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Original Article

Aromatherapeutic essential oils and their pharmaceutical combinations: Tools for inhibition of quorum sensing activity and biofilm formation of human pathogens*

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ABSTRACT

Background and Aims: Aromatherapy, as one of the complementary therapies, uses essential oils as the main therapeutic agents to treat several diseases. In the present study, it was aimed to investigate inhibition of quorum sensing (QS) and biofilm formation of aromatherapeutic essential oils (AEOs) and their pharmaceutical combinations (PC-I and PC-II).
Methods: The anti-QS potential of AEOs were determined using the biosensor strains *Chromobacterium violaceum* ATCC 12472 and *Pseudomonas aeruginosa* PAO1. Anti-QS activity was detected by agar-well diffusion and violacein pigment inhibition assays. Blocking of PAO1 swim and swarm motilities and biofilm formation was also performed.
Results: Most of the AEOs demonstrated highly active (>95%) violacein pigment inhibition. Additionally, they inhibited swarming (40.34%-72.80%) and swimming (20.06%-50.08%) motilities of PAO1. Moreover, the majority of AEOs also decreased the biofilm formation, particularly on *P. aeruginosa* and *S. aureus*.
Conclusion: Consequently, aromatherapeutic formulations might be a complementary or prophylactic cure for infectious disease by their anti-QS and antibiofilm activities rather than just antimicrobial effects.

Keywords: Aromatherapeutic essential oils, Antibiofilm, quorum sensing, *Pseudomonas aeruginosa* PAO1, Synergistic effect

INTRODUCTION

Increasingly becoming fundamental in the global health system due to better patient tolerance, renewability and better biodegradability, aromatherapy is one of the complementary therapies, which uses essential oils to cure or support the cure of numerous physical or mental problems including bacterial or viral born infectious diseases, respiratory, digestive and urinary problems, headache, depression, insomnia, muscular pain, as well as skin ailments such as acne and dermatitis (Ali et al., 2015; Yan, Wang, Cruz Flores, & Su, 2019). The therapeutic effects of essential oils have been attributed to their chemical components mainly terpenoids as well as nonterpenoid compounds such as phenols, esters and oxides. Essential oils can be mostly obtained from dried

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flowers, leaves, fruits, roots, barks or whole aerial parts of plants by using several techniques including water or steam distillation, solvent extraction, supercritical fluid and subcritical water extractions. In addition to the advantages of their use, with their well-known antibacterial and antiviral properties, they are claimed to be very good complementary products and alternatives to anti-pathogenic drugs and antibiotics (Boire, Riedel, & Parrish, 2013; Yap, Yiap, Ping, & Lim, 2014).

Over the past decade, the uncontrollable spread of bacteria that are simultaneously resistant to various drugs have increased in the community because of the inappropriate use of antibiotics. With antibiotic resistance, these bacteria could cause wider infection control problems such as treatment failure, increased fatality rate, and dispersion of resistant bacteria from hospital to community. Biofilm formation, in which bacteria stick to each other and to a surface, also creates a serious problem in medical facilities, inducing resistant hospital infection. Moreover, biofilm formation represents the main indicator of bacterial infection, especially those caused by devices such as catheters, prosthetic valves, orthopedic devices, etc. Therefore, the eradication of the biofilm matrix from these kinds of surfaces becomes extremely difficult (Li et al., 2018; Vipin, Mujeeburahiman, Ashwini, Arun, & Rekha, 2019; Yap et al., 2014; Zhang et al., 2019; Zhang et al., 2018).

