also inhibited important virulence factors like pyocyanin, rhamnolipids, and LasA protease. Additionally, limonene played a role in interrupting quorum sensing communication, as it inhibited violacein biosynthesis by *Chromobacterium violaceum* and decreased acyl-homoserine lactone (AHL) synthesis in *P. aeruginosa*. High-throughput molecular analysis confirmed massive downregulation of pivotal quorum sensing genes (*lasI/R* and *rhlI/R*), ultimately pointing to limonene's remarkable property of interfering with bacterial signaling circuitry. These results highlight limonene as a multi-target pharmacological agent against *P. aeruginosa*, representing a promising alternative to classical antibiotics. More studies are required to determine its mechanisms of action, effects on other quorum sensing regulated genes, and responsiveness in clinical settings. These findings add to the mounting evidence supporting the use of natural compounds against antibiotic resistance and biofilm-associated infections.

Cite this article as: Qaralleh, H. (2024) Limonene as a Multi-Target Antibiofilm and Quorum Sensing Inhibitor Against *Pseudomonas aeruginosa*. Journal of Basic and Applied Research in Biomedicine, 10(1): 80-88. 10.51152/jbarbiomed.v10i1.245

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic notorious pathogen implicated in a wide array of health care-associated infections among compromised immunity. Its remarkable resistance to multiple antibiotics, together with the ability to form robust biofilms, presents serious treatment challenges in clinical settings (Zhao et al., 2024). Biofilms are structured bacterial communities encased in a self-produced extracellular matrix that raises the bacterial survival by increasing bacteria resistance to antimicrobial agents. Biofilm formation in *P. aeruginosa* is closely related to pathogenicity, and this is mediated by quorum sensing, a method of cell-cell communication that regulates genes involved in both biofilm formation and virulence (Yin et al., 2022).

This development of multidrug-resistant (MDR) strains of *P. aeruginosa* has raised the demand for finding alternative therapeutic options. Extraordinary attention has been directed towards plant-based natural compounds because their promotion as antimicrobial and antibiofilm agents has shown promises (Er-rahmani et al., 2024). Limonene is one of the major monoterpene constituents found in the essential oils from the peels of citrus species showing wide ranges of biological activities, such as antimicrobial, antioxidant, anti-inflammatory, and anticancer activities. Most importantly, the antimicrobial effects of limonene are very significant because limonene has been reported for its efficacy against various microorganisms such as bacteria, fungi, and viruses. Recent studies have highlighted its utility in combating infectious diseases by disrupting microbial growth and survival mechanisms (Gupta et al., 2021). Besides, limonene is rich in antioxidant and anti-inflammatory properties, adding to its therapeutic potential in mitigating oxidative stress and chronic inflammation (Eddin et al., 2021). Besides that, its anticancer effects lie in the induction of apoptosis and inhibition of tumor growth, further expanding the scope of its medical relevance (Jia et al., 2013; Yu et al., 2018).

Especially intriguing is the interference of limonene with quorum sensing -a very important mechanism by which bacteria control biofilm formation and the production of virulence factors. Thus, limonene could theoretically have an effect on reducing the pathogenicity of *P. aeruginosa* and other biofilm-producing bacteria by interfering with their quorum sensing systems (Luciardi et al., 2021). Nevertheless, considering all its promising properties, little is known about the impact of limonene on *P. aeruginosa* biofilm development and quorum sensing regulated virulence factors. This knowledge gap, therefore, could be targeted against novel therapeutic strategies for microbial communication and biofilm-associated infections.

The study focused on the sub-MIC effects of limonene against *P. aeruginosa* on the inhibition of biofilm formation and disruption of quorum sensing systems. Molecular and biochemical assays were carried out to investigate how limonene modulates quorum sensing regulated gene expression and production of virulence factors. Such findings might be the doorway to alternative or adjunctive therapies to diminish infections caused by *P. aeruginosa* and the rise of antibiotic resistance.

MATERIALS AND METHODS

Limonene

R-(+)-D-Limonene ($C_{10}H_{16}$, M.W. 136.42) was purchased from Sigma-Aldrich.

Bacterial strains

A beta-lactamase-resistant *Pseudomonas aeruginosa* strain was isolated from the urine specimen of a patient diagnosed with a urinary tract infection. The isolate was prepared in pure culture and identified using standard biochemical and culture methods. Strain confirmation and determination of its antibiotic resistance

profile were performed using the Biomérieux VITEK® 2 system. *Chromobacterium violaceum* ATCC 12472 was purchased from the American Type Culture Collection.

Antibacterial activity

Disk diffusion method

The antibacterial potential of limonene was assessed using the disc diffusion technique (ALrawashdeh et al., 2019). To perform the assay, Muller-Hinton agar was seeded with 100 µL of a *P. aeruginosa* suspension standardized to 10^8 CFU/mL (0.5 McFarland standard). A sterile disc impregnated with 1 mg of limonene was gently placed on the agar surface. Discs loaded with 10% DMSO and cefotaxime (30 µg/disc) were included as negative and positive controls, respectively. The plates were incubated at 37°C for 24 hours to allow for bacterial growth and potential inhibition. Following incubation, the diameter of the inhibition zones around the discs was measured in millimeters, providing a comparative evaluation of limonene's antibacterial activity.

Minimum Inhibitory Concentration (MIC)

To quantitatively evaluate the antibacterial efficacy of limonene, its minimum inhibitory concentration (MIC) was determined using a 96-well microplate method, as adapted from (Jaafreh et al., 2019). Each well was loaded with 200 µL of Mueller-Hinton Broth (MHB), followed by serial dilutions of limonene to achieve concentrations ranging from 10 mg/mL to 0.01 mg/mL. Subsequently, 10 µL of a standardized *P. aeruginosa* suspension, containing 10^4 CFU/mL, was introduced into each well. A solution of DMSO was prepared under the same conditions as a negative control, while additional wells containing the extract alone or bacteria without the extract served as control setups. The plate was incubated at 37°C for 24 hours to facilitate bacterial growth. The MIC was determined as the lowest concentration of limonene that completely inhibited visible bacterial growth, highlighting the compound's ability to suppress *P. aeruginosa* proliferation under defined conditions.

Biofilm inhibitory assay and Minimum Biofilm Inhibitory Concentration (MBIC)

The anti-biofilm activity of limonene against *P. aeruginosa* was assessed using the crystal violet staining method, as described by (Qaralleh, 2023). Various concentrations of limonene were prepared in a 96-well microplate containing Mueller-Hinton Broth (MHB) inoculated with *P. aeruginosa* culture. The plate was incubated following the same protocol as the MIC procedure. After incubation, the plate contents were discarded, and the wells were washed three times with phosphate-buffered saline (PBS) to remove non-adherent cells. Crystal violet was added to each well and allowed to stain the biofilm for 15 minutes, after which the wells were emptied and washed thrice with PBS to remove excess dye. Ethanol was added as a decolorizing agent and incubated for 15 minutes, and the dissolved stain was transferred to a new 96-well plate. The absorbance at 590 nm was recorded to quantify the biofilm mass. The percentage of biofilm inhibition was calculated relative to the untreated control, and the concentration of limonene that resulted in 50% biofilm inhibition was identified as the minimum biofilm inhibitory concentration (MBIC50%).

Cell viability

The tetrazolium salt 2,3,5-triphenyl-tetrazolium chloride (TTC) assay was employed to evaluate the effect of limonene on the viability of cells within *P. aeruginosa* biofilms, following the method described by (Gordya et al., 2017). A 96-well microplate was prepared as per the MIC and crystal violet assay protocols. After 24 hours of incubation, the contents of the wells were discarded, and the wells were thoroughly washed to remove non-adherent cells. A TTC solution, prepared using MHB supplemented with 0.2% glucose and 50 µL of TTC (5 mg/mL), was added to each well in a total volume of 200 µL. The plate was incubated at 37°C with agitation at 150 rpm for 6 hours to allow for the reduction of TTC by metabolically active cells. Following incubation, the absorbance at 405 nm was measured to quantify the viable cells within the biofilm. The percentage of viable cells was calculated, providing a measure of the impact of limonene on biofilm cell viability.

