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# Ciprofloxacin- and gentamicin-mediated inhibition of *Pseudomonas aeruginosa* biofilms is enhanced when combined the volatile oil from *Eucalyptus camaldulensis*

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## ABSTRACT

Pseudomonas biofilms cause therapeutic failures in many clinical infections. This study investigated the combined actions of the volatile oil from *Eucalyptus camaldulensis* and antibiotics (ciprofloxacin and gentamicin) against planktonic and biofilm populations of *Pseudomonas aeruginosa*. Further, the quorum sensing genes of *P. aeruginosa,* grown as biofilms, were also investigated. The minimum inhibitory concentrations (MICs) and biofilm inhibitory concentrations (BICs) of gentamicin, ciprofloxacin, and/or the volatile oil of *Eucalyptus camaldulensis* were determined against planktonic and biofilm populations of clinical *P. aeruginosa* isolates. Further, the fractional inhibitory concentrations (FICs) were determined for the antibiotics and volatile oil using a modified checkerboard assay. The bacterial genomic DNA was extracted from biofilm-producing isolates and the target quorum sensing genes were amplified using polymerase chain reaction. Out of 96 biofilm-producing isolates, 43 (44.8%), 29 (30.2%), and 24 (25%) were, respectively, strong, intermediate, and weak biofilm producers. The ciprofloxacin BICs were 30–200-fold higher than the ciprofloxacin MICs. Ciprofloxacin and the volatile oil demonstrated a synergistic effect against both planktonic and biofilm populations. Although the volatile oil and gentamicin also demonstrated a synergistic effect against planktonic cells, only an additive effect was observed against biofilm cells. There was a significant relationship between the frequency of quorum sensing genes, rhlLR and lasLR, and the MICs of piperacillin, with *P* values of 0.043 and 0.032, respectively. The ciprofloxacin MIC was significantly correlated with the presence of lasLR (*P* = 0.031) and the ceftazidime MIC was correlated with the presence of lasLR (*P* = 0.023). The volatile oil from *E. camaldulensis* leaves has antibacterial effects on *P. aeruginosa* biofilms, alone and in combination with ciprofloxacin. There is a high correlation between the presence of quorum sensing genes (LaslR and rhlLR) and biofilm production, and with pipracillin resistance.

**Keywords:**

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## INTRODUCTION

*Pseudomonas aeruginosa,* a widely occurring Gram-negative bacillus, is an opportunistic pathogen that often grows as a biofilm. A biofilm is a community of microbes that is typically attached to a surface and encased in an extracellular matrix; the component cells display characteristics that are unique from those exhibited by their planktonic counterparts. *P. aeruginosa* is known to produce robust biofilms that may cause severe problems [1]. A variety of cellular components, including flagella, type IV pili, extracellular DNA, and extracellular polysaccharides, contribute to bacterial attachment to surfaces [2], including to a variety of medical devices. Once growing as biofilm, the unique characteristics of the sessile cells of bacteria further complicate their eradication, potentially leading to the development of chronic infections [3]. Many of these unique characteristics are encoded by quorum sensing (QS) genes, such as those belonging to the *P. aeruginosa* acyl homoserine lactone (AHL) system encoded by the *lasR-lasI* and *rhlR-rhlI* gene pairs [4].

Despite antibiotic therapy advancements, antibiotic-resistant infections are becoming increasingly common and problematic [5]. The most important factor influencing the emergence and spread of antibiotic resistance is the excessive exposure of bacteria to antibiotics [6]. In light of scientific progress in understanding the medicinal properties of plants, such natural antibiotic sources have received increased interest because of their low toxicity, pharmacological activities, and economic viability. To increase antimicrobial efficacy against multi-drug-resistant bacteria, efforts are being made to examine new molecules that can reduce the minimum inhibitory concentration (MIC) of antibiotics against clinically important bacteria. Essential oils have been demonstrated to have such effects when combined with some antibiotics, including aminoglycosides such as amikacin [7].