Biofilm formation and bacterial virulence are correlated with quorum sensing (QS), a process of bacterial cell to cell communication in which cells regulate the transcription of the specific genes responsible for the production of antibiotics, biofilm differentiation, cell division, bioluminescence and the other virulence features (Ahmad, Viljoen, & Chenia, 2015; Brun, Bernabè, Filippini, & Piovan, 2019; Bali, Erkan Türkmen, Erdönmez, & Sağlam, 2019). The QS system permits bacteria to assess their population density via the production and sensing of QS signaling molecules called N-acyl homoserine lactones (AHLs) and oligopeptides in Gram-negative and Gram-positive bacteria, respectively (Y. Zhang et al., 2018). Blocking QS signaling molecules or the bacterial QS system is considered as a significant alternative strategy for controlling persistent infections due to bacterial resistance and a promotive target to discover the anti-infective properties of natural products (Vasavi, Arun, & Rekha, 2014; Ahmad et al., 2015; Doğan, Gökalsın, Şenkardeş, Doğan, & Sesal, 2019;). Therefore, the objective of our research was to evaluate the anti-QS activity of aromatherapeutic essential oils (AEOs) and their combinations via *Chromobacterium violaceum* ATCC 12472 and *Pseudomonas aeruginosa* PA01 as biosensor strains and their antibiofilm effects against Gram-negative and Gram-positive human pathogens.

MATERIAL AND METHODS

Preparation of aromatherapeutic essential oils (AEOs) and their pharmaceutical combinations

Eleven aromatherapeutic essential oils (AEOs) of *Cedrus atlantica* (Endl.) G.Manetti ex Carrière (cedrus), *Citrus aurantium* L. *var. bergamia* (bergamot), *Citrus limon* L. (lemon), *Citrus sinensis* L. (orange), *Eugenia caryophyllus* (Spreng.) Bullock & S.G.Harrison (clove), *Eucalyptus globulus* Labill. (eucalyptus), *Lavandula angustifolia* Mill. (lavender), *Melaleuca alternifolia* (Maiden & Betche) Cheel. (tea tree), *Mentha piperita* L. (mint), *Rosmarinus officinalis* L. (rosemary), *Thymus vulgaris* L. (thyme) and their pharmaceutical combinations were applied on QS biosensor strains and human pathogens to detect their anti-QS and antibiofilm activities. AEOs were purchased from Florame (St. Remy de Provence, France). The combinations of these AEOs were coded as pharmaceutical composition-I (PC-I) and II (PC-II). PC-I is the combination of thyme:bergamot:lemon:tea tree:lavender:mint essential oils (1:2:4:5:5:5) whereas, PC-II is the combination of thyme:tea tree essential oils (1:1). The stock solution of AEOs was prepared in dimethyl sulfoxide (DMSO) and diluted as 0.05% DMSO when used in the experiments.

Bacteria and culture conditions

The bacterial strains were obtained from the American Type Culture Collection (ATCC, USA). Gram-positive human pathogen bacteria: *Bacillus cereus* American Type Culture Collection (ATCC 6633), *Staphylococcus aureus* (ATCC 29213), *Staphylococcus epidermidis Wild-type*, *Enterococcus faecalis* (ATCC 29212), *Micrococcus luteus* (ATCC 7468), and Gram-negative human pathogen bacteria: *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumoniae* (ATCC 700603), *Klebsiella oxytoca* (ATCC 43165), *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (ATCC 14074), *Serratia marcescens* (ATCC 27117), *Acinetobacter baumannii* (ATCC 19606) and *Proteus mirabilis* (ATCC 7002) were used to detect the antibiofilm activity of AEOs. The wild-type strain *Chromobacterium violaceum* (ATCC 12472) used as a biosensor strain for anti-QS activity was a kind gift by Prof. Dr. Robert Mclean from the Department of Biology, Texas State University-San Marcos, USA. *P. aeruginosa* PAO1 was also kindly gifted by Daniel Lopez, PhD from the National Centre for Biotechnology (CNB), Autonomous University of Madrid, Spain. The bacterial cultures were grown in Brain Heart Infusion (BHI) broth medium (Merck, Germany) at 37°C for 24h. *C. violaceum* 12472 and PAO1 were cultivated in Luria–Bertani broth (LB) medium (Sigma-Aldrich, USA, pH=7.0) at 30°C and 37°C for 24 h, respectively. All strains were subcultured until the optical density (OD) of 0.4 at 600 nm was reached.