Light microscopy observation

To observe the effect of limonene on *P. aeruginosa* biofilm formation, glass coverslips were placed at the base of 24-well plates to serve as surfaces for biofilm development. Treated bacterial cultures with limonene concentrations of 0 (control), 0.039, and 0.078 mg/mL were loaded into the wells. The plates were then incubated at 37°C for 48 hours to allow biofilm formation. Following incubation, the coverslips were carefully removed from the wells, gently washed with phosphate-buffered saline (PBS) to remove non-adherent cells, and subsequently stained with crystal violet for 1 minute. The stained coverslips were rinsed to remove excess dye, air-dried, and examined under a light microscope (Optika, Italy).

Effect of Limonene on Biofilm Development Stages and Related Factors

The impact of limonene on various stages of *P. aeruginosa* biofilm development and associated factors, including aggregation, surface hydrophobicity, swarming motility, and extracellular polymeric substance (EPS) production, was assessed. For each assay, *P. aeruginosa* cultures were treated with limonene at sub-MIC concentrations (0.078, 0.039, and 0 mg/mL) and incubated at 37°C for the required duration (typically 24 hours). Cultures were then processed according to specific methods to assess the targeted factor, with appropriate controls included to ensure reliability.

The effect of limonene on *P. aeruginosa* aggregation was assessed using the protocol by Shanks et al. (2008). Treated cultures were incubated at 37°C for 24 hours, and the absorbance at 600 nm (A_{600}) was measured as the initial reading. Following 1 minute of vortexing, a second A_{600} reading was taken. The percentage of aggregation was calculated using the formula:

$$percentage\ of\ aggregation = \frac{Abs(2) - Abs(1)}{Abs(2)} \times 100\%$$

Where Abs(2) represents the second absorbance reading (after vortexing), and Abs(1) is the initial absorbance reading.

Surface hydrophobicity was determined using the *n*-hexadecane partitioning method (Krishnan et al., 2012). Cultures treated with limonene were incubated for 24 hours, and the initial A_{600} was recorded. Equal volumes (1.5 mL) of the culture and *n*-hexadecane were mixed, allowed to stand for 15 minutes, and the aqueous phase's A_{600} was measured. Hydrophobicity was calculated as:

$$percentage\ of\ hydrophobicity = \frac{Abs(1) - Abs(2)}{Abs(1)} \times 100\%$$

Where Abs(1) represents the initial absorbance reading, and Abs(2) is the aqueous phase absorbance reading.

Swarming motility was evaluated on agar plates containing limonene (Sagar et al., 2022). Swarming agar with limonene at concentrations of 0.078, 0.039, and 0 mg/mL was prepared. A 1-µL inoculum of *P. aeruginosa* was placed at the center of each plate, and after 48 hours of incubation at 37°C, the diameter of the motility zone was measured in millimeters.

EPS production was assessed using the phenol-sulfuric acid method (Razack et al., 2011). Cultures treated with limonene were incubated at 37°C for 24 hours. After incubation, a portion of the culture was mixed with cold ethanol and stored at 4°C for 24 hours to precipitate EPS. The precipitate was centrifuged, dissolved in deionized water, and treated with a mixture of 5% cold phenol and concentrated H_2SO_4 (1:1:5). The absorbance at 490 nm was measured to quantify EPS, and the percentage reduction in EPS production was calculated relative to the untreated control.

Effect of Sub-MIC Concentrations of Limonene on *P. aeruginosa* Virulence Factors: Pyocyanin, Rhamnolipids, and LasA Protease

The influence of limonene at sub-MIC concentrations (0.078 and 0.039 mg/mL) on the virulence factors of *P. aeruginosa* was assessed. General procedures involved treating bacterial cultures

with limonene at specific concentrations and incubating them under standard conditions (24 hours, 37°C). Following incubation, the cultures were centrifuged, and the supernatants from the treated samples were used for all tests. Each virulence factor was then evaluated using specific methodologies tailored to the target factor.

To quantify pyocyanin production, 7.5 mL of the blue-green supernatant was subjected to liquid-liquid extraction with 4.5 mL of chloroform. The chloroform layer was then mixed with 1.5 mL of 0.2N hydrochloric acid, producing a pink solution. The absorbance at 520 nm was measured, and the percentage of pyocyanin inhibition was calculated (Hossain et al., 2017).

To quantify rhamnolipid production, 1 mL of the supernatant underwent liquid-liquid extraction with 3 mL of diethyl ether. The ether layer was then collected and evaporated using a rotary evaporator. The residue was dissolved in 200 µL of distilled water, followed by the addition of 900 µL of 0.18% orcinol in 53% H_2SO_4. The solution was boiled for 30 minutes, and the absorbance at 421 nm was recorded to calculate the percentage of rhamnolipid inhibition (Luo et al., 2017).

To determine LasA protease activity, 1 mL of the supernatant was mixed with 5 mL of casein solution (0.65% in 50 mM Tris-HCl). The mixture was incubated at 37°C for 30 minutes, and the reaction was terminated with 5 mL of pre-chilled 10% trichloroacetic acid. After further incubation at 35°C for 30 minutes and centrifugation, the supernatant was reacted with 5 mL of 0.5M sodium carbonate and 1 mL of Folin's reagent. After a 30-minute incubation at 35°C, the absorbance at 660 nm was measured to calculate the percentage of protease activity inhibition (Hossain et al., 2017).

Effect of Limonene on the Quorum Sensing Systems of *Chromobacterium violaceum* and *Pseudomonas aeruginosa*

Anti-Quorum Sensing Activity Against *Chromobacterium violaceum*

The anti-quorum sensing potential of limonene against *C. violaceum* was initially assessed using the well diffusion method. An aliquot of 100 µL of *C. violaceum* culture containing 10^6 CFU/mL was evenly spread on LB agar plates. A well was then created at the center of the plate, and 100 µL of DMSO containing 1 mg of limonene was added to the well. The plates were incubated at 37°C for 24 hours, and the diameter of the inhibition zone was measured in millimeters to evaluate the disruption of quorum sensing.

In addition, violacein production, a marker of quorum sensing, was quantified to further assess limonene's anti-quorum sensing activity. Treated cultures of *C. violaceum* (0.078, 0.039, and 0 mg/mL of limonene) were centrifuged, and the supernatant was discarded. The cell pellet was resuspended in 1 mL of DMSO and incubated at room temperature for 3 hours. Following another centrifugation, the violacein-containing DMSO was collected, and its absorbance at 575 nm was recorded. The percentage of violacein inhibition was calculated to provide a quantitative measure of limonene's impact on quorum sensing (Chen *et al*., 2022).

Inhibition of Acyl Homoserine Lactone (AHL) Production

The effect of limonene on acyl homoserine lactone (AHL) production in *P. aeruginosa* was evaluated using a modified method from (Lahiri et al., 2021). Cultures of *P. aeruginosa* treated with 0.078, 0.039, and 0 mg/mL of limonene were incubated at 37°C for 24 hours. After incubation, the cultures were centrifuged, and a portion of the supernatant was extracted with ethyl acetate. The extract was collected after 10 minutes, concentrated using a rotary evaporator, and 40 µL of the concentrated solution was mixed with 50 µL of a 1:1 solution of hydroxylamine (2M) and NaOH (3.5M). Subsequently, 90 µL of a 1:1 mixture of ferric chloride (10% in 4M HCl) and 95% ethanol was added. The absorbance at 520 nm was measured to determine the level of AHL inhibition. The percentage of AHL inhibition was calculated, providing insight into limonene's ability to disrupt quorum sensing communication in *P. aeruginosa*.

