

## Aromatherapeutic Antibacterials: Comparative Study of 40 Essential Oils and Their Biofilm Inhibition in *Pseudomonas aeruginosa* ATCC9027

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### ABSTRACT

Growing evidence suggests that biofilm formation in *Pseudomonas aeruginosa* (PA) results from quorum sensing, reducing bacterial sensitivity to antibiotics and fostering the development of antibiotic-resistant strains. Addressing the need for a compound that can inhibit quorum sensing signals to mitigate biofilm formation in PA, this study screened 40 essential oils for their potential as quorum sensing inhibitors against PA ATCC9027. Utilizing the agar well diffusion assay, the antibacterial activity of these essential oils was compared with levofloxacin, revealing that 25 out of 40 essential oils exhibited antibacterial activity against PA ATCC9027. The highest antibacterial activity was exhibited by essential oils from *Cinnamomum burmannii* (Cinnamon Bark Oil), *Cananga odorata* (Ylang-ylang Oil), and *Eucalyptus globulus* (Eucalyptus Oil), confirmed by respective MIC values of 0.09765% (v/v), 0.390625% (v/v), and 1.5625% (v/v), and MBC values of 0.09765% (v/v), > 1.5625% (v/v), and > 1.5625% (v/v). Growth curve inhibition assay followed by crystal violet biofilm formation assay revealed that only Ylang-ylang Oil exhibited biofilm formation inhibition at 1/4 MIC concentration. Docking analysis of compounds from these essential oils against quorum sensing enzymes (LasR, PqsR, and QscR) revealed that Ylang-ylang Oil compounds exhibited the highest binding affinity. In conclusion, among the 40 essential oils tested, 25 show potential as antibacterials against PA ATCC9027, with Ylang-ylang Oil standing out as a promising candidate for quorum sensing inhibitor development. The use and development of quorum sensing inhibitors from Ylang-ylang Oil compounds are expected to reduce the burden of antibiotic resistance, especially in *Pseudomonas aeruginosa*.

## 1. Introduction

*Pseudomonas aeruginosa* (PA) is a gram-negative bacterium reported as one of the leading causes of the highest mortality rates, often isolated/found in ICU settings. It can induce both acute and chronic infections in patients with compromised immune systems or burn injuries (Gupte *et al.* 2021; Wang *et al.* 2021). PA can synthesize adhesive substances, secrete proteases to break down and invade host cells, and produce virulence factors such as pyocyanin to defend itself against the host immune system. This phenomenon is commonly observed in acute infections (Allen *et al.* 2005;

Wang *et al.* 2021). In chronic infections, PA can form biofilms, which are slimy polymeric matrices created by a group of bacteria acting as a protective shield against antibiotics. It has been reported that biofilm formation can result in a 100-fold increase in antibiotic resistance (Hall and Mah 2017).

Quorum sensing (QS) is a communication system among bacteria that utilizes small diffusible molecules as signaling agents. There are three known QS systems, two of which employ N-acylhomoserine lactones (AHLs) as signaling molecules, known as the Las and Rhl systems. The third system, called Pqs, connects these two systems (Li *et al.* 2022). Due to the crucial role of QS systems in virulence factor production and biofilm formation in PA, it is considered an ideal target for developing anti-infective drugs, particularly against PA. This is

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because Quorum Sensing Inhibitors (QSIs) can reduce bacterial toxicity without affecting growth and enhance the sensitivity of bacterial biofilms to antibiotic treatment (Hentzer and Givskov 2003; Munir *et al.* 2020). Several identified QSIs from synthetic compounds include halogenated furanones (Hentzer *et al.* 2002), benzothiazole (Gabr *et al.* 2015), isatin-b-thiocarbonylhydrazones (Gabr *et al.* 2018), phenylalanine arginyl b-naphthylamide (El-Shaer *et al.* 2016), and aspirin (El-Mowafy *et al.* 2014). Additionally, natural compounds such as zingerone (Kim *et al.* 2015), curcumin (Tyagi *et al.* 2015), alginates (He *et al.* 2014), and 1H-pyrrole-2-carboxylic acid (Hassan *et al.* 2016) have also been recognized as QSIs.

Essential oils have been widely used in traditional medicine due to their antibacterial, antifungal, antiviral, and insecticidal activities, as well as their immune system-boosting properties. Some essential oils exhibit antimicrobial activity by disrupting the bacterial cell membrane or cell wall, triggering cell lysis and the release of bacterial content (Ganesh and Rai 2016). Despite the extensive use of essential oils as antibacterial agents, only a few studies have reported their activity as QSIs against PA. Recent research has identified essential oils with QSI activity against PA, including essential oils from *Citrus paradisi* (grapefruit (Luciardi *et al.* 2020), *Citrus reticulata* (mandarin orange) (Luciardi *et al.* 2016), *Melaleuca alternifolia* (tea tree) (Noumi *et al.* 2018), *Carum carvi* (caraway) (Fekry *et al.* 2022), *Murraya koenigii* (Ganesh and Rai 2016). However, these studies have limitations, mainly in determining the QSI activity of each essential oil separately in each study. The objective of this study is to thoroughly evaluate the antibacterial effectiveness of 40 varieties of essential oils before establishing their potential as Quorum Sensing Inhibitors (QSIs). Upon discovering essential oil candidates with the strongest quorum-sensing inhibition activity against PA, further research will focus on isolating and identifying their active compounds. These compounds can then be developed against PA and potentially other bacteria, either as a standalone agent or in combination with antibiotics or other agents.