Minimum inhibitory concentrations (MICs) of AEOs

Minimum inhibitory concentrations (MICs) of the AEOs against human pathogens and biosensor strains were performed using the broth microdilution method (Zgoda & Porter, 2001). Briefly, 100µL of essential oils were diluted in sterile 96-well microplates in 95 µL of Brain Heart Infusion (BHI) broth and 5 µL of the tested bacteria (10^8 CFU/mL) were added to each well. The final volume in the wells was 200 µL and the microplates were incubated at 30°C or 37°C for 24 hours. The 96-well microtiter plates were then measured in a microplate reader at 600 nm according to the control to determine the growth inhibition of the essential oil on the microorganisms. Gentamicin (10µg/ml) (Sigma, Saint Louis, USA) was used as the positive antibiotic control.

Anti-quorum sensing (Anti-QS) assays qualitative detection: Agar well diffusion method

Anti-QS activity of AEOs, at the sub-MIC of 0.4% v/v was performed with the biosensor strain ***C. violaceum*** ATCC 12472 (Zahin et al., 2010). Briefly, Luria–Bertani (LB) agar plates were

inoculated with 0.1 ml of overnight bacterial cultures and wells of 6 mm diameter were opened at the bottom of soft agar. Each oil was added into the wells and the petri dishes were left for incubation at 30 °C for 48 hours. QS inhibitions of each oil were detected as an opaque zone with loss of purple pigmentation around each well. The measurements were made from the outer edge of the disks to the edge of the opaque zones suggesting anti-QS inhibition.

Quantitative detection: Violacein pigment inhibition assay

Inhibitory effects of violacein pigment production by AEOs at the sub-MIC of 0.4% v/v were also measured spectrophotometrically (Blosser & Gray, 2000). Briefly, each oil was added to 200 µL of bacterial culture and incubated at 30°C until complete pigmentation was achieved in the blank, i.e., the untreated culture. First, 200 µL of treated (test) and untreated cultures (control) were placed in a microcentrifuge tube and lysed by addition of 200 µL of 10 % SDS, vortexed for 5 sec. and incubated at room temperature for 5 min. Subsequently, 900 µL of water-saturated butanol (50 mL n-butanol mixed with 10 mL distilled water) was added to the cell lysate, followed by vortexing for 5 sec. and centrifugation at 13,000×g for 5 min. The upper (butanol) phase containing the violacein was collected and the absorbance was read at 585 nm in UV-Vis spectrophotometer. The percentage of violacein inhibition was calculated using the following formula:

Violacein inhibition (%) = (A_{585nm}(Control)–A_{585nm}(Test))/(A_{585nm}(Control)) x100

Motility assays of *Pseudomonas aeruginosa* PAO1 strain

The swimming and swarming motility assays were performed using a previously described, slightly modified method (Packiavathy, Agilandeswari, Musthafa, Pandian, & Ravi, 2012). In the swimming assay, 5 µL of overnight culture of *P. aeruginosa* PAO1 (A600 nm=0.4) was point inoculated at the center of an agar medium consisting of 1% tryptone, 0.5% NaCl and 0.3% agar with 0.0125% v/v sub-MIC concentration of the materials. For swarming assays, the agar medium comprised 1% peptone, 0.5% NaCl, 0.5% agar and 0.5% filter-sterilized D-glucose with the same sub-MIC concentration. The plates were then incubated at 37°C in an upright position for 16 h. The reduction in swimming and swarming migration was recorded by measuring the swimming and swarming zones of the bacterial cells after 16 h compared to the negative controls.

Antibiofilm activity assay

Antibiofilm effects of AEOs at the sub-MIC of 0.0125% v/v were performed in 96-well U-bottom polystyrene microtiter plates according to the slightly modified method (O'Toole & Kolter, 1998). An overnight culture of *C. violaceum* ATCC 12472 was diluted 1:100 with LB broth and grown for another hour. After the addition of each oil and the combinations, 100 µL of the culture was pipetted into the wells of the microtiter plates and the plates were incubated for 24 h at 30°C. Then, the medium was removed and washed with 1×PBS buffer in triplicate. The plates were dried at 65°C in a universal oven and then 100 µL of a 1% m/V crystal violet aqueous solution was added. The stain was allowed to fix at room temperature for 20 min, after which the dye was removed from the wells by washing thoroughly with sterile water. For the quantification of the attached biomass, the bound dye was dissolved with 30% acetic acid solution, and the absorbance was determined at 595 nm. Inhibitor-mediated reduction of biofilm formation was assessed by comparing it to the control without the oils, and the standard antibiotic amoxicillin (2µg/ml) was also used as a positive control. Amoxicillin and gentamicin (10µg/ml) were used as positive controls since they are widely used in infectious diseases and their effectiveness is known. The percentage inhibition of biofilm was calculated as:

Biofilm inhibition (%) = (Control OD595nm-Test OD595 nm/ Control OD595 nm)x100

Statistical analysis

The results of all the experiments were performed in triplicate and repeated at least twice. All values are expressed as means ± standard deviations (SD). Statistical analyses were performed using the statistical program SPSS version 20.0 (Statistical Package for the Social Sciences). Differences among means were performed by analysis of variance (ANOVA) and averages were compared using Bonferroni test. Differences at *$p<0.05$, ** $p<0.01$ and ***$p<0.001$ were considered to indicate statistical significance.

RESULTS AND DISCUSSION

Minimal inhibitory concentrations of AEOs and their pharmaceutical combinations

Minimal inhibitory concentration (MIC) values of AEOs and their pharmaceutical combinations (PC-I and PC-II) were detected to select the sub-MIC concentrations to study their effects on QS and biofilm inhibition. The MIC values of AEOs against biosensor strains and human pathogens were found to be in the ranges of 0.025%- 1.6% (v/v) and 0.2%- 1.6% (v/v), respectively (Table 1). The majority of AEOs was detected to inhibit bacterial growth (Table 1). Eucalyptus (its main components: 1,8-cineole and α-pinene), clove (eugenol, eugenyl acetate, and β-caryophyllene) AEOs and the combinations (PC-I and PC-II) showed higher antimicrobial effect than gentamycin as a positive control on *S. typhimurium*, indicating the lower MIC values (<1.2% v/v). Eucalyptus, lavender (its main components: linalyl acetate, linalool and cis-ß-ocimene), mint (menthol and menthone), and clove AEOs also displayed higher growth inhibition than gentamycin on *S. marcescens*, indicating lower MIC values (<0.3% v/v) (Table 1). Orange (its main components: limonene and myrcene) and lemon (limonene, ß-pinene and γ-terpinene) AEOs exhibited the best growth inhibition with the MIC value of 0.2% (v/v) on *S. aureus*. Furthermore, rosemary AEO, including the main components of 1,8-cineole, β-pinene and α-pinene, displayed the best antimicrobial effect on *S. epidermidis* and *A. baumannii* (Table 1). Except lavender and rosemary AEOs, *P. aeruginosa* ATCC 27853 demonstrated as the most resistant strain against all AEOs; however, *P. aeruginosa* PAO1 as the QS biosensor strain was found to be the most susceptible to all AEOs. The MIC results revealed that AEOs exhibited more effective inhibition against Gram positive bacterial growth than Gram negatives (Table 1). The antibacterial properties of essential oils have mostly been reported as being more effective against Gram positive

than Gram negative bacteria (Bharti et al., 2020; Pellegrini et al., 2014). Due to the existence of hydrophobic lipopolysaccharide in the outer membrane structure of Gram-negative bacteria, their external membrane could be impermeable to AEOs (Zgurskaya, López, & Gnanakaran, 2015). Therefore, our results are in agreement with these reports (Pellegrini et al., 2014; Zgurskaya et al., 2015; Bharti et al., 2020).

where thyme (40.33±1.15 mm) and tea tree (32.33±2.52 mm) AEOs presented the highest activity with larger zones (Figure 1). Our qualitative QS results were more encouraging than the study by Mokhetho et al., in which they observed an anti-QS activity with the highest diameter zones of 5.50±1.10 mm (Mokhetho, Sandasi, Ahmad, Kamatou, & Viljoen, 2018).

Table 1. MIC values of aromatherapeutic essential oils on pathogen bacteria and biosensor strains.