Effect of limonene of Quorum sensing related genes

A Gentier 48E RT-PCR system (Xi'an Tianlong Science and Technology, China) was employed to examine the impact of limonene (0.078 mg/mL) on the expression of quorum-sensing genes (*lasI*, *lasR*, *rhlI*, and *rhlR*). Total RNA was extracted using the Easy Pure RNA Kit (Haidian District, Beijing, China), and RNA concentrations were determined using a UV/VIS Nano Spectrophotometer (MicroDigital, Korea). First-strand cDNA synthesis was carried out using the Easy Script First-Strand cDNA Synthesis SuperMix (Haidian District, Beijing, China). SYBR Green PCR Master Mix was used for RT-PCR, along with primers specified in Table 1. All analyses were performed in triplicate. The housekeeping gene *rpoD* served as an internal control to normalize target gene expression levels, and relative fold changes in expression were calculated using the ΔΔCt method. Fold changes for each target gene were compared to the untreated control group (Banerjee *et al*., 2017).

Table 1: list of primers used in this study

Gene	Type	Primer sequence
LasI	Forward	5'-TTG CTC GCC GCA CAT CTG-3'
	Reverse	5'-ATC ATC TTC TCC ACG CCT AC-3'
LasR	Forward	5'-ACC GTT TCA TAG AGT CGG TC-3'
	Reverse	5'-ACC ACT GCA ACA CTT CCT TC-3'
rhlI	Forward	5'-TCT TCA TCG AGA AGC TGG GC-3'
	Reverse	5'-ACG ATG TAG CGG GTT TGC G-3'
rhlR	Forward	5'-TGA TGT CCA ACC CGG TCT G-3'
	Reverse	5'-TCG AAC TTC TTC TGG ATG TTC-3'
rpoD	Forward	5'-ACG AGG AAG AAG AAA GCG-3'
	Reverse	5'-ACC AGC TTG ATC GGC ATG-3'

Statistical analysis

The statistical analysis to determine significant differences between the tested groups and the untreated control group was conducted using GraphPad Prism 8 software. One-way ANOVA was employed to analyze the data, and significance levels were denoted as *, **, or ***, corresponding to p-values of <0.05, <0.01, and <0.001, respectively.

RESULTS

Antibacterial

Table 2: inhibition zones of Limonene (1 mg/disc), Cefotaxime (30 µg/disc), and DMSO (10%), and the MIC value (mg/mL) of limonene against *P. aeruginosa*

	Inhibition zone (mm)	MIC (mg/mL)
Limonene (1 mg/disc)	0.0	0.63
Cefotaxime (30 µg/disc)	16.5±1.0	-
DMSO (10%)	0.0	-

The antibacterial activity of limonene against *P. aeruginosa* was studied by disc diffusion method and determination of MIC (Table 2). Limonene at 1 mg/disc did not show any inhibition zone in the disc diffusion assay, just like the negative control DMSO at 10%, but its determined MIC value was 0.63 mg/mL. In contrast, the positive control cefotaxime at 30 µg/disc gave an inhibition zone of 16.5 mm and was thus a very effective inhibitor of bacterial growth. Overall, these results suggest that while limonene has no detectable activity in the disc diffusion assay, it is inhibiting *P. aeruginosa* at its MIC and thus may have potential as an antibacterial agent under certain conditions.

Effect of limonene on *P. aeruginosa* biofilm

The crystal violet technique was adapted to measure the capability of limonene against the formation of biofilm by *P. aeruginosa*. Limonene exhibited marked inhibition against *P. aeruginosa* biofilm formation compared to the control group, as seen in Figure 1. Limonene at the established MIC value of 0.63 mg/mL inhibited the growth of biofilm at 97.18%. Similarly, higher concentrations such as 10, 5, 2.5, and 1.25 mg/mL concentration showed very high activity with more than 99% inhibition. Even at lower concentrations of 0.3 mg/mL, inhibition was high at 98.31%, while for 0.16 mg/mL, the percentage was 93.4%. It declined at 0.02 mg/mL with inhibition at 45.72% and reduced further at 0.01 mg/mL to 19.99% yet higher compared to that of the control. The present study demonstrated limonene's potential in inhibiting *P.*

aeruginosa biofilm formation, hence proving as efficient anti-biofilm agents.

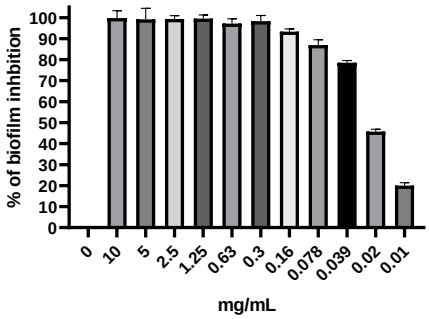


Figure 1: Percentage inhibition of *P. aeruginosa* biofilm by limonene (10–0.01 mg/mL)

Cell viability

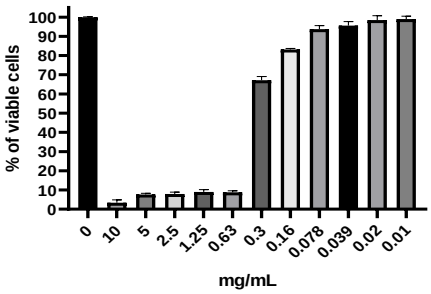


Figure 2: Percentage of viable *P. aeruginosa* cells in biofilm treated with limonene (10–0.01 mg/mL)

The effect of limonene on *P. aeruginosa* viable cells, as assessed through the TTC assay, reveals a marked reduction in cell viability at concentrations of 0.63 mg/mL and higher (Figure 2). Specifically, 0.63 mg/mL results in 8.76% viable cells compared to the control (100% viability). Concentrations ranging from 1.25 mg/mL to 10 mg/mL maintain cell viability below 10%, suggesting that the observed antibiofilm activity at these levels stems primarily from bacterial growth inhibition. Conversely, lower concentrations (e.g., 0.3 mg/mL and below) exhibit higher viability rates, with 67.18% at 0.3 mg/mL and 83.19% at 0.16 mg/mL. This indicates that the antibiofilm activity at these lower concentrations is likely due to disruption of biofilm formation rather than bactericidal effects. Thus, at 0.63 mg/mL and above, the antibiofilm activity correlates with significant reduction in cell viability, while at lower concentrations, the activity is achieved without heavily impacting cell viability. As a result, concentrations of 0.078 and 0.039 mg/mL have been selected for further analysis.

Light microscopy observation

The impact of limonene on *Pseudomonas aeruginosa* biofilm formation was visually assessed using light microscopy at concentrations of 0, 0.078, and 0.039 mg/mL (Figure 3). In the untreated control group, a dense and robust biofilm structure was observed, characterized by aggregated bacterial clusters and extensive extracellular matrix. This indicates a fully developed biofilm, typical of *P. aeruginosa* under standard conditions. At a concentration of 0.078 mg/mL, limonene caused a noticeable disruption in biofilm integrity. The bacterial clusters appeared less dense, and the extracellular matrix was significantly reduced, suggesting a strong inhibitory effect on biofilm formation. At 0.039 mg/mL, the disruption of the biofilm was less pronounced compared to the higher concentration but still evident. The bacterial clusters were more scattered and smaller, with a visible reduction in biofilm thickness and matrix compared to the control. These results demonstrate a dose-dependent inhibitory effect of limonene on *P. aeruginosa* biofilm formation, supporting its potential as an antibiofilm agent. The light microscopy images further corroborate the quantitative findings, emphasizing the structural disruption induced by limonene at sub-MIC levels.