## 2. Materials and Methods

### 2.1. Essential Oils: Source, Preparation, and Profiling

The essential oils employed in this study are commercial products branded under SESMU,

adhering to standardization and accompanied by a Certificate of Analysis (COA). The COA data, including compound components used for quality control assessed through gas chromatography, were used to compare essential oils for profiling purposes. The essential oils were prepared using a 50% DMSO solvent for the antibacterial screening test. In other experiments, the essential oils were formulated into a macroemulsion with a composition of essential oil: Tween 80: water for injection at a ratio of 1:3:12.

### 2.2. Antibacterial Screening via Agar Well Diffusion

We applied the agar well diffusion method to evaluate the antibacterial potential of 40 essential oils against *Pseudomonas aeruginosa* strain R. Hugh 813 (ATCC 9027). PA (0.5 McFarland  $\sim 10^8$  cfu/ml) was streaked on Mueller-Hinton agar plates. Essential oils, diluted at a 1:1 (v/v) ratio in DMSO (50%), were dispensed into six wells in each petri dish containing 50  $\mu$ L of the diluted essential oil. DMSO (50%) and levofloxacin solution (5  $\mu$ g/50  $\mu$ L) were used as negative and positive controls, respectively. Following incubation at  $35 \pm 2^\circ\text{C}$  for 18 hours, antibacterial activity was gauged by measuring inhibition zone diameters. The antibacterial effectiveness of essential oils against PA ATCC9027 is represented as a percentage. This percentage is calculated by comparing the measured inhibition zone diameters of each essential oil to those of the positive control (levofloxacin) in each petri dish.

### 2.3. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Three out of the 40 tested essential oils, namely *Cinnamomum burmannii* (Cinnamon Bark Oil), *Cananga odorata* (Ylang-ylang Oil), and *Eucalyptus globulus* (Eucalyptus Oil), exhibited the highest potential in the agar well diffusion assay. These were then selected for MIC was determined with a microbroth dilution technique as described by the Clinical and Laboratory Standards Institute (CLSI 2003). The first column of the microplate served as the negative control, containing 100  $\mu$ L of growth medium (Mueller-Hinton Broth media) and 100  $\mu$ L of essential oil-free emulsion. The second column acted as the positive control, with 100  $\mu$ L of essential oil-free emulsion and 100  $\mu$ L of microbial test suspension (PA ATCC9027). The remaining wells were filled with 100  $\mu$ L of growth medium. The third column received 100  $\mu$ L of essential oil emulsion or

levofloxacin with a predetermined concentration, with subsequent transfers to the fourth column until the twelfth column completed the dilution series. Each test well-received 100  $\mu$ L of microbial test suspension. The microplate was incubated at  $35\pm 2^\circ\text{C}$  for 16–18 hours, and the MIC was determined as the lowest concentration without observable microbial growth, visually compared to the negative control in terms of clarity. For MBC determination, 10  $\mu$ L of sterile Mueller-Hinton agar medium was poured into a sterile petri dish and allowed to solidify. In all wells indicating clarity (MIC concentration and above), an Ose needle was immersed and then streaked on the surface of Mueller Hinton agar. The petri dishes were subsequently incubated at  $35\pm 2^\circ\text{C}$  for 16–18 hours. The MBC value was determined from the concentration that showed no colony growth on the agar plates. MIC determinations were conducted in quadruplicate, while MBC determinations were performed in triplicate.

#### 2.4. Evaluation of Growth Inhibition

We examined the growth-inhibiting effects of three highly potent essential oils against PA ATCC9027. The experiment began by diluting overnight PA ATCC9027 cultures at a 1:100 ratio into fresh Mueller-Hinton Broth (MHB) media, using a microplate setup similar to MIC determination. In the microplate, the initial column was the negative control, consisting of 100  $\mu$ L of Mueller-Hinton Broth media and 100  $\mu$ L of essential oil-free emulsion. The second column acted as the positive control, containing 100  $\mu$ L of essential oil-free emulsion and 100  $\mu$ L of microbial test suspension (PA ATCC9027). For the test column, essential oil emulsions at concentrations of 1/2, 1/4, and 1/8 MIC were added to a 96-well microplate. Each group was replicated 8 times (8 wells). Incubation was carried out at  $35\pm 2^\circ\text{C}$ , and the absorbance (optical density [OD]) was measured at 630 nm using the ELISA reader BK-EL10C (Biobase, Shandong, China) at intervals of 0, 3, 6, 9, 12, and 22 hours for each well.

#### 2.5. Crystal Violet Biofilm Inhibition Assay

The inhibitory effect on static biofilm formation was assessed using the crystal violet staining method with the PA ATCC9027 strain, following established procedures with slight modifications (Au-O'Toole, 2011; Mastoor *et al.* 2022). In brief, overnight cultures of PA ATCC9027 were diluted 1:100 into fresh MHB media. The positive control consisted of

100  $\mu$ L of essential oil-free emulsion and 100  $\mu$ L of microbial test suspension (PA ATCC9027). Essential oil emulsion was added at concentrations of 1/4 and 1/8 MIC before static incubation at  $37^\circ\text{C}$  for a week. After incubation, plates were thoroughly washed to remove planktonic cells, and adherent cells were measured by crystal violet (0.25% w/v) staining. The optical density (OD) was measured at 630 nm using the ELISA reader BK-EL10C (Biobase, Shandong, China). The tests were carried out with three replicates for each concentration of essential oil.