With the continued emergence of new antibiotic-resistant strains of many medically important bacteria, including *P. aeruginosa*, novel strategies for countering these bacteria, including when they grow in resistant biofilms, need to be developed. This study investigated the antibacterial action of a volatile oil extracted from *Eucalyptus camaldulensis*, alone and in combination with selected antibacterial agents, to inhibit biofilm-producing isolates of *P. aeruginosa*. The relationships between the presence of QS

genes and antimicrobial agent MICs were also investigated.

## Materials and methods

### Essential oil extraction

*E. camaldulensis* leaves were harvested from locally grown plants and rinsed free of any adherent soil. Clean, dried leaves (100 g) were suspended in 500 mL of distilled water and boiled for 3 h; the collected water vapor was condensed and collected. The oil was separated from the aqueous phase of the condensate and kept in tightly sealed vials, at 4° C, until used.

The components of the volatile oil were identified, using gas chromatography-mass spectroscopy at the Ministry of Science and Technology (Baghdad, Iraq). Prior to use in any experiments, a volume of volatile oil was dissolved in 20 volumes of dimethyl sulfoxide and diluted with distilled water to yield a 1% solution.

### Bacterial isolates

Bacterial isolates were obtained from patients admitted to the Urology and Dermatology Departments of the Al-Ramadi Teaching Hospital (Ramadi, Iraq) and from outpatients visiting private clinics in the city. All samples were collected between October 2019 and February 2020 and were from patients of both sexes (mean age, 29 ± 17.8 years). The study specimens were obtained from catheter-acquired urinary tract and wound infections as well as from patients with burns and otitis media.

All procedures involving human participants were approved by the University of Anbar Ethical Approval Committee (authentication no. 112, October 29, 2019). Written informed consent was provided by all adult participants or by the parent or guardian of any minor.

### Microbiology culture

*P. aeruginosa* isolates were recovered following growth on selective media (cetrimide agar containing 2.2% nalidixic acid) and were confirmed based on colony morphology, pigment formation on selective media, and the results of specific growth tests (indole, methyl red, Voges Proskauer, citrate utilization, gelatin liquefaction, and growth at 42° C ) [8].

### Biofilm production

The ability of the isolates to form biofilms was initially determined using a qualitative microtiter plate assay. Briefly, this test involved the inoculation of a standardized bacterial inoculum into the wells of a 96-well microtiter plate. After overnight incubation, methanol was added to each well for 15 min; thereafter, the plates were drained and allowed to air dry. Crystal violet was added to each well for 5 min, prior to being rinsed out of the wells. Acetic acid (160 µL) was added to each well and the optical density of each well was read at 570 and 630 nm [9]. The optical density (OD) at 630 nm was used to classify the ability of an isolate to form a biofilm as weak (OD < 1.078), intermediate (OD = 1.078–2.156), or strong (OD > 2.156).

Thereafter, a quantitative assay of biofilm formation on alginate beads was performed, as previously described [10]. Briefly, sodium alginate beads were prepared and stored at 4° C for up to one week, prior to use, as previously described [11]. Thereafter, biofilms were grown on the alginate beads. First, the bacteria were grown overnight in brain heart infusion (BHI) broth and diluted in fresh medium to a density of approximately $10^4$ colony forming units (CFU)/mL and added (600 µL) to the wells of 48-well culture plates. Prepared alginate beads (200 µL) were added to the wells and the plates were incubated, with agitation (150 rpm), at 37° C for the desired time.

A dissolution buffer was prepared from a 10× stock solution ($NaCO_3$ [5.3 g] and citric acid [5.2 g] in 100 mL of distilled $H_2O$). Following incubation for the desired period, the beads were separated and rinsed with sterile deionized water (600 µL), added to a tube containing dissolution buffer (2 mL), and crushed. The resuspended bacteria were serially diluted and plated on BHI agar to determine the bacterial numbers. Throughout the process, sterile beads were incubated in BHI broth to confirm the absence of contamination. Note: alginate beads freshly colonized by bacteria for 24 h were rinsed and used in subsequent experiments; hereafter, these are referred to as colonized alginate beads.