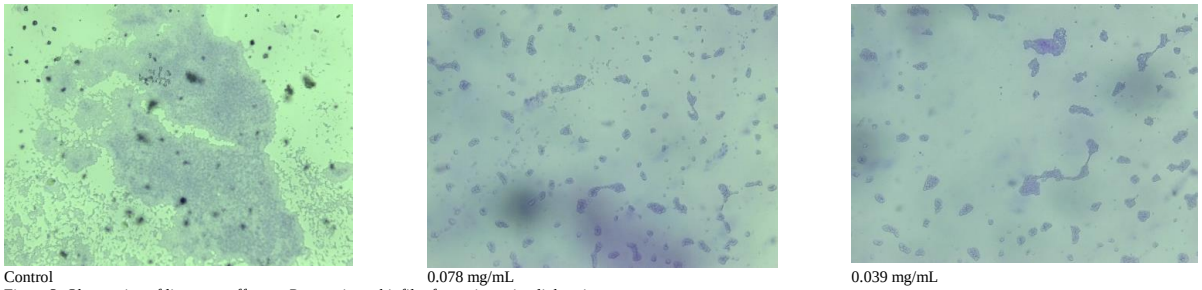
Effect of Limonene on Biofilm Development Stages and Related Factors

The effect of limonene on various stages of biofilm development and related factors, including aggregation, hydrophobicity, swarming motility, and EPS production, was evaluated (Figure 4). Specifically, limonene's impact on *P. aeruginosa* aggregation demonstrates a significant reduction compared to the control (0 mg/mL), which shows 41.41% aggregation (Figure 4A). At a concentration of 0.078 mg/mL, aggregation decreases significantly ($p < 0.01$) to 25.56%. Similarly, at 0.039 mg/mL, aggregation is reduced ($p < 0.05$) to 31.44%. These findings suggest that limonene effectively disrupts bacterial aggregation, an important factor in biofilm formation.

The results of the effect of limonene on *P. aeruginosa* surface hydrophobicity reveal a marked reduction compared to the control group (Figure 4B). In the control (0 mg/mL), surface hydrophobicity is 61.39%, demonstrating *P. aeruginosa*'s natural ability to maintain a hydrophobic cell surface, which facilitates aggregation and biofilm formation. Treatment with 0.039 mg/mL of limonene reduces hydrophobicity to 52.63%, indicating a moderate decline. At 0.078 mg/mL, hydrophobicity significantly decreases to 35.73%, suggesting a substantial disruption in cell surface properties that may impair biofilm formation and related processes.

The results of the effect of limonene on *P. aeruginosa* swarming motility indicate a noticeable reduction in the swarming zone diameter compared to the control (Figure 4C). In the control (0 mg/mL), the swarming zone measures 41.00 mm, representing normal motility. Treatment with 0.039 mg/mL of limonene reduces the swarming zone to 33.67 mm, signifying a moderate inhibition of motility. At the higher concentration of 0.078 mg/mL, the swarming zone is further diminished to 27.50 mm, highlighting a significant impairment in swarming motility. These findings suggest that limonene effectively disrupts *P. aeruginosa* motility, a critical factor in biofilm formation and virulence.

The effect of limonene on the ability of *P. aeruginosa* to produce extracellular polymeric substances (EPS) reveals a significant inhibition compared to the control group (Figure 4D). At 0.039 mg/mL, EPS production is reduced by 24.06%, indicating a moderate decrease. When the concentration is increased to 0.078 mg/mL, the inhibition increases to 34.59%, demonstrating a stronger disruption of EPS production. These results suggest that limonene effectively interferes with EPS synthesis, which is essential for biofilm stability and development.



Control 0.078 mg/mL 0.039 mg/mL

Figure 3: Observation of limonene effect on P. aeruginosa biofilm formation using light microscope.

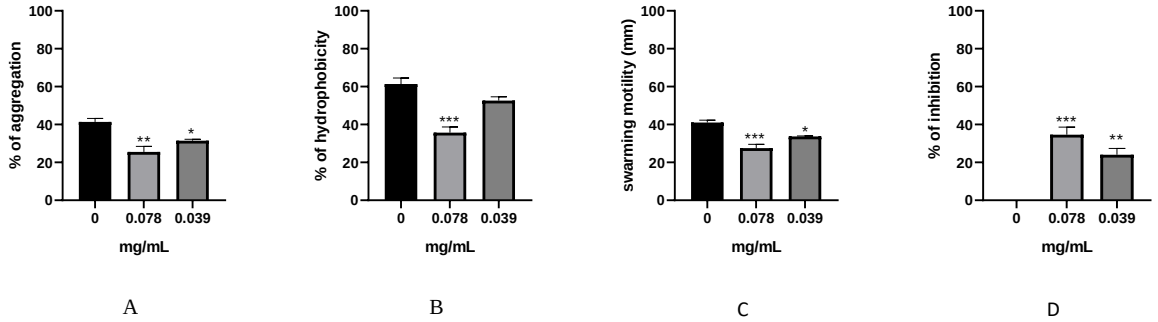


Figure 4: Effect of limonene (0.078 and 0.039 mg/mL) on biofilm development stages and related factors, including (A) aggregation, (B) surface hydrophobicity, (C) swarming motility, and (D) EPS production.

Effect of Sub-MIC Concentrations of Limonene on *P. aeruginosa* Virulence Factors: Pyocyanin, Rhamnolipids, and LasA Protease

The effect of sub-MIC concentrations of limonene on *P. aeruginosa* virulence factors, including pyocyanin, rhamnolipids, and LasA protease, was evaluated (Figure 5). The results for pyocyanin production reveal a substantial reduction compared to the control group (Figure 5A). At 0.039 mg/mL of limonene, pyocyanin production decreases to 25.94%, reflecting a moderate inhibition. At the higher concentration of 0.078 mg/mL, pyocyanin production is significantly inhibited, dropping to 58.95%. These findings suggest that limonene effectively reduces pyocyanin production, a key virulence factor associated with *P. aeruginosa* pathogenicity.

The effect of limonene on *P. aeruginosa* rhamnolipid production demonstrates its capability to disrupt this essential virulence factor (Figure 5B). In the control group (0 mg/mL), rhamnolipid production remains uninhibited. However, exposure to 0.039 mg/mL of limonene results in a notable decrease, with a 19.35% inhibition. At the higher concentration of 0.078 mg/mL, the inhibition significantly increases to 49.75%, reflecting limonene's enhanced ability to interfere with rhamnolipid synthesis. These findings underscore limonene's potential in mitigating virulence factor production in *P. aeruginosa*.

The effect of limonene on *P. aeruginosa* LasA protease production highlights its inhibitory potential against this virulence factor (Figure 5C). In the control group (0 mg/mL), LasA protease production remains fully active, with no inhibition observed (0%). Treatment with 0.039 mg/mL of limonene results in a modest inhibition of 9.90%, suggesting an initial disruption in protease activity. At a higher concentration of 0.078 mg/mL, the inhibition significantly increases to 35.76%, indicating a substantial reduction in LasA protease production. These results demonstrate limonene's effectiveness in targeting *P. aeruginosa* virulence mechanisms.

Effect of Limonene on the Quorum Sensing Systems of *C. violaceum* and *P. aeruginosa*

The effect of limonene on quorum sensing systems was assessed in *Chromobacterium violaceum* and *P. aeruginosa* using both qualitative and quantitative approaches. Qualitative analysis, conducted via a well diffusion assay, demonstrated that limonene at 1 mg/well produced a clear inhibition zone measuring 10.5 mm (Figure 6A), indicating its effectiveness as a quorum sensing inhibitor.

Quantitatively, limonene's impact on violacein production, a quorum sensing-regulated pigment in *C. violaceum*, revealed notable inhibition (Figure 6B). At a concentration of 0.039 mg/mL, violacein production decreased by 9.53%, indicating a mild disruption of quorum sensing. When the concentration was increased to 0.078 mg/mL, violacein production inhibition reached 33.00%, showing a significant reduction.