	Biosensor Strains		Gram negative human pathogens							Gram positive human pathogens				
Aromatherapeutic Essential Oils % (v/v)/ Biosensor Strains/ Human Pathogen Bacteria	***C. violaceum* ATCC 12472**	***P. aeruginosa* PAO1**	***A. baumannii* ATCC 19606**	***E. coli* ATCC 25922**	***K. oxytoca* ATCC 43165**	***K. pneumoniae* ATCC 700603**	***P. aeruginosa* ATCC 27853**	***P. mirabilis* ATCC 7002**	***S. thyphimurium* ATCC 14074**	***S.marcescens* ATCC 27117**	***B. cereus* ATCC 6633**	***S. epidermidis* wt.**	***S. aureus* ATCC 29213**	***E. faecalis* ATCC 29212**	***M. luteus* ATCC 7468**
PC-I	1,6	0.025	0,8	0,8	0,8	1,6	-	1,6	02	0,8	04	0,8	04	0,8	04
PC-II	1,6	0.025	04	04	0,8	0,8	-	0,8	0,8	0,8	0,8	04	04	04	04
Eucalyptus	1,6	01	1,6	0,8	1,6	0,8	-	1,6	08	02	0,8	0,8	0,8	1,6	0,8
Bergamot	0,8	0.025	-	0,8	-	0,8	-	-	-	-	0,8	0,8	0,8	1,6	0,8
Cedrus	-	0.025	-	1,6	-	-	-	-	-	-	1,6	-	-	-	-
Lavender	1,6	01	-	0,8	-	04	1,6	-	-	02	04	0,8	04	04	04
Orange	1,6	0.025	-	0,8	-	0,8	-	-	-	-	04	0,8	02	-	0,8
Mint	1,6	005	-	1,6	1,6	0,8	-	1,6	-	02	0,8	0,8	04	-	0,8
Tea Tree	0,8	005	0,8	04	1,6	0,8	-	08	1,6	04	0,8	0,8	04	08	04
Thyme	-	005	04	04	0,8	0,8	-	1,6	1,6	04	04	0,8	04	08	04
Lemon	-	0.025	-	04	04	1,6	-	-	-	-	1,6	04	02	0,8	0,8
Clove	0,8	005	1,6	0,8	1,6	0,8	-	1,6	0,8	02	0,8	1,6	0,8	1,6	0,8
Rosemary	0,8	01	02	04	1,6	04	16	-	-	-	08	02	0,8	0,8	0,8
Gentamycin	N.D	N.D	01	004	016	016	12	008	12	03	03	01	0.025	008	0.025

N.D: Not detected.

Inhibition of quorum sensing (QS) formation in *C. violaceum* 12472

In this study, QS inhibition of *C. violaceum* 12472, a biosensor strain, by AEOs was assessed qualitatively using the agar-well diffusion method shown in Figure 1. and Table 2. The synthesis of purple pigment violacein by the strain was comprised by QS. The indicators of QS inhibition were loss of its purple pigmentation and the formation of opaque halos around the wells including the AEOs. All AEOs, except cedrus AEO, exhibited a colorless, opaque zone of different diameters, which inferred that they displayed detectable anti-QS effects at the sub-MIC concentration of 0.4%v/v (Figure 1). The QS inhibitory diameters of AEOs on violacein production varied from 10.00±1.00 mm to 40.33±1.15mm (Table 2). In this study, the qualitative QS results of AEOs were found on all tested AEOs, except cedrus AEO, which displayed significant QS inhibition zones on *C. violaceum* 12472 in the agar well diffusion assay

Quantitative QS inhibition of *C. violaceum* 12472 was also measured spectrophotometrically using AEOs at the sub-MIC concentration of 0.4% v/v. Excluding cedrus AEO, all the AEOs showed significant ($p<0.01$ and $p<0.001$) inhibitory effect on violacein production without inhibition of bacterial growth (Figure 2). Anti-QS effects of the AEOs ranged from 33.01±4.70% to 99.96±0.02%. The inhibition of violacein by orange (33.01%±4.70) and lemon (34.10%±2.20) AEOs was significant ($p<0.01$) but less than the others. Eucalyptus AEO exhibited a remarkable anti-QS effect with the value of 87.96%±1.13 ($p<0.01$), which was much better than the anti-QS potentials of orange and lemon AEOs (Figure 2). The AEOs with high QS inhibitory effects were PC-I (98.10%±0.77), PC-II (98.34%±0.42), bergamot (98.37%±0.60), thyme (98.28%±0.47), lavender (99.20%±0.47), mint (96.77%±0.46), tea tree (99.38%±0.17), clove (99.86%±0.03) and rosemary (99.96%±0.02) AEOs (Figure 2).