### Biofilm growth on alginate beads

The growth rate of each isolate, on alginate beads, was determined by transferring colonized beads, washed with sterile distilled water, to fresh BHI media in new 48-well plates, in triplicate. The plates were incubated under the previously described conditions for varying lengths of time. After growth for the desired period, the beads were removed from the wells, washed with sterile distilled water, and added to a tube of dissolution buffer. The resultant number of cells were determined, as described above.

In a similar manner, the effect of ciprofloxacin on biofilm was assessed by placing washed beads, colonized with the one of two selected *P. aeruginosa* isolates, into triplicate wells containing either distilled water or an aqueous solution of ciprofloxacin (800 µg/mL [isolate 16] or 900 µg/mL [isolate 14] ciprofloxacin). The plates were again incubated under the previously defined conditions for various periods, up to 24 h. The number of sessile cells were determined by removing the beads, washing them, re-suspending the bacteria in dissolution buffer, and enumerating the cells by growth on BHI agar [10].

### Antimicrobial susceptibility

The antibiotic susceptibilities of selected isolates were determined using a Kirby-Bauer test, as described by the Clinical and Laboratory Standards Institute (CLSI) [12], and using an epsilometer [E-test, 13].

### Minimum inhibitory concentration (MIC)

The resazurin microtiter plate assay, with slight modifications, was used to determine the MIC of the volatile oil. Briefly, 100 µL of Mueller Hinton broth (MHB) was added to each well of a 96-well microtiter plate. Aliquots (100 µL) of the 1% volatile oil were added to the wells in the first row of the microtiter plate (1:2 dilution). The volatile oil was then serially diluted through the remaining wells (maximum dilution, 1:256). A bacterial suspension (10 µL, containing $10^6$ CFU) was added to each well; the plate was covered and incubated overnight at 37° C. Following incubation, 15 µL of resazurin solution was added to each well, and the plates were incubated for an additional 3 h. The MIC was determined to be lowest volatile oil concentration that prevented a detectable color change [14].

Ciprofloxacin and gentamicin (maximum concentrations, 250 µg/mL) MICs were determined in a similar manner.

### Biofilm inhibitory concentration (BIC)

Mueller Hinton broth (MHB, 600 µL) was mixed with an equal volume of 1% volatile oil in a test tube. Serial dilutions in the volatile oil were made in BHI-containing tubes to create 1:2 through 1:256 dilutions of the volatile oil. Aliquots (600 µL) of the volatile oil dilutions were added to triplicate wells in a 48-well plate. Colonized alginate beads (200 µL), prepared as described above, were placed in each well. Each plate was tightly covered and incubated at 37° C (agitating at 150 rpm) for 24 h. Upon conclusion of incubation, the beads were transferred to the dissolution buffer and the bacterial numbers quantitated on MH agar, as described above [15].

An analogous method was used to determine the BICs of ciprofloxacin and gentamicin (100–1600 µg/mL).

### Checkerboard assays

Standard checkerboard assays were setup, in 96-well plates, to determine the MICs of the test agents against planktonic cells. The assays involved serial dilutions of the volatile oil (1% solution) in one direction (1:2 – 1:256 dilutions) and serial dilutions of the chosen antibiotics (gentamicin and ciprofloxacin, in separate plates) in the perpendicular direction; all dilutions were prepared in MHB. According to the CLSI recommendations, the stock antibiotic solutions were at least double the previously determined MICs for the target organism. Equal volumes (100 µL) of the antibiotic and volatile oil dilutions were then added to a different 96-well plate, preserving the same orders of the antibiotic and volatile oil dilutions. For the planktonic cells, 10 µL of bacteria (suspended in MHB to a density approximately equal to a 0.5 McFarland standard) were added to each well. After the addition of the bacteria, the plates were covered and incubated at 37° C for 24 h. Thereafter, 15 µL of resazurin was added to each well and the wells without color changes were noted. The fractional inhibitory concentration (FIC) of each antimicrobial was used to determine the FIC index (FICI).