Additionally, the effect of limonene on *P. aeruginosa* production of N-Acyl Homoserine Lactones (AHL) reveals its capability to interfere with quorum sensing (Figure 6C). At 0.039 mg/mL, AHL production is inhibited by 8.72%, indicating a mild disruption of quorum sensing. When the concentration is increased to 0.078 mg/mL, inhibition significantly rises to 26.39%, demonstrating a stronger suppression of these crucial signaling molecules. These results highlight limonene's potential to effectively impair quorum-sensing mechanisms in *P. aeruginosa*, contributing to its anti-virulence activity.

Effect of limonene of Quorum sensing related genes

The effect of limonene on *P. aeruginosa* quorum-sensing gene expression reveals significant downregulation compared to the control (untreated) (Figure 7). The relative expression of *lasI*, which is responsible for signal synthesis, decreases from 1 in the control to 0.26267 with limonene treatment. Similarly, lasR, the receptor gene for *lasI*-regulated signals, is reduced to 0.17809. For the rhl quorum-sensing system, *RhII*, involved in synthesizing *rhl*-regulated signals, experiences a moderate reduction in expression to 0.67144, while RhIR, the receptor gene for *rhl*-regulated signals, shows a dramatic decrease to 0.09035. These findings highlight that limonene strongly suppresses quorum-sensing gene expression, effectively disrupting critical bacterial communication pathways essential for virulence and biofilm formation in *P. aeruginosa*.

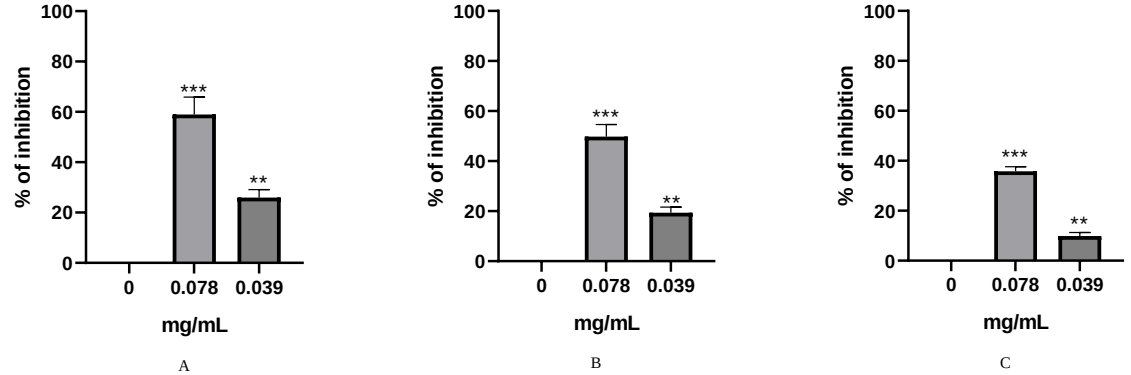
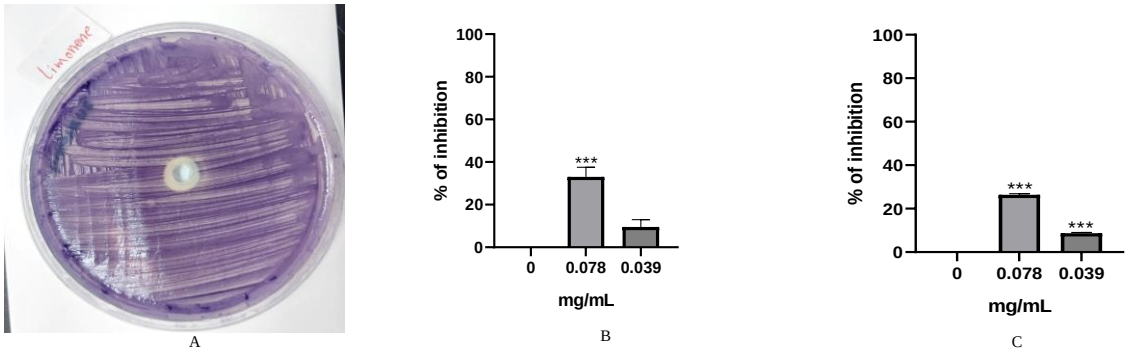


Figure 5: Effect of limonene (0.078 and 0.039 mg/mL) on *P. aeruginosa* virulence factors, including (A) pyocyanin, (B) rhamnolipids, and (C) LasA protease



A B C

Figure 6: Effect of limonene on the quorum sensing systems of *Chromobacterium violaceum*, shown by (A) zone of inhibition and (B) violacein production, and (C) on *Pseudomonas aeruginosa* AHL production.

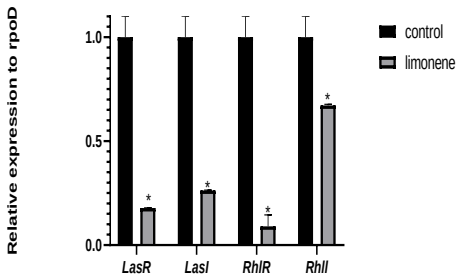


Figure 7: Effect of limonene (0.078 mg/mL) on quorum sensing genes (*lasI/R* and *rhlI/R*)

DISCUSSION

The growing prevalence of antibiotic-resistant *P. aeruginosa* poses a serious public health concern, underscoring the urgent need for alternative approaches that target virulence mechanisms rather than bacterial viability (Chinemerem Nwobodo et al., 2022). Biofilm formation and quorum sensing play pivotal roles in *P. aeruginosa*'s pathogenicity, enabling its resistance to antimicrobial agents and evasion of host immune responses (Tuon et al., 2022). This study underscores the potential of limonene, a monoterpene derived from citrus essential oils, as a versatile agent that effectively inhibits biofilm development, attenuates virulence factors, and disrupts quorum sensing systems, offering a promising alternative for managing *P. aeruginosa* infections.

Limonene demonstrated a notable inhibitory effect on *P. aeruginosa* biofilm formation. While it did not produce a measurable inhibition zone in the disc diffusion assay, likely due to its volatile nature or limited solubility (Balouiri et al., 2016), its minimum inhibitory concentration (MIC) was established at 0.63 mg/mL, representing the concentration necessary to suppress bacterial growth. These results are consistent with prior studies indicating that natural compounds, such as monoterpenes, exhibit antimicrobial activity by disrupting bacterial cell membranes (Moo et al., 2021). Interestingly, reports on the MIC of limonene against *P. aeruginosa* vary significantly. Wang et al. (2018) identified an MIC of 10 mL/L, while Costa et al. (2019) reported an MIC of 512 µg/mL (Costa et al., 2019; J. N. Wang et al., 2018). These variations are likely attributable to differences in experimental methodologies, bacterial strains, or environmental conditions, emphasizing the need for standardized protocols when assessing the antimicrobial efficacy of natural compounds (Balouiri et al., 2016).

Interestingly, even at sub-MIC concentrations, limonene exhibited potent antibiofilm activity, inhibiting biofilm formation by over 97% at 0.078 mg/mL. This indicates that limonene primarily disrupts biofilm development stages rather than exerting bactericidal effects. Such a mechanism has critical clinical implications, as targeting biofilms could improve the efficacy of existing antibiotics in treating *P. aeruginosa* infections (Sarkar et al., 2014). Additionally, studies have shown that *Citrus limon* essential oils, with limonene as the predominant component (60%), significantly inhibit biofilm formation by *P. aeruginosa* at concentrations between 0.1 and 4 mg/mL, achieving reductions of up to 65% (Luciardi et al., 2021). These findings highlight the potential of limonene and citrus-derived essential oils as complementary agents in combating biofilm-associated infections.

Biofilm formation is a complex, multi-stage process that includes initial adhesion, aggregation, maturation, and eventual dispersion (Ma'aitah, 2024). This study demonstrated that limonene significantly inhibited the early stages of biofilm development, notably aggregation and surface hydrophobicity. The reduction in aggregation percentages reflects impaired bacterial clustering, which is vital for biofilm maturation. Additionally, the observed decrease in surface hydrophobicity suggests that limonene disrupts bacterial adhesion to surfaces, a critical initial step in biofilm formation (Habimana et al., 2014). These findings highlight limonene's potential to interfere with the foundational processes of biofilm development, presenting a promising strategy for biofilm-related infection control.