#### 2.6. *In Silico* Anti-Quorum Sensing Activity

Gathered from the Certificate of Analysis (COA), the compounds utilized for assessing the quality control of three essential oils, recognized for their potent antibacterial properties from prior experiments, were acquired. These compounds were then employed as ligands in the molecular docking analysis. The 3D structures of these compounds were downloaded from the PubChem Compound page and converted to PDB format using Chimera (Pettersen *et al.* 2004). Protein targets from the RCSB Protein Data Bank (PDB codes 2UVO, 4JVC, and 6CC0) were chosen as docking receptors, and the active sites of the receptors were cleared of all bound ligands and water molecules. The standard protocol using AutoDockVina (Trott and Olson 2010) was applied to dock these compounds at the active site of receptors, where the grid box was analyzed from the native ligand using Discovery Studio 2021 Client. The coordinates for the centers (x, y, z) and sizes along all axes for the grid boxes were determined for the following receptors: LasR (PDB ID 2UVO) with center coordinates of  $x = 23.710750$ ,  $y = 16.636667$ ,  $z = 79.439458$ ; PqsR (PDB ID 4JVC) with center coordinates of  $x = -52.864187$ ,  $y = -1.716625$ ,  $z = 10.120938$ ; QscR (PDB ID 6CC0) with center coordinates of  $x = -76.492950$ ,  $y = -9.502200$ ,  $z = 12.195000$ . The size in all directions for the grid boxes was set to 20. The conformation with the most favorable (lowest) binding energy was selected to analyze the interaction between the target receptor and ligand in Discovery Studio 2021 Client.

#### 2.7. Statistical Analysis

Unless stated otherwise, all results were expressed as mean  $\pm$  standard deviations for each sample, and treatments were conducted in triplicates. Statistical analyses and data visualization were

carried out using R version 4.2.1 in RStudio. T-tests with Bonferroni adjustment were employed for antibacterial screening tests and biofilm formation inhibition assays. The Wicoxon test, followed by the Benjamini-Hochberg adjustment method, was utilized for analyzing growth curve inhibition assays. P-values < 0.05 were deemed statistically significant.

### 3. Results

#### 3.1. Profiling 40 Varieties of Essential Oils

The essential oils utilized in this study were commercially branded SESMU products, each accompanied by a Certificate of Analysis (COA) attesting to their standardization. Detailed in Appendix 1 is the list of essential oils used in the antibacterial screening. Leveraging the data from the COA, a comparative analysis of compounds identified by gas chromatography (GC) was conducted across the various essential oils.

This study involved a thorough analysis of 40 essential oil varieties, as depicted in Figure 1A, showcasing compound distribution used for quality control via gas chromatography. The graph provides insights into essential oil quality and composition. Additionally, Figure 1B presents a clustering heatmap, visually representing similarities in quality control compound contents among essential oils. The clustering pattern offers a clear overview of relationships between different varieties, aiding in categorization and analysis. This comprehensive profiling formed the basis for evaluating antibacterial efficacy and quorum sensing inhibition in PA, emphasizing the intricate link between essential oil composition and antibacterial properties. The profiling information of these essential oils can also be used to distinguish *in silico* activity towards quorum sensing regulatory proteins in the following experiments.

#### 3.2. Agar Well Diffusion Test Against PA ATCC9027

The agar diffusion method was employed to assess the antibacterial potential of 40 essential oils against PA ATCC9027. Petri dishes containing PA ATCC9027 were treated with essential oils, and after 18 hours of incubation, inhibition zones were observed and measured. The results were compared to the positive control (levofloxacin). A schematic illustration of the plate used in the agar well diffusion assay is depicted in Figure 2A. Zone of inhibition diameters are shown in

Figure 2B. We observed that the negative control, DMSO 50%, exhibited minimal inhibition zones (mean  $\pm$ 3 mm surrounding wells), and we normalized by subtracting the zone of inhibition in the essential oils group from the observed zone of inhibition in the negative control. To account for variations in bacterial counts between petri dishes, we designed the inhibition zone activity test by adding levofloxacin positive control to each petri dish. This allowed us to obtain the inhibition zone as a percentage of the observed zone of inhibition in each petri dish.

In Figure 2C, 25 of the 40 tested essential oils exhibited inhibition zone diameters, signifying antibacterial activity against PA ATCC9027. Notably, oils showing significant inhibition zones include *Cinnamomum burmannii* (Cinnamon Bark Oil), *Cananga odorata* (Ylang-ylang Oil), *Eucalyptus globulus* (Eucalyptus Oil), *Magnolia champaca* (Magnolia Oil), *Boswellia carterii* (Frankincense Oil), *Plumeria alba* (Frangipani Oil), *Melaleuca alternifolia* (Tea Tree Oil), *Origanum majorana* (Marjoram Oil), *Artemisia dracunculus* (Tarragon Oil), *Melaleuca cajuputi* (Cajuput Oil), *Rosmarinus officinalis* (Rosemary Oil), *Citrus aurantium bergamia* (Bergamot Oil), *Pelargonium graveolens* (Geranium Oil), *Mentha spicata* (Spearmint Oil), *Myristica fragrans* (Nutmeg Oil), *Juniper virginiana* Linne (Cedarwood Oil), *Alpinia malaccensis* (Alpinia Oil), *Vanilla planifolia* (Vanilla Oil), *Citrus paradisi* (Grapefruit Oil), *Salvia sclarea* L. (Clary Sage Oil), *Foeniculum vulgare* (Sweet Fennel Oil), *Lavandula* L. (Lavender Oil), *Piper nigrum* L. (Black Pepper Oil), *Citrus sinensis* (Orange Oil), and *Citrus medica limonum* (Lemon Oil).