$$\text{FIC (A)} = \frac{MIC(A)\ in\ combination\ with\ antibiotic\ (B)}{MIC(A) alone}$$

$$\text{FIC (B)} = \frac{MIC(B) in\ combination\ with\ volatile\ oil(A)}{MIC(B) alone}$$

A, volatile oil; B, antibiotic. The FICI is represented by FIC(A) + FIC(B). A FICI ≤ 0.5 indicates synergy, whereas values of 0.5–4 indicate an additive effect (no interaction between drugs) and values >4 indicate an antagonistic effect [16].

For the biofilm test, antibiotic (gentamicin and ciprofloxacin) and volatile oil dilutions were prepared in a similar manner as for the planktonic cell assay. Equal volumes of antibiotic and volatile oil (600 µL, total) were placed in 48-well plates, representing the various combinations of antibiotic and oil concentrations. Colonized alginate beads, prepared as described previously, were added to the wells and the plates were covered and incubated overnight at 37° C, with agitation at 150 rpm. Following incubation, the beads were removed, rinsed, and placed in dissolution solution. The bacteria were resuspended, diluted, and grown on MH agar plates to determine the number of surviving cells. The FICI was calculated in a manner analogous to that reported, above.

### Polymerase chain reaction (PCR) analysis of quorum sensing genes

Bacterial DNA was extracted from 96 biofilm producing isolates of *P. aeruginosa* using DNA extraction kits and an automatic nucleic acid extraction system (Samaga, Cepheid, Italy) [17]. The recovered DNA was quantitated, as previously described [18]. The PCR involved the use of *lasI*, *lasR*, *rhlI*, and *rhlR* primers (Alpha DNA, Montreal, QC, Canada), listed in Table 1; the primers were reconstituted in deionized water at concentrations of 10 pmol/µL. Additionally, the PCR also required the use of the Go Taq master mix (Promega, Madison, WI), which contained Taq DNA polymerase, 400 µM (each) dATP, dGTP, dCTP, dTTP, and 3-mM $MgCl_2$, in a reaction buffer (pH 8.5). The PCR was carried out in a 25-µL volume (containing master mix [12.5 µL], yellow and blue loading dye, and 2.5 µL of primer, 5 µL of the target DNA (10 ng), and nuclease-free water (2.5 µL). The thermal cycler (Esco, Portland, OR, USA) was programmed to perform 33 cycles of denaturation (1 min at 94° C), annealing (45 s at 61.9° C), and extension (1 min at 72° C); a final extension step of 10 min at 72° C completed the reaction. The PCR products were separated using agarose (1.5%) gel electrophoresis and stained to enable to determine their sizes [19].

**Table 1**. Primer sequences for the target *lasLR* and *rhlLR* genes.

| Target Gene | | Primer Sequences (5'-3') | Size (bp) |
|---|---|---|---|
| ***lasL*** | F | GTGTTCAAGGAGC GCAAAGG | 238 |
| | R | AACGGCTGAGTTC CCAGATG | |
| ***lasR*** | F | TCGAACATCCGGT CAGCAAA | 128 |
| | R | GTTCACATTGGCT TCCGAGC | |
| ***rhlI*** | F | CCGTTGCGAACGA AATAGCG | 308 |
| | R | CAGTTCGACCATC CGCAAAC | |
| ***rhlR*** | F | TCGCTCCAGACCA CCATTTC | 284 |
| | R | GACGGAGGCTTTT TGCTGTG | |

### Statistical analysis

An analysis of variance (ANOVA) was completed on the results of the experiments involving alginate beads. T-tests were used to evaluate differences between means, and the Pearson's correlation coefficient was used to determine the correlation between biofilm production and QS genes. The statistical analyses of the final data were processed using SPSS (ver. 19.0, SPSS, Chicago, IL, USA).