Swarming motility, a key factor for biofilm expansion and surface colonization, was significantly inhibited by limonene. This effect is likely linked to the downregulation of rhamnolipid production, as rhamnolipids function as biosurfactants that facilitate bacterial motility (Bhadra et al., 2023). Supporting this, previous studies have demonstrated that targeting rhamnolipid synthesis effectively suppresses swarming motility and biofilm formation in *Pseudomonas aeruginosa (O'May & Tufenkji, 2011)*. By concurrently affecting these interconnected pathways, limonene provides a holistic approach to disrupting biofilm development, making it a promising candidate for managing biofilm-associated infections.

Limonene also significantly inhibited extracellular polymeric substance (EPS) production, a critical component of the biofilm matrix. EPS provides structural stability to biofilms and shields the embedded bacteria from external stressors, including antibiotics and host immune responses (Singh et al., 2021). The observed reduction in EPS production suggests that limonene disrupts biofilm integrity, potentially increasing the susceptibility of bacterial cells to environmental challenges. These findings align with previous studies that emphasize the role of natural compounds in inhibiting EPS synthesis as an effective strategy for weakening biofilm resilience (Kashi et al., 2024). This highlights limonene's potential to target the biofilm matrix, a key factor in *P. aeruginosa*'s pathogenicity.

Virulence factors such as pyocyanin, rhamnolipids, and LasA protease are central to *P. aeruginosa*'s pathogenicity, facilitating tissue damage, immune evasion, and biofilm resilience. In this study, limonene exhibited a dose-dependent reduction in the production of these virulence factors at sub-MIC concentrations. Pyocyanin, a blue-green pigment, is known to generate reactive oxygen species (ROS) that damage host tissues and impair immune responses (Mudaliar & Bharath Prasad, 2024). Limonene's ability to inhibit pyocyanin production highlights its potential to mitigate oxidative stress-related damage, thereby reducing the severity of infections (Liao et al., 2012). These findings position limonene as a promising agent for targeting virulence factors, contributing to a multifaceted approach to managing *P. aeruginosa* infections.

Rhamnolipids are critical for maintaining biofilm architecture and facilitating motility, such as swarming (Rather et al., 2021). Limonene's ability to reduce rhamnolipid production not only disrupts biofilm stability but also suppresses swarming motility, significantly impairing *P. aeruginosa*'s capacity to colonize surfaces (Deforet, 2023). Additionally, the observed decrease in LasA protease activity suggests that limonene attenuates tissue invasion and the degradation of host proteins, further limiting the pathogenic potential of *P. aeruginosa*. These results align with previous research demonstrating that natural compounds targeting quorum sensing regulated pathways can effectively reduce virulence factor production in *P. aeruginosa (García-Reyes et al., 2020)*. This highlights limonene's role as a multi-target agent capable of mitigating key mechanisms underlying *P. aeruginosa* pathogenicity.

Quorum sensing is a critical cell-to-cell communication mechanism that regulates gene expression associated with biofilm formation and virulence (Warrier et al., 2021). This study highlights limonene's potential to disrupt quorum sensing systems in both *P. aeruginosa* and *C. violaceum*. The significant inhibition of violacein production in *C. violaceum* underscores limonene's effectiveness in interfering with AHL-mediated signaling pathways. Violacein, a pigment regulated by quorum sensing, serves as a reliable indicator of quorum sensing inhibition (Bouyahya et al., 2022; Gonzales et al., 2024). These findings demonstrate limonene's capacity to target quorum sensing regulated pathways, offering a promising approach for mitigating virulence and biofilm formation in pathogenic bacteria.

In *P. aeruginosa*, limonene effectively downregulated the expression of key quorum sensing genes, including *lasI/R* and *rhlI/R*. The *lasI* gene encodes the enzyme responsible for synthesizing N-(3-oxododecanoyl)-homoserine lactone (3-oxo-C12-HSL), the signaling molecule of the Las system, while *lasR* encodes its receptor. These genes regulate numerous virulence factors and are critical for biofilm maturation (Kumar et al., 2022). Similarly, the *rhlI* gene is responsible for producing N-butyryl-homoserine lactone (C4-HSL), the signaling molecule of the Rhl system, while *rhlR* encodes its receptor. The Rhl system modulates biofilm structure, rhamnolipid production, and additional virulence factors like pyocyanin (Cocotl-Yañez et al., 2020). By downregulating these quorum sensing genes, limonene disrupts the hierarchical quorum sensing signaling cascade, which is crucial for coordinated bacterial behavior. This disruption leads to reduced biofilm formation and a significant decrease in virulence. These findings are consistent with prior research identifying quorum sensing inhibition as a promising strategy for managing *P. aeruginosa* infections (Brindhadevi et al., 2020). Limonene's ability to target these pathways positions it as a potential therapeutic agent in combating bacterial pathogenicity.

Limonene's antimicrobial and anti-quorum sensing activities appear to be mediated through several mechanisms. Its lipophilic structure allows it to integrate into bacterial membranes, disrupting their integrity and permeability (Lin et al., 2024). This membrane disruption may impair the function of membrane-bound quorum sensing receptors, thereby hindering signal transduction. Furthermore, limonene's ability to modulate gene expression suggests that it interferes with intracellular signaling pathways, disrupting quorum sensing regulated processes (R. Wang et al., 2018). The observed reduction in N-Acyl Homoserine Lactone (AHL) production aligns with the hypothesis that limonene directly or indirectly targets AHL synthases, suppressing the synthesis of these critical signaling molecules. By affecting both membrane structure and intracellular signaling, limonene exhibits a multifaceted approach to quorum sensing inhibition, making it a promising agent for disrupting bacterial communication and reducing virulence in *P. aeruginosa*.

The findings from this study highlight limonene's potential as a versatile therapeutic agent against *P. aeruginosa*. By targeting quorum sensing systems and biofilm-associated pathways, limonene represents a promising alternative to traditional antibiotics, particularly for managing multidrug-resistant infections. One of its key advantages is the ability to attenuate virulence factors without exerting bactericidal pressure, which significantly reduces the risk of resistance development, a critical challenge with conventional antimicrobial therapies (Ahmedi et al., 2022; Gambino et al., 2022). These characteristics position limonene as a valuable component in the development of innovative strategies for combating bacterial infections, particularly in the era of rising antibiotic resistance.

CONCLUSION

Limonene demonstrates substantial promise as an anti-virulence and antibiofilm agent against *P. aeruginosa*. Its multifaceted mechanisms ranging from inhibiting biofilm formation to attenuating virulence factor production and disrupting quorum sensing systems underscore its potential as a versatile therapeutic option. Further research is essential to fully elucidate its mechanism of action, particularly by studying additional genes and employing advanced techniques to explore its effects on bacterial pathways. Moreover, assessing the toxicity profile of limonene is critical to ensure its safety and viability for clinical applications. These findings add to the growing body of evidence supporting the use of natural compounds as innovative strategies to address the escalating challenge of antibiotic-resistant pathogens. Limonene's ability to target bacterial virulence without exerting bactericidal pressure provides a promising avenue for developing sustainable and effective therapies.

Funding Sources

This study was not supported by any sponsor or funder.