Conversely, several essential oils showed no inhibition zones indicating either no antibacterial activity against PA ATCC9027 or extremely minimal activity undetected in the experiment. These oils included *Piper cubeba* (Cubeb Oil), *Anthemis nobilis* (Roman Chamomile Oil), *Syzygium aromaticum* (Clove Bud Oil), *Curcuma domestica* (Turmeric Oil), *Pogostemon cablin* benth (Patchouli Oil), *Cymbopogon flexuosus* (Lemongrass Oil), *Zingiber officinale* (Ginger Oil), *Mentha piperita* (Peppermint Oil), *Aetoxylan sympetalum* (Aetoxylan Oil), *Origanum vulgare* (Origanum Oil), *Vetiveria zizanioides* (Vetiver Oil), *Citrus reticulata* (Tangerine Oil), *Cymbopogon martini* (Palmarosa Oil), *Litsea cubeba* (Litsea Cubeba Oil), and *Citrus aurantium* (Neroli Oil).

The findings reveal significant variations in antimicrobial activity among the tested essential oils, indicating diverse inhibitory potential. For subsequent experiments, three essential oils with



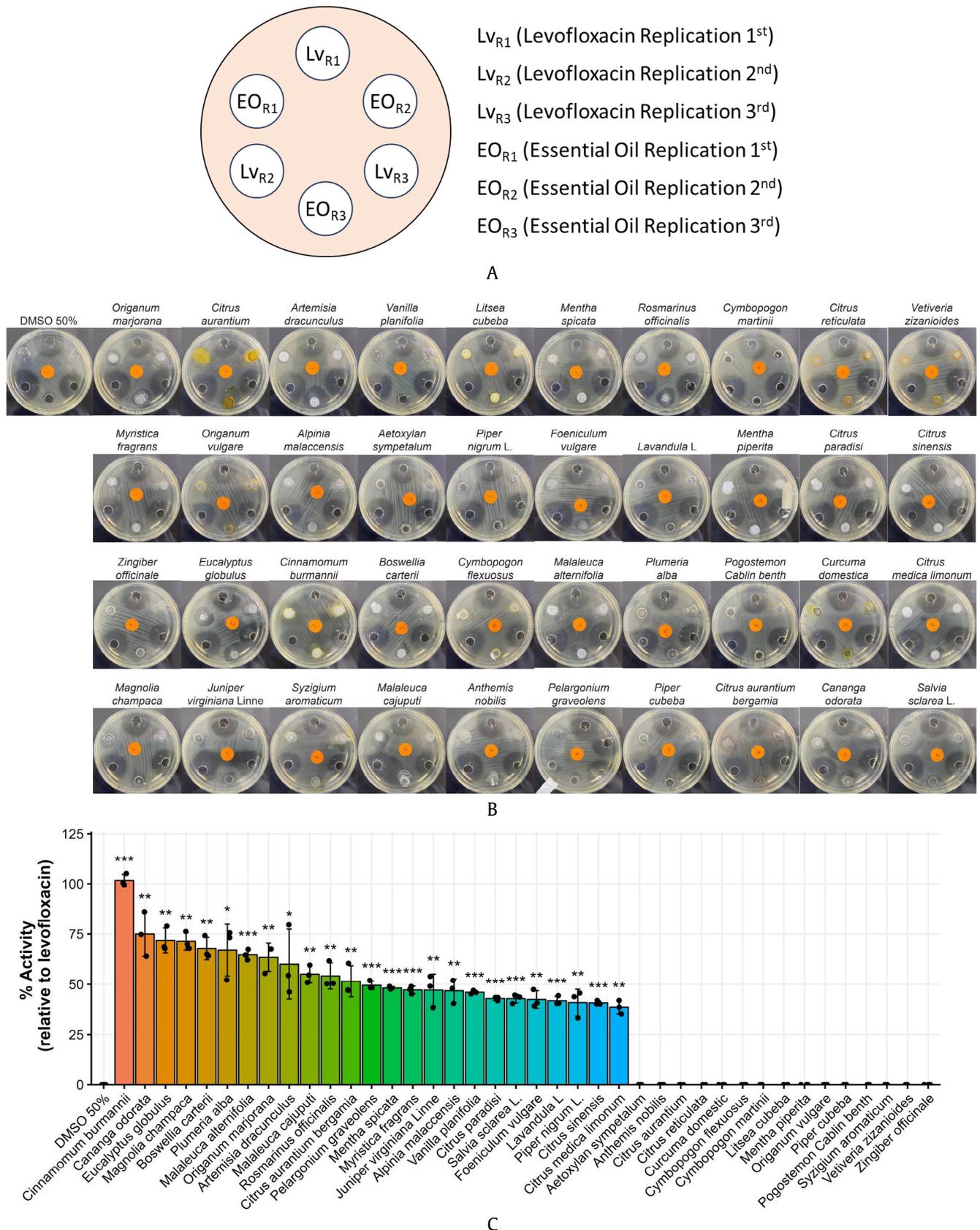


Figure 2. Evaluation of antibacterial activity of 40 essential oils using the agar well diffusion assay against *Pseudomonas aeruginosa* ATCC9027. (A) Schematic figure of plate used in agar well diffusion assay, (B) presentation of the inhibition zone diameters for the 40 essential oils, (C) comparative analysis of the antibacterial activities of the 40 essential oils against the levofloxacin control

the highest antibacterial activity will be selected to identify their potential as quorum-sensing inhibitors against PA ATCC9027.