### Results

The clinically derived isolates of *P. aeruginosa* (n = 138) included 96 (69.5%) that produced biofilms. In the alginate bead experiments, artificial biofilms were formed on the alginate beads. The 24-h growth rate of the sessile cells was significantly lower than that of the planktonic cells, for all isolates tested.

After cultivating *P. aeruginosa* overnight on alginate beads in BHI broth containing 800 and 900 µg/mL of

ciprofloxacin for isolates 16 and 14, respectively, there was complete growth inhibition. However, when the bacteria were actively growing on the alginate beads and forming biofilms, there was growth and after 4 h of incubation; the ciprofloxacin concentrations had negligible effects on bacterial growth. For both isolates, the development of growth occurred between 4 and 8 h. However, the growth was sharply reduced by 12 h for isolate no. 16 and by 24 h for isolate no. 14 (Fig. 1).

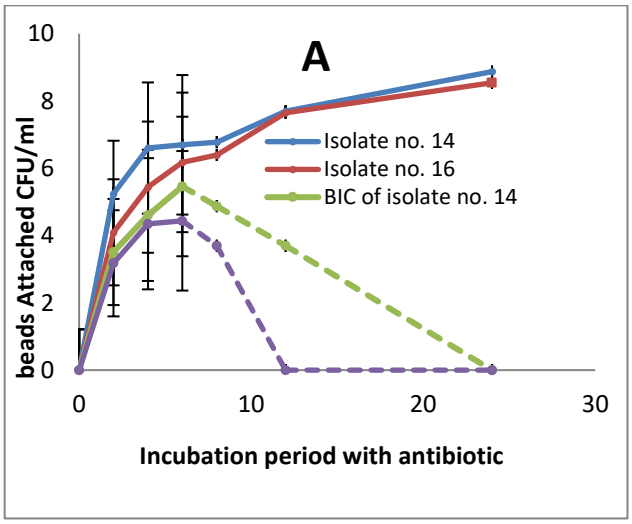


**Figure 1**. Stable biofilm formation within 24 hours. Mean colony forming unit (CFU)/mL counts are plotted against growth time.

Cultures were set up from diluted overnight cultures (~$10^4$ CFU/mL). Triplicate bead samples were collected at 2, 4, 6, 8, 12, and 24 hours. A: Without ciprofloxacin, and With ciprofloxacin (800 µg/mL for isolate no. 16, 900 µg/mL for isolate no. 14), B: With combined (400 and 300 µg/mL for isolates no. 24 and 26 respectively) ciprofloxacin and *E. camaldulensis* volatile oil (0.048 and 0.096 U/mL for isolates no. 14 and 16, respectively).

All patient isolates of *P. aeruginosa* were tested for antibiotic sensitivity (ciprofloxacin and gentamicin) using the Kirby Bauer disk diffusion and E-test assays, using criteria established by CLSI [17] (Table 2). From the results of the quantifiable biofilm formation assay, the various *P. aeruginosa* isolates were classified as strong, weak, or moderate biofilm producers.

**Table 2.** Resistance of clinical *Pseudomonas aeruginosa* isolates to selected antibiotics.

| Antibiotics | *P. aeruginosa* isolates | | | | | |
|---|---|---|---|---|---|---|
| | Resistant | | Intermediate | | Sensitive | |
| | No. | % | No. | % | No. | % |
| **Aztreonam** | 19 | 19.8 | 35 | 36.5 | 42 | 43.7 |
| **Tobramycin** | 10 | 10.5 | 26 | 27 | 60 | 62.5 |
| **Piperacillin** | 6 | 6.2 | 8 | 8.4 | 82 | 85.4 |
| **Meropenem** | 21 | 21.9 | 15 | 15.6 | 60 | 62.5 |
| **Ciprofloxacin** | 20 | 20.8 | 15 | 15.6 | 61 | 63.6 |
| **Gentamicin** | 8 | 8.4 | 46 | 47.9 | 42 | 43.7 |
| **Ceftazidime** | 19 | 19.8 | 8 | 8.3 | 69 | 71.9 |

The GC/MS analysis of the *E. camaldulensis* volatile oil is presented in Fig. 2. Ten major compounds were identified, comprising 73.11% of the volatile oil. These compounds were identified as terpinene (13.31%), cineole (12.48%), cephrol (11.82%), terpineol (8.74%), pentanol (7.04%), citronellol (6.83%), mentha (5.89%), neoclovene (3.36%), nerol (2.15%), and ocimene (1.49%).