Data Availability

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding authors.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

References

Ahmedi, S., Pant, P., Raj, N., and Manzoor, N. (2022). Limonene inhibits virulence associated traits in Candida albicans: In-vitro and in-silico studies. *Phytomedicine Plus*, **2**(3), 100285. https://doi.org/10.1016/j.phyplu.2022.100285

ALrawashdeh, I., Qaralleh, H., Al-limoun, M., and Khleifat, K. (2019). Antibactrial Activity of Asteriscus graveolens Methanolic Extract: Synergistic Effect with Fungal Mediated Nanoparticles against Some Enteric Bacterial Human Pathogens. *Journal of Basic and Applied Research in Biomedicine*, **5**(2), 89–98. https://doi.org/10.51152/jbarbiomed.v5i2.40

Balouiri, M., Sadiki, M., and Ibnsouda, S. K. (2016). Methods for in vitro evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis*, **6**(2), 71–79. https://doi.org/https://doi.org/10.1016/j.jpha.2015.11.005

Banerjee, M., Moulick, S., Bhattacharya, K. K., Parai, D., Chattopadhyay, S., and Mukherjee, S. K. (2017). Attenuation of Pseudomonas aeruginosa quorum sensing, virulence and biofilm formation by extracts of Andrographis paniculata. *Microbial Pathogenesis*, **113**, 85–93. https://doi.org/10.1016/j.micpath.2017.10.023

Bhadra, S., Chettri, D., and Kumar Verma, A. (2023). Biosurfactants: Secondary Metabolites Involved in the Process of Bioremediation and Biofilm Removal. *Applied Biochemistry and Biotechnology*, **195**(9), 5541–5567. https://doi.org/10.1007/s12010-022-03951-3

Bouyahya, A., Chamkhi, I., Balahbib, A., Rebezov, M., Shariati, M. A., Wilairatana, P., Mubarak, M. S., Benali, T., and El Omari, N. (2022). Mechanisms, anti-quorum-sensing actions, and clinical trials of medicinal plant bioactive compounds against bacteria: a comprehensive review. *Molecules*, **27**(5), 1484. https://doi.org/10.3390/molecules27051484

Brindhadevi, K., LewisOscar, F., Mylonakis, E., Shanmugam, S., Verma, T. N., and Pugazhendhi, A. (2020). Biofilm and

Quorum sensing mediated pathogenicity in Pseudomonas aeruginosa. *Process Biochemistry*, **96**, 49–57. https://doi.org/10.1016/j.procbio.2020.06.001

Chen, J., Hsiao, H.-C., Hou, Y.-C., Hsieh, C.-W., Hsu, H.-Y., Chen, H.-Y., and Lin, S.-P. (2022). Improvement in violacein production by utilizing formic acid to induce quorum sensing in Chromobacterium violaceum. *Antioxidants*, **11**(5), 849. https://doi.org/10.3390/antiox11050849

Chinemerem Nwobodo, D., Ugwu, M. C., Oliseloke Anie, C., Al-Ouqaili, M. T. S., Chinedu Ikem, J., Victor Chigozie, U., and Saki, M. (2022). Antibiotic resistance: The challenges and some emerging strategies for tackling a global menace. *Journal of Clinical Laboratory Analysis*, **36**(9), e24655. https://doi.org/10.1002/jcla.24655

Cocotl-Yañez, M., Soto-Aceves, M. P., González-Valdez, A., Servín-González, L., and Soberón-Chávez, G. (2020). Virulence factors regulation by the quorum-sensing and Rsm systems in the marine strain Pseudomonas aeruginosa ID4365, a natural mutant in lasR. *FEMS Microbiology Letters*, **367**(12), fnaa092. https://doi.org/10.1093/femsle/fnaa092

Costa, M. D. S., Rocha, J. E., Campina, F. F., Silva, A. R. P., Da Cruz, R. P., Pereira, R. L. S., Quintans-Júnior, L. J., De Menezes, I. R. A., De S. Araújo, A. A., De Freitas, T. S., Teixeira, A. M. R., and Coutinho, H. D. M. (2019). Comparative analysis of the antibacterial and drug-modulatory effect of d-limonene alone and complexed with β-cyclodextrin. *European Journal of Pharmaceutical Sciences*, **128**, 158–161. https://doi.org/10.1016/j.ejps.2018.11.036

Deforet, M. (2023). Long-range alteration of the physical environment mediates cooperation between swarming colonies. *Environmental Microbiology*, **25**(8), 1451–1464. https://doi.org/10.1111/1462-2920.16373

Eddin, L. B., Jha, N. K., Meeran, M. F. N., Kesari, K. K., Beiram, R., and Ojha, S. (2021). Neuroprotective Potential of Limonene and Limonene Containing Natural Products. In *Molecules* (Vol. 26, Issue 15, p. 4535). https://doi.org/10.3390/molecules26154535

Er-rahmani, S., Errabiti, B., Matencio, A., Trotta, F., Latrache, H., Koraichi, S. I., and Elabed, S. (2024). Plant-derived bioactive compounds for the inhibition of biofilm formation: a comprehensive review. *Environmental Science and Pollution Research*, **31**(24), 34859–34880. https://doi.org/10.1007/s11356-024-33532-2

Gambino, E., Maione, A., Guida, M., Albarano, L., Carraturo, F., Galdiero, E., and Di Onofrio, V. (2022). Evaluation of the Pathogenic-Mixed Biofilm Formation of Pseudomonas aeruginosa/Staphylococcus aureus and Treatment with Limonene on Three Different Materials by a Dynamic Model. In *International Journal of Environmental Research and Public Health* (Vol. 19, Issue 6). https://doi.org/10.3390/ijerph19063741

García-Reyes, S., Soberón-Chávez, G., and Cocotl-Yanez, M. (2020). The third quorum-sensing system of Pseudomonas aeruginosa: Pseudomonas quinolone signal and the enigmatic PqsE protein. *Journal of Medical Microbiology*, **69**(1), 25–34. https://doi.org/10.1099/jmm.0.001116

Gonzales, M., Jacquet, P., Gaucher, F., Chabrière, É., Plener, L., and Daudé, D. (2024). AHL-Based Quorum Sensing Regulates the Biosynthesis of a Variety of Bioactive Molecules in Bacteria. *Journal of Natural Products*, **87**(4), 1268–1284. https://doi.org/10.1021/acs.jnatprod.3c00672

Gordya, N., Yakovlev, A., Kruglikova, A., Tulin, D., Potolitsina, E., Suborova, T., Bordo, D., Rosano, C., and Chernysh, S. (2017). Natural antimicrobial peptide complexes in the fighting of antibiotic resistant biofilms: Calliphora vicina medicinal maggots. *PLoS One*, **12**(3), e0173559. https://doi.org/10.1371/journal.pone.0173559

Gupta, A., Jeyakumar, E., and Lawrence, R. (2021). Journey of limonene as an antimicrobial agent. *Journal of Pure & Applied Microbiology*, **15**(3), 1094–1110. https://doi.org/10.22207/JPAM.15.3.01

Hossain, M. A., Lee, S.-J., Park, N.-H., Mechesso, A. F., Birhanu, B. T., Kang, J., Reza, M. A., Suh, J.-W., and Park, S.-C. (2017). Impact of phenolic compounds in the acyl homoserine lactone-mediated quorum sensing regulatory pathways. *Scientific Reports*, **7**(1), 10618. https://doi.org/10.1038/s41598-017-10997-5

Jaafreh, M., Khleifat, K. M., Qaralleh, H., and Al-limoun, M. O. (2019). Antibacterial and Antioxidant Activities of Centeurea damascena Methanolic Extract. *Journal of Basic and Applied Research in Biomedicine*, **5**(1), 55–63.