### 3.3. Antibacterial Activities of Potential Essential Oils

Based on the observation of the inhibitory test, it was found that *Cinnamomum burmannii* (Cinnamon Bark Oil), *Cananga odorata* (Ylang-ylang Oil), and *Eucalyptus globulus* (Eucalyptus Oil) are three essential oils that exhibit the largest inhibition zones. Subsequently, the minimal concentrations of these essential oils required to start inhibiting or killing PA ATCC9027 were determined using the broth microdilution method. The MIC and MBC of levofloxacin were also determined to serve as a reference for the sensitivity of the used bacteria. The observed MIC for levofloxacin was 0.5 µg/ml, which aligns with the quality control test requirements (EUCAST Quality Control) for *Pseudomonas aeruginosa* with a MIC range of 0.5–4.0 µg/ml. The detailed values of MIC and MBC observations are presented in Table 1.

### 3.4. Determination of Growth Curve Inhibition at sub-MIC doses

The inhibition activity profiling against the growth of PA ATCC9027 was conducted after the administration of essential oils at their sub-MIC concentrations. This was aimed at understanding the toxicity profile of the essential oils at their sub-MIC concentrations. The data obtained from this toxicity profile will serve as a basis for further observations on the inhibitory activity against biofilm formation, as determined subsequently. Periodic observations were made using an ELISA reader instrument, with optical density reflecting the quantity of microbes in each well being monitored. For statistical analysis, comparison of each time point were made by comparing the normalized absorbances with positive control. The observed p-value are provided in Table 2.

The results of normalized absorbance measurements are depicted in Figure 3. Notably, both *Cinnamomum burmannii* and *Eucalyptus globulus* essential oils exhibited a dose-dependent inhibition of bacterial growth, starting as early as the third hour, even at the minimal concentration of 1/8 MIC. In contrast, the growth inhibitory effects of *Cananga odorata* essential oil were only evident at the sixth

Table 1. MIC and MBC observations

Essential oil or compound	MIC (% v/v)	MBC (% v/v)
Levofloxacin	0.05*	0.8*
<i>Cinnamomum burmannii</i>	0.09765	0.09765
<i>Cananga odorata</i>	0.390625	> 1.5625
<i>Eucalyptus globulus</i>	1.5625	> 1.5625

\*Concentration of levofloxacin in % (w/v)

Table 2. Statistical significance values derived from the growth curve analysis

Essential oil	hours	p-value		
		1/2 MIC	1/4 MIC	1/8 MIC
<i>Cinnamomum burmannii</i>	3	***	**	**
	6	***	***	***
	9	***	***	***
	12	***	***	***
	22	***	***	*
<i>Cananga odorata</i>	3	ns	ns	ns
	6	***	***	***
	9	***	***	***
	12	***	***	***
	22	***	ns	ns
<i>Eucalyptus globulus</i>	3	***	**	ns
	6	***	***	***
	9	***	***	***
	12	***	***	***
	22	***	***	ns

hour. Additionally, at the 22<sup>nd</sup> hour mark, *Cananga odorata* essential oil was the only one that did not show significant growth inhibition compared to the positive control.

### 3.5. Assessment of Biofilm Inhibition Activity

Given the study's aim to pinpoint potential essential oils with promise as antiquorum sensing inhibitors, we delved into the impact of essential oils on activating the quorum sensing signal pathway, specifically biofilm formation. To directly gauge essential oil efficacy in hindering biofilm development in PA ATCC9027, we introduced essential oils at concentrations of 1/4 and 1/8 MIC to microplates containing PA ATCC9027 in MHB media. After incubation for a week, the resulting biofilm was subjected to staining with crystal violet, and its quantification was performed using spectrophotometry.

The findings, illustrated in Figure 4, highlight *Cananga odorata* essential oil as the sole significant inhibitor of biofilm formation in PA ATCC9027. Nevertheless, at a 1/8 MIC concentration, *Cananga odorata* essential oil does not demonstrate inhibitory activity. From these results, it can be inferred that

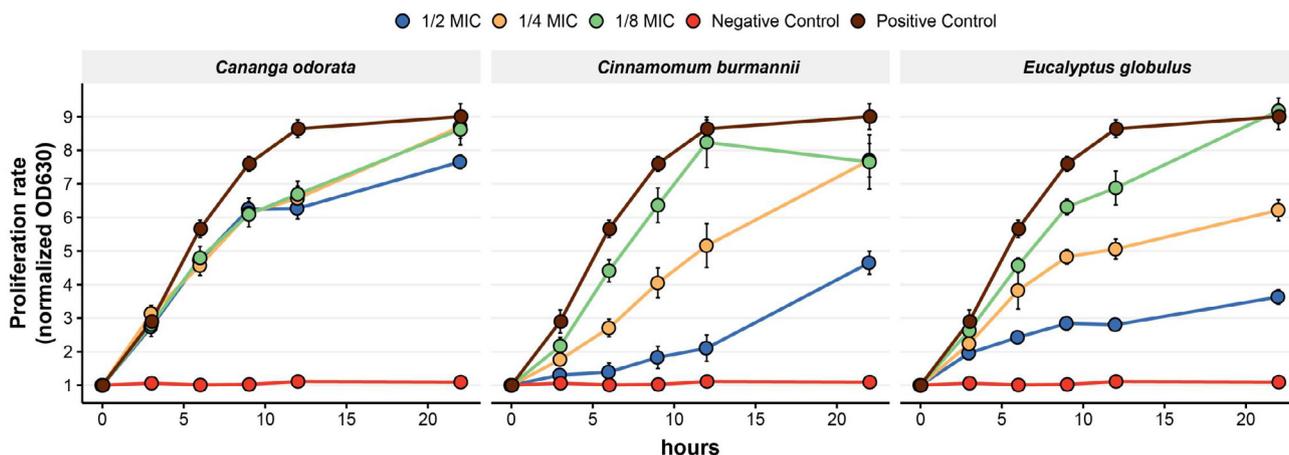


Figure 3. Growth curve Inhibition analysis at sub-MIC concentration of essential oils. The negative control consists of Mueller-Hinton Broth media and essential oil-free emulsion. The positive control contains essential oil-free emulsion and microbial test suspension (*Pseudomonas aeruginosa* ATCC9027)