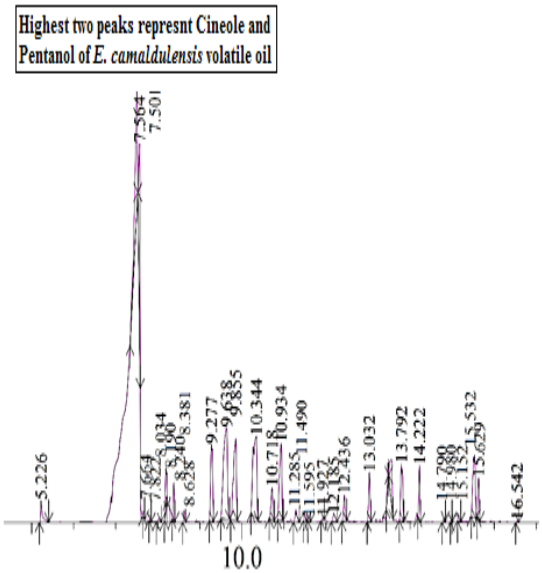


**Figure 2.** Gas chromatography-mass spectrometry analysis of *Eucalyptus camaldulensis* volatile oil.

Checkerboard analyses of the antimicrobial effects of the antibiotics and volatile oil against planktonic and biofilm cells of two *P. aeruginosa* isolates demonstrated the synergistic effect of the volatile oil and ciprofloxacin combination against both planktonic and sessile cells of

bacteria (isolate 14). However, combinations of volatile oil and gentamicin were generally observed to have additive antimicrobial effects; the combination was observed to have a synergistic antimicrobial effect against planktonic cells of isolate 14 (Table 3).

**Table 3.** Checkerboard assay results for selected antibiotics and the essential oil against two isolates (nos. 14 and 16) of *Pseudomonas aeruginosa*.

| | **Planktonic bacteria** | | | | | **Biofilm bacteria** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Oil + CIP** | | | | | | | | | | |
| Isolate no. | MIC DN (U/mL) (Oil) | MIC µg/mL (CIP) | FIC DN (U/mL) (Oil) | FIC µg/mL (CIP) | Activity | BIC DN (U/mL) (Oil) | BIC µg/mL (CIP) | FIC DN (U/mL) (Oil) | FIC µg/mL (CIP) | **Activity** |
| 14 | (3.125) | 31.25 | (0.781) | 0.97 | S | (12.5) | 900 | (0.048) | 400 | **S** |
| 16 | 1/32 (3.125) | 3.906 | 1/256 (0.3906) | 0.24 | S | 1/16 (6.25) | 800 | 1/1024 (0.0967) | 300 | **S** |
| **Oil + GENT** | | | | | | | | | | |
| | MIC DN (U/mL) (Oil) | MIC µg/mL (GENT) | FIC DN (U/mL) (Oil) | FIC µg/mL (GENT) | Activity | BIC DN (U/mL) (Oil) | BIC µg/mL (GENT) | FIC DN (U/mL) (Oil) | FIC µg/mL (GENT) | **Activity** |
| 14 | 1/32 (3.125) | 3.9 | 1/256 (0.39) | 1.95 | S | 1/8 (12.5) | 900 | 1/32768 (0.003) | 1000 | **A** |
| 16 | 1/32 (3.125) | 0.97 | 1/64 (1.562) | 0.48 | A | 1/16 (6.25) | 100 | 1/512 (1.953) | 300 | **A** |

CIP, ciprofloxacin; GENT, gentamicin; Oil, volatile oil; S, synergy; A, antagonism; MIC, minimum inhibitory concentration; BIC, biofilm inhibitory concentration; FIC, fractional inhibitory concentration; DN, dilution number.