Jia, S.-S., Xi, G.-P., Zhang, M., Chen, Y.-B., Lei, B., Dong, X.-S., and Yang, Y.-M. (2013). Induction of apoptosis by D-limonene is mediated by inactivation of Akt in LS174T human colon cancer cells. *Oncol Rep*, **29**(1), 349–354. https://doi.org/10.3892/or.2012.2093

Kashi, M., Noei, M., Chegini, Z., and Shariati, A. (2024). Natural compounds in the fight against Staphylococcus aureus biofilms: a review of antibiofilm strategies. *Frontiers in Pharmacology*, **15**, 1491363. https://doi.org/10.3389/fphar.2024.1491363

Krishnan, T., Yin, W.-F., and Chan, K.-G. (2012). Inhibition of quorum sensing-controlled virulence factor production in Pseudomonas aeruginosa PAO1 by ayurveda spice clove (Syzygium Aromati cum) bud extract. *Sensors*, **12**(4), 4016–4030. https://doi.org/10.3390/s120404016

Kumar, L., Patel, S. K., Kharga, K., Kumar, R., Kumar, P., Pandohee, J., Kulshresha, S., Harjai, K., and Chhibber, S. (2022). Molecular Mechanisms and Applications of N-Acyl Homoserine Lactone-Mediated Quorum Sensing in Bacteria. In *Molecules* (Vol. 27, Issue 21, p. 7584). https://doi.org/10.3390/molecules27217584

Lahiri, D., Nag, M., Dutta, B., Dey, S., Mukherjee, D., Joshi, S. J., and Ray, R. R. (2021). Antibiofilm and anti-quorum sensing activities of eugenol and linalool from Ocimum tenuiflorum against Pseudomonas aeruginosa biofilm. *Journal of Applied Microbiology*, **131**(6), 2821–2837. https://doi.org/10.1111/jam.15171

Liao, J.-C., Deng, J.-S., Chiu, C.-S., Hou, W.-C., Huang, S.-S., Shie, P.-H., and Huang, G.-J. (2012). Anti-inflammatory activities of Cinnamomum cassia constituents in vitro and in vivo. *Evidence-Based Complementary and Alternative Medicine*, **2012**, 429320. https://doi.org/10.1155/2012/429320

Lin, H., Li, Z., Sun, Y., Zhang, Y., Wang, S., Zhang, Q., Cai, T., Xiang, W., Zeng, C., and Tang, J. (2024). D-Limonene: Promising and Sustainable Natural Bioactive Compound. *Applied Sciences*, **14**(11), 4605. https://doi.org/10.3390/app14114605

Luciardi, M. C., Blázquez, M. A., Alberto, M. R., Cartagena, E., and Arena, M. E. (2021). Lemon oils attenuate the pathogenicity of Pseudomonas aeruginosa by quorum sensing inhibition. *Molecules*, **26**(10), 2863. https://doi.org/10.3390/molecules26102863

Luo, J., Dong, B., Wang, K., Cai, S., Liu, T., Cheng, X., Lei, D., Chen, Y., Li, Y., and Kong, J. (2017). Baicalin inhibits biofilm formation, attenuates the quorum sensing-controlled virulence and enhances Pseudomonas aeruginosa clearance in a mouse peritoneal implant infection model. *PloS One*, **12**(4), e0176883. https://doi.org/10.1371/journal.pone.0176883

Ma'aitah, S. (2024). Pseudomonas aeruginosa biofilm formation: Antibiofilm Strategies and conventional methods of evaluation. *Journal of Basic and Applied Research in Biomedicine*, **10**(1), 11–28. https://doi.org/10.51152/jbarbiomed.v10i1.234

Moo, C.-L., Osman, M. A., Yang, S.-K., Yap, W.-S., Ismail, S., Lim, S.-H.-E., Chong, C.-M., and Lai, K.-S. (2021). Antimicrobial activity and mode of action of 1,8-cineol against carbapenemase-producing Klebsiella pneumoniae. *Scientific Reports*, **11**(1), 20824. https://doi.org/10.1038/s41598-021-00249-y

Mudaliar, S. B., and Bharath Prasad, A. S. (2024). A biomedical perspective of pyocyanin from Pseudomonas aeruginosa: its applications and challenges. *World Journal of Microbiology and Biotechnology*, **40**(3), 90. https://doi.org/10.1007/s11274-024-03889-0

O'May, C., and Tufenkji, N. (2011). The swarming motility of Pseudomonas aeruginosa is blocked by cranberry proanthocyanidins and other tannin-containing materials. *Applied and Environmental Microbiology*, **77**(9), 3061–3067. https://doi.org/10.1128/AEM.02677-10

Qaralleh, H. (2023). Chemical Composition and Quorum Sensing Inhibitory Effect of Nepeta curviflora Methanolic Extract against ESBL Pseudomonas aeruginosa. *Journal of Pharmacopuncture*, **26**(4), 307–318. https://doi.org/10.3831/KPI.2023.26.4.307

Rather, M. A., Gupta, K., and Mandal, M. (2021). Microbial biofilm: formation, architecture, antibiotic resistance, and control strategies. *Brazilian Journal of Microbiology*, **52**, 1701–1718. https://doi.org/10.1007/s42770-021-00624-x

Razack, S. A., Vijayagopal, V., and Viruthagiri, T. (2011). Production of Exopolysaccharides by P. aeruginosa, P. fluorescens and Bacillus sp. *IUP Journal of Biotechnology*, **5**(4), 14–19.

Sagar, P. K., Sharma, P., and Singh, R. (2022). Inhibition of quorum sensing regulated virulence factors and biofilm formation by Eucalyptus globulus against multidrug-resistant Pseudomonas aeruginosa. *Journal of Pharmacopuncture*, **25**(1), 37–45. https://doi.org/10.3831/KPI.2022.25.1.37

Sarkar, R., Chaudhary, S. K., Sharma, A., Yadav, K. K., Nema, N. K., Sekhoacha, M., Karmakar, S., Braga, F. C., Matsabisa, M. G., and Mukherjee, P. K. (2014). Anti-biofilm activity of Marula–a study with the standardized bark extract. *Journal of Ethnopharmacology*, **154**(1), 170–175.

Singh, S., Datta, S., Narayanan, K. B., and Rajnish, K. N. (2021). Bacterial exo-polysaccharides in biofilms: role in antimicrobial resistance and treatments. *Journal of Genetic Engineering and Biotechnology*, **19**, 1–19. https://doi.org/10.1186/s43141-021-00242-y

Tuon, F. F., Dantas, L. R., Suss, P. H., and Tasca Ribeiro, V. S. (2022). Pathogenesis of the Pseudomonas aeruginosa biofilm: A review. *Pathogens*, **11**(3), 300. https://doi.org/10.3390/pathogens11030300

Wang, J. N., Chen, W. X., Chen, R. H., and Zhang, G. F. (2018). Antibacterial activity and mechanism of limonene against Pseudomonas aeruginosa. *J. Food Sci. Technol*, **39**(7), 1–5. https://doi.org/10.13386/j.issn1002-0306.2018.07.001

Wang, R., Vega, P., Xu, Y., Chen, C.-Y., and Irudayaraj, J. (2018). Exploring the anti-quorum sensing activity of a d-limonene nanoemulsion for Escherichia coli O157:H7. *Journal of Biomedical Materials Research Part A*, **106**(7), 1979–1986. https://doi.org/10.1002/jbm.a.36404

Warrier, A., Satyamoorthy, K., and Murali, T. S. (2021). Quorum-Sensing Regulation of Virulence Factors in Bacterial Biofilm. *Future Microbiology*, **16**(13), 1003–1021. https://doi.org/10.2217/fmb-2020-0301

Yin, R., Cheng, J., Wang, J., Li, P., and Lin, J. (2022). Treatment of Pseudomonas aeruginosa infectious biofilms: Challenges and strategies. *Frontiers in Microbiology*, **13**, 955286. https://doi.org/10.3389/fmicb.2022.955286

Yu, X., Lin, H., Wang, Y., Lv, W., Zhang, S., Qian, Y., Deng, X., Feng, N., Yu, H., and Qian, B. (2018). D-limonene exhibits antitumor activity by inducing autophagy and apoptosis in lung cancer. *OncoTargets and Therapy*, **11**, 1833–1847. https://doi.org/10.2147/OTT.S155716

Zhao, Y., Xu, H., Wang, H., Wang, P., and Chen, S. (2024). Multidrug resistance in Pseudomonas aeruginosa: genetic control mechanisms and therapeutic advances. *Molecular Biomedicine*, **5**(1), 62. https://doi.org/10.1186/s43556-024-00221-y