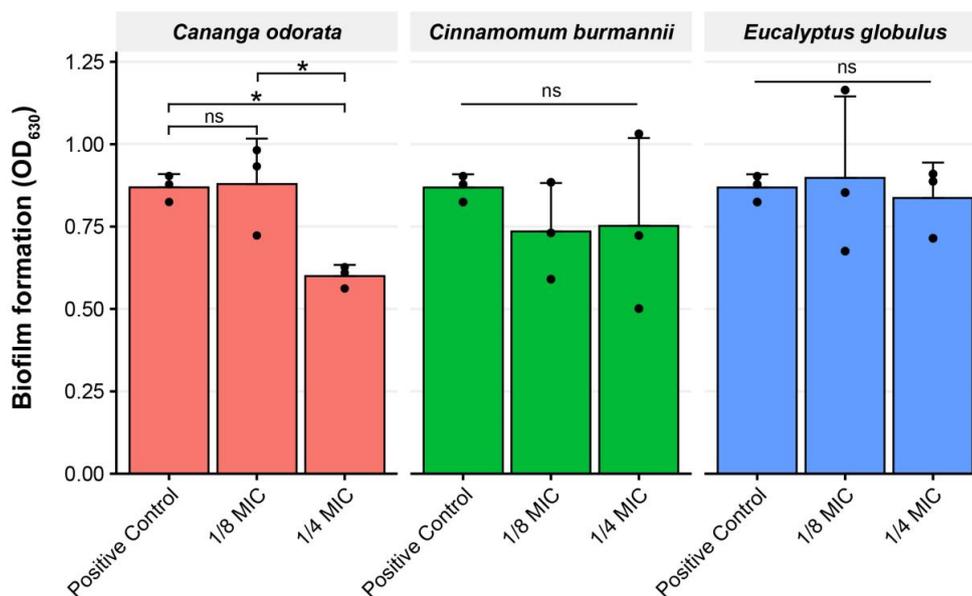


Figure 4. Assessment of essential oils' inhibitory effect on biofilm formation in *Pseudomonas aeruginosa* ATCC9027. The positive control contains essential oil-free emulsion and microbial test suspension (*Pseudomonas aeruginosa* ATCC9027)

*Cananga odorata* essential oil, specifically at a sub-MIC concentration of 1/4 MIC, effectively inhibits biofilm formation in PA ATCC9027 without inducing noteworthy toxicity to the bacteria.

### 3.6. Binding Affinity Analysis of Identified Compounds in Essential Oils to Quorum Sensing Enzyme Regulators via Targeted Docking

Molecular docking was conducted to explore the binding modes of compounds identified in essential oils with enzymes known to function as regulators

or participate in quorum sensing in *Pseudomonas aeruginosa*. These enzymes include LasR (PDB ID 2UV0), PqsR (PDB ID 4JVC), and QscR (PDB ID 6CC0). According to the available CoA data, compounds identified in *Cinnamomum burmannii* essential oils consist of Cinnamaldehyde (C<sub>9</sub>H<sub>8</sub>O), while those in *Eucalyptus globulus* essential oils include 1,8-Cineole. *Cananga odorata* essential oil contains four identified compounds based on CoA: trans-Caryophyllene, (E,E)-alpha-farnesene, Benzyl Benzoate, and Methyl p-cresol.

Remarkably, among the six compounds subjected to docking with the three proteins, the highest affinity was found in the compounds derived from *Cananga odorata* essential oil. The molecular docking scores and residual amino acid interactions of compounds derived from essential oils against *Pseudomonas aeruginosa* LasR (PDB ID 2UV0) are outlined in Table 3. Regarding the LasR protein, benzyl benzoate demonstrated a binding affinity of -9.7 kcal/mol, establishing hydrogen bonds with residues Tyr-56, Thr-75, and Thr-115 in the protein. Additionally, benzyl benzoate was capable of forming Residual Hydrophobic/Pi-Cation/Pi-Anion/Pi-Alkyl Interactions with residues, including Leu-36, Asp-73, Tyr-64, Trp-88, Ala-105, and Leu-110.

Table 4 displays the outcomes of molecular docking scores and residual amino acid interactions for compounds derived from essential oils against *Pseudomonas aeruginosa* QscR (PDB ID 6CC0). Notably,

benzyl benzoate also demonstrated the highest affinity for QscR, with a binding affinity of -10.3 kcal/mol, forming hydrogen bonds with residues Ser-38 and Tyr-66 in the QscR protein. Residual Hydrophobic/Pi-Cation/Pi-Anion/Pi-Alkyl Interactions were also observed with residues Phe-54, Asp-75, Ile-77, Val-78, Trp-90, Ala-105, Phe-101, and Ile-110. Regarding the PsqR protein, a compound component in *Cananga odorata* essential oil, trans-Caryophyllene, was also found to bind with the highest affinity compared to the other five compounds tested, with an affinity of -7.8 kcal/mol. It formed Residual Hydrophobic/Pi-Cation/Pi-Anion/Pi-Alkyl Interactions with residues Leu-208 and Ile-236 in the PsqR protein. Table 5 presents the molecular docking scores and residual amino acid interactions for compounds derived from essential oils against *Pseudomonas aeruginosa* PqsR (PDB ID 4JVC).