In our research, *lasLR* and *rhlLR* system genes were observed (Fig. 3). The PCR results showed that 98% of the *P. aeruginosa* isolates that produce biofilm, carried the *laslR* gene and 94% possessed the *rhlLR* gene. An analysis of the association of the QS genes with the ability of the isolates to form biofilms demonstrated a significant correlation of both genes with biofilm formation (*lasIR*, $P = 0.04$; *rhlIR*, $P = 0.038$). There was a significant association between resistance to ciprofloxacin and the presence of *lasLR* ($P = 0.031$).

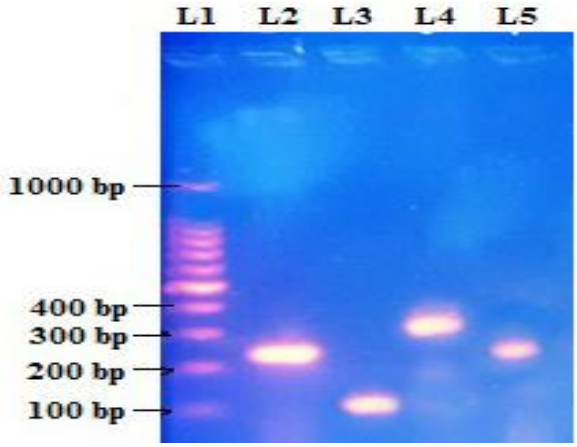


**Figure 3.** Agarose (1.5%) gel electrophoresis of quorum sensing gene polymerase chain reaction products. L1: 100 bp ladder, L2: *lasL* gene (238 bp), L3: *lasR* gene (128 bp), L4: *rhlL* gene (308 bp), L5: *rhlR* gene (284 bp).

## Discussion

The emergence of drug-resistant pathogens has become a crucial infectious disease problem, worldwide. Among resistant pathogens, *P. aeruginosa* is one species that frequently displays broad-spectrum drug resistance, making it a major public health concern. To date, the search for potential agents effective against sessile cells of bacteria remains a particular challenge [20].

In our study, ciprofloxacin exhibited antimicrobial activity against planktonic and sessile *P. aeruginosa* cells, but the sessile cells exhibited resistance to ciprofloxacin levels that were 30–200-fold higher than the MIC needed to inhibit planktonic cell growth. Agarwal et al [21] concluded that four-fold higher ciprofloxacin concentrations were required to inhibit sessile cells than were required to inhibit planktonic *P. aeruginosa* cells. Similar to our results, Bueno [22] concluded that sessile cells of microbes were up to 1000-fold less susceptible to antibiotics than were the corresponding planktonic cells.

One potential solution to the resistance issue and, particularly, to the side effects associated with the use of elevated antibiotic concentrations involves combining antibiotics with medicinal herb extracts to bolster their activity. The focus of the present study was an *in vitro* examination of the antimicrobial action of the volatile oil of *E. camaldulensis*, combined with ciprofloxacin. The interactions of the oil and the antibiotics (both ciprofloxacin and gentamicin, independently) were examined using the checkerboard method. The obtained FICI values indicated the existence of a synergistic effect between the volatile oil and ciprofloxacin against both planktonic and sessile cells of *P. aeruginosa*; the oil/gentamicin combination generally demonstrated additive effects.

The synergistic interactions between ciprofloxacin and essential/volatile oils could lead to new treatment strategies involving reduced antibiotic doses. In our study,

the synergistic action of the volatile oil and ciprofloxacin was observed using the checkerboard assay. The results support the suggestion of Knezevic et al. [23] that the antimicrobial activity of *E. camaldulensis* essential oil is synergistic with that of ciprofloxacin against the bacteria in their study. The observed results may reflect the action of the components of plant volatile oils that function as natural, broad-spectrum antimicrobials. These components may function by inhibiting adenosine triphosphate formation, disturbing membrane permeability, and changing the proton motive forces within membranes. Some essential oils also function as quorum sensing inhibitors, leading to decreased formation of biofilm and virulence factors [24, 25].