Table 2. Qualitative QS inhibition of the aromatherapeutic essential oils (AEOs) by agar well diffusion method.

Aromatherapeutic essential oils (0.4 v/v)	Violacein Inhibition Zones (mm)
PC-I	16.67±1.15[a]
PC-II	22.00±2.00[b]
Eucalyptus	15.33±1.15[a]
Bergamot	20.50±1.50[a,b]
Cedrus	-
Lavender	22.50±1.00[a,b]
Orange	10.00±1.00[c]
Mint	21.33±1.53[a,b]
Tea Tree	32.33±2.52[d]
Thyme	40.33±1.15[e]
Lemon	10.67±1.15[c]
Clove	23.00±2.65[b]
Rosemary	20.00±2.00[a,b]

The values of violacein inhibition zone (mm) represent averages ± standard deviations (SD) for triplicate experiments. Values in the same column with different superscripts are significantly different ($p<0.05$).

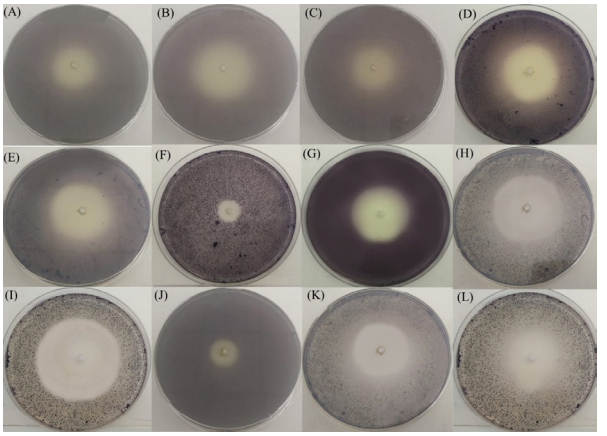

Figure 1. Violacein inhibition of *Chromobacterium violaceum* ATCC 12472 by AEOs. The opaque zones with loss of purple pigmentation around the wells show violacein inhibition (A)PC-I, (B) PC-II, (C) Eucalyptus EO, (D) Bergamot EO, (E) Lavender EO, (F) Orange EO, (G) Mint EO, (H) Tea tree EO, (I) Thyme EO, (J) Lemon EO, (K) Clove EO, (L) Rosemary EO.

In quantitative results, the combinations and individual EOs bergamot, lavender, mint, tea tree, thyme, clove and rosemary were found to have highly active QS inhibition (>95%), but showed no statistical difference between each other and the AEO combinations ($p>0.05$). These findings demonstrated that since QS inhibition of AEO combinations was not stronger than individual AEOs, the QS inhibition potentials of AEOs are mostly associated with their major constituents such as menthol, thymol, carvacrol, eugenol, geraniol and geranial. Therefore, our results are in agreement with previous reports (Cáceres, Hidalgo, Stashenko,

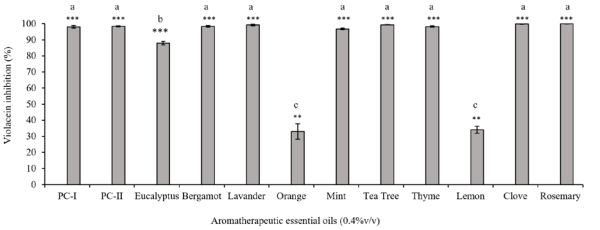

Figure 2. Quantitative analysis of violacein pigment inhibition of *Chromobacterium violaceum* CV12472 treated with the aromatherapeutic essential oils (AEOs) and combinations (PC-I and PC-II). Bars represent the mean of three independent experiments±SD (ANOVA). **Statistically different from the control (**$p<0.01$, ***$p<0.001$). Values with different superscripts are significantly different from each AEO ($p<0.05$).