Table 3. Molecular docking scores and residual amino acid interactions of essential oils-derived compounds against *Pseudomonas aeruginosa* LasR (PDB ID 2UV0)

Ligands	Sources	Affinity (kcal/mol)	H-bond	Residual hydrophobic/Pi-Cation/Pi-Anion/Pi-Alkyl interaction
C <sub>9</sub> H <sub>8</sub> O (Cinnamaldehyde)	<i>Cinnamomum burmannii</i>	-7.4	-	Trp-88, Phe-101, Ala-105, Leu-110
C <sub>10</sub> H <sub>18</sub> O (1,8-Cineole)	<i>Eucalyptus globulus</i>	-6.8	-	Leu-40, Tyr-47, Ala-50, Val-76, Ala-127
C <sub>15</sub> H <sub>24</sub> (trans-Caryophyllene)	<i>Cananga odorata</i>	-7.5	-	Leu-336, Ala-50, Ile-52, Ala-127
C <sub>15</sub> H <sub>24</sub> ((E,E)-alpha-farnesene)	<i>Cananga odorata</i>	-8.3	-	Leu-36, Leu-40, Ala-50, Tyr-56, Tyr-64, Val-76, Trp-88, Phe-101
C <sub>14</sub> H <sub>12</sub> O <sub>2</sub> (Benzyl Benzoate)	<i>Cananga odorata</i>	-9.7	Tyr-56, Thr-75, Thr-115	Leu-36, Asp-73, Tyr-64, Trp-88, Ala-105, Leu-110
C <sub>8</sub> H <sub>10</sub> O (Methyl p-cresol)	<i>Cananga odorata</i>	-6.4	Thr75, Thr-115, Ser-129	Leu-36, Ile-52, Asp-73, Tyr-64, Trp-88, Ala-127

Table 4. Molecular docking scores and residual amino acid interactions of essential oils-derived compounds against *Pseudomonas aeruginosa* QscR (PDB ID 6CC0)

Ligands	Sources	Affinity (kcal/mol)	H-bond	Residual hydrophobic/Pi-Cation/Pi-Anion/Pi-Alkyl interaction
C <sub>9</sub> H <sub>8</sub> O (Cinnamaldehyde)	<i>Cinnamomum burmannii</i>	-7.1	-	Phe-54, Val-78
C <sub>10</sub> H <sub>18</sub> O (1,8-Cineole)	<i>Eucalyptus globulus</i>	-5.8	-	Phe-54, Tyr-52, Val-78
C <sub>15</sub> H <sub>24</sub> (trans-Caryophyllene)	<i>Cananga odorata</i>	-4.4	-	Phe-54, Val-78, Met-127
C <sub>15</sub> H <sub>24</sub> ((E,E)-alpha-farnesene)	<i>Cananga odorata</i>	-8.2	-	Phe-54, Tyr-58, Tyr-66, Val-78, Leu-82, Met-127
C <sub>14</sub> H <sub>12</sub> O <sub>2</sub> (Benzyl Benzoate)	<i>Cananga odorata</i>	-10.3	Ser-38, Tyr-66	Phe-54, Asp-75, Ile-77, Val-78, Trp-90, Ala-105, Phe-101, Ile-110
C <sub>8</sub> H <sub>10</sub> O (Methyl p-cresol)	<i>Cananga odorata</i>	-6.6	-	Tyr-52, Phe-54, Tyr-66, Val-78, Met-127

Table 5. Molecular docking scores and residual amino acid interactions of essential oils-derived compounds against *Pseudomonas aeruginosa* PqsR (PDB ID 4JVC)

Ligands	Sources	Affinity (kcal/mol)	H-bond	Residual hydrophobic/Pi-Cation/Pi-Anion/Pi-Alkyl interaction
C <sub>9</sub> H <sub>8</sub> O (Cinnamaldehyde)	<i>Cinnamomum burmannii</i>	-5.6	-	Leu-197, Leu-208, Ile-236
C <sub>10</sub> H <sub>18</sub> O (1,8-Cineole)	<i>Eucalyptus globulus</i>	-5.2	-	Val-211, Tyr-258
C <sub>15</sub> H <sub>24</sub> (trans-Caryophyllene)	<i>Cananga odorata</i>	-7.8	-	Leu-208, Ile-236
C <sub>15</sub> H <sub>24</sub> ((E,E)-alpha-farnesene)	<i>Cananga odorata</i>	-6.8	-	Ala-168, Leu-208, Ile-236, Ile-263
C <sub>14</sub> H <sub>12</sub> O <sub>2</sub> (Benzyl Benzoate)	<i>Cananga odorata</i>	-7.0	-	Pro-129, Ala-168, Leu-197, Leu-207, Leu-208, Phe-221, Ile-236
C <sub>8</sub> H <sub>10</sub> O (Methyl p-cresol)	<i>Cananga odorata</i>	-4.9	Ser-196, Leu-208, Arg-209	Leu-197, Phe-221, Ile-236