The volatile oil investigated in this study was composed of several components, including terpinene, cineole, cephrol, terpineol, pentanol, citronellol, mentha, neoclovene, nerol, and ocimene. Nerol, one of the main components, was previously demonstrated to have potent antibacterial activity against pathogenic bacteria [26] Terpineol and cineol have been demonstrated to cause cell wall structure alterations that result in bacterial cell death [27, 28].

Further, the present study demonstrated that gentamicin and the volatile oil combination dispersed *P. aeruginosa* biofilms. The combination demonstrated a more pronounced effect in decreasing both biofilm and planktonic cell numbers than did either the antibiotic or oil, alone, after short- or long-term treatment. The combination was more effective against planktonic cells than against sessile cells, in terms of the FIC. These results support the suggestion of Al-Snaf [29] that the positively charged aminoglycosides bind to the negatively charged polymers in the biofilm matrix, potentially slowing antibiotic penetration. Volatile oils have been shown to penetrate biofilms due to their ability to disrupt the negatively charged cell membranes of surface-attached microbes, potentially making the biofilm cells more accessible to some antibiotics [30].

The QS system in *P. aeruginosa* depends upon the presence of AHLs and the *Las* and *rhl* genes. The expression of certain virulence genes can be triggered by specific stimuli, including bacterial cell density or iron limitation. An additional level of complexity is added by the third QS system in these bacteria: the *P. aeruginosa* quinolone signal (PQS) system. The PQS system is interconnected with AHL-dependent signaling and can also be triggered by iron limitation within the bacterial population [31].

In our study, the PCR results showed that the frequency of QS genes in our clinical isolates was high, with 94–98% of the biofilm-forming isolates possessing *rhLR* and/or *LaslR*. QS is a regulation mechanism that enables bacteria to regulate their population size through responses to intracellular concentrations of freely diffusible sensing molecules that are continuously produced by the cells. *LasI* and *rhlI* encode homoserine lactone synthesis, whereas *lasR* and *rhlR* encode transcriptional activators (*LasR* and *RhlR*) that respond to their respective signal molecules and activate transcription of *lasI* and *rhlI*. The two systems work sequentially, with the *RhlR*/*RhlI* system being subordinate to the *LasR*/*LasI* system. The QS systems control more than 200 genes, including those responsible for pathogenesis [32].

Our results showed that the *LasR*/*LasI* genes are associated with MICs value of ciprofloxacin. This observation agrees with those reported by Skindersoe et al [33]. That group reported that ciprofloxacin decreases the expression of a range of QS-regulated virulence factors. The underlying mechanism may be mediated by changes in membrane permeability, thereby influencing the flux of N-3-oxo-dodecanoyl-l-homoserine lactone. The biofilms of *P. aeruginosa* QS mutants have less antibiotic resistance than that of their wild-type parents, and QS inhibition has been shown to promote the eradication of biofilms by antimicrobial treatments.

## Conclusion

This study suggested that the BICs for selected antimicrobial agents were 30–200-fold higher than the MICs needed to inhibit planktonic cell growth. The volatile oil extracted from *E. camaldulensis* leaves was also shown to have marked antibacterial effects on *P. aeruginosa*. Further, a combination of ciprofloxacin and the volatile oil demonstrated synergistic antimicrobial activity, enhancing the antimicrobial action of both compounds against early biofilm formation. Finally, our findings showed that the isolates tested in the susceptibility experiments are strong biofilm producers. Further, the QS genes that control biofilm production were correlated with antibiotic resistance in the studied clinical isolates.

## Data Availability

The data underlying the findings of this study will be available from the corresponding author, upon reasonable request.

## Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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