Torres, & Ortiz, 2020; Husain et al., 2015; Mokhetho et al., 2018; Raut & Karuppayil, 2014). Moreover, anti-QS effect of eucalyptus AEO (87.96%±1.13) was in line with an earlier report (Luís, Duarte, Gominho, Domingues, & Duarte, 2016) while the violacein inhibitions of orange (33.01%±4.70) and lemon (34.10%±2.20) AEOs were more effective than previous reports (Kerekes et al., 2013; Mukherji & Prabhune, 2014). Although there are some reports about AEOs with anti-QS activity (Husain et al., 2015; Mokhetho et al., 2018; Alibi et al., 2020; Cáceres et al., 2020), our results also reveal that AEOs with high anti-QS effects (96.77%-99.96%) could be used as promising anti-QS compounds.

Inhibition effect of AEOs on swarming and swimming motility and biofilm formation of *P. aeruginosa* PAO1

P. aeruginosa PAO1 is a swarming opportunistic Gram-negative pathogen that mostly causes nosocomial infections by forming permanent biofilms. To create an efficient infection, it synthesizes many virulence factors like biofilm formation, swarming and swimming motility via the quorum sensing (QS) process (Ilic-Tomić et al., 2017; Önem, Tüzün, & Akkoç, 2021). In the present study, the inhibition effect of AEOs and their pharmaceutical combinations (PC-I and PC-II) on motility ability and biofilm formation of *P. aeruginosa* PAO1 was tested at the sub-MIC concentration (0.0125% v/v). The motility inhibition results showed that all AEOs significantly ($p<0.05$) blocked the swarming and swimming motility of PAO1 in the inhibition rate value of 40.34%-72.80% and 20.06%-50.08%, respectively. The swimming and swarming inhibition effects of each essential oil were mostly higher than the combinations, PC-I and PC-II (Table 3). Lavender and thyme AEOs presented the best inhibition on the motility ability of PAO1 with the lowest swarming (15.84±1.20 mm) and swimming zones (37.23±1.24 mm), respectively. In the swarming inhibition activity, after lavender AEO, eucalyptus and orange AEOs also exhibited high effects with the inhibition values of 68.68% and 70.05%, respectively (Table 3). Previous studies showed that *E. globulus* essential oil (EO) at 100 µg/ml, *R. officinalis* EO at 0.02% (v/v) and *M. piperita* EO at 3% (v/v) reduced the swarming motility of PAO1 in the inhibition values of 25%, 61.53% and 81.3%, respectively (Bai A & Vittal, 2014; Husain et al., 2015; Merghni et al., 2018). Compared to these studies, in our results, each EO and their combinations at low concentration (0.0125% v/v) presented remarkable anti-swarming activity (Table 3). Furthermore, a review study showed that *Thymus vulgaris*, *Lavandula angustifolia*, and

Table 3. Inhibition effect of AEOs (0.0125% v/v) on swimming and swarming motilities of *Pseudomonas aeruginosa* PAO1.

Aromatherapeutic Essential Oils	Swarming Zone Diameter (mm)	Swarming inhibition (%)	Cell Viability (Log CFU/ml)	Swimming Zone Diameter (mm)	Swimming inhibition (%)	Cell Viability (Log CFU/ml)
PC-I	33.15±0.23	43.09	6.28	55.18±1.17	24.67	7.02
PC-II	28.48±1.30	51.10	6.32	53.24±1.26	27.32	6.98
Eucalyptus	18.17±1.65	68.80	6.42	60.02±1.30	20.06	6.97
Bergamot	26.33±0.45	54.79	6.27	43.18±1.06	25.21	6.86
Cedrus	27.87±1.35	52.15	6.23	50.07±1.20	28.81	7.12
Lavender	15.84±1.20	72.80	6.28	45.19±1.00	38.31	6.93
Orange	17.56±0.50	70.05	6.24	51.27±0.69	30.01	6.91
Mint	30.06±1.25	50.09	6.51	50.02±0.55	32.88	6.88
Tea Tree	34.75±0.50	40.34	6.53	54.71±0.75	25.32	6.97
Thyme	27.38±1.23	53.00	6.17	37.23±1.24	50.08	6..89
Lemon	20.34±1.38	66.79	6.78	51.46±0.25	30.05	7.10
Clove	23.18±1.38	60.20	6.18	46.81±1.02	36.10	7.16
Rosemary	32