#### 4. Discussion

The development of natural compound agents as quorum-sensing inhibitors is essential to prevent the proliferation of bacterial strains resistant to antibiotics. These agents are anticipated to effectively impede quorum sensing signaling, thereby preventing the formation of biofilm phenotypes within infecting bacterial populations. This inhibition allows antibiotics to access and target infecting bacteria more effectively. This study identifies natural compounds from 40 essential oils to assess their activity as quorum-sensing inhibitors against PA ATCC9027. The comprehensive profiling of these essential oil varieties uncovered distinct compound distributions, offering valuable insights into their quality and composition. The agar well diffusion assay revealed distinct antibacterial activities among the tested essential oils. Notably, 25 out of the 40 essential oils exhibited significant inhibition zones against PA ATCC9027, indicating substantial antibacterial efficacy. Prominent oils include *Cinnamomum burmannii* (Cinnamon Bark Oil), *Cananga odorata* (Ylang-ylang Oil), *Eucalyptus globulus* (Eucalyptus Oil), and others. Due to the pronounced antibacterial activity exhibited by these three essential oils, the discussion is focused on these particular oils.

The results of this study are in line with a previous report indicating that essential oil from cinnamon (*Cinnamomum zeylanicum*) bark has bacteriostatic and bactericidal effects against *P. aeruginosa* PAO1 at 0.125% (v/v) (Elcocks *et al.* 2020). It's important to note that this report used a different *Cinnamomum* species and a different bacterial strain than those used in this study, so it's crucial to recognize that there is no prior report on the antibacterial activity of *C. burmannii* bark essential oil against PA ATCC9027. Despite the difference in cinnamon species and the tested bacteria, the compound contents within the same organism

family are generally similar. Therefore, their activity against bacteria from the same organism family is expected to be relatively consistent. The results obtained in this study reveal that the bacteriostatic and bactericidal activities of *C. burmannii* bark essential oil are identical, both at 0.09765% (v/v). The antibacterial activity of cinnamon essential oil is attributed to its component, cinnamaldehyde. Cinnamaldehyde has been reported to exhibit antibacterial and anti-quorum sensing activities against *Pseudomonas aeruginosa* PAO1 (Kavanaugh and Ribbeck 2012; Subhaswaraj *et al.* 2018).

Furthermore, the study revealed that *E.s globulus* essential oil derived from its twigs and leaves exhibited antibacterial activity against PA ATCC9027, with MIC and MBC values of 1.5625% (v/v) and >1.5625% (v/v), respectively. This finding aligns with previous reports indicating that *E. globulus* essential oil, along with its major component 1,8-cineole, possesses antibacterial activity against *P. aeruginosa* (Damjanović-Vratnica *et al.* 2011; Sagar *et al.* 2022). Additionally, 1,8-cineole has been reported to affect bacterial biofilm formation and pathogenicity by suppressing the expression of the luxS gene in *E. coli* O101 (Wang *et al.* 2022). Against *P. aeruginosa* PAO1, 1,8-cineole from *Musa paradisiaca* has also exhibited inhibitory activity against quorum sensing and virulence factor production (Karupppiah *et al.* 2021).

Biofilm formation is critical to PA pathogenicity, contributing to increased antibiotic resistance. The assessment of essential oils' impact on biofilm formation revealed that *C. odorata* essential oil is a significant inhibitor, particularly at a 1/4 MIC concentration. This suggests the potential of *C. odorata* essential oil to disrupt biofilm development without inducing noteworthy toxicity to the bacteria. Ylang-ylang oil inhibited biofilm formation by *E. coli* ATCC 25922, *S. aureus* ATCC 6538, *S. epidermidis* clinical

isolated strain, and *Candida albicans* ATCC10231 (Tadtong et al. 2012; Lee et al. 2014). Research on *C. odorata* essential oil, especially against PA ATCC9027, has not been extensively reported. In this study, molecular docking analysis focused on identifying the binding affinity of essential oil-derived compounds to quorum sensing enzymes (LasR, QscR, and PqsR). Among the compounds, benzyl benzoate from *C. odorata* essential oil demonstrated the highest binding affinity to these enzymes. In line with previous studies, benzyl benzoate has been identified as a potential quorum sensing inhibitor, exhibiting a 37.7% suppression of biofilm formation at a concentration of 100 µg/ml in *P. aeruginosa* PAO1 (Chen et al. 2019). However, there is no report regarding the mechanism by which benzyl benzoate exhibits anti-biofilm activities. This *in-silico* discovery confirms the biofilm formation inhibition assay results, indicating that among the three major antibacterial candidates with the potential as anti-quorum sensing inhibitors that inhibit these enzymes, *Cananga odorata* essential oil is the most promising. From these results, it can also be inferred that a potential mechanism through which essential oils may interfere with quorum sensing pathways in PA is by interacting with these enzymes.

Essential oils, particularly from *Cinnamomum burmannii*, *Cananga odorata*, and *Eucalyptus globulus*, hold promise as antibacterial agents, with *Cananga odorata* essential oil, in particular, emerging as a quorum sensing inhibitor. Future research should concentrate on isolating and identifying specific bioactive compounds responsible for these effects. This discovery holds the potential for developing strategies to combat biofilm-related infections. It contributes to our understanding of the antimicrobial properties of essential oils and also underscores their potential as alternative or adjunctive therapies in combating PA infections. In conclusion, this study highlights 25 essential oils with the potential to be developed as antibacterial agents, with *Cananga odorata* (Ylang-ylang Oil) demonstrating significant activity in inhibiting biofilm formation in PA. This finding has significant value for advancing antimicrobial and anti-quorum sensing strategies derived from essential oils.

### Conflict of Interest

The authors declare no conflicts of interest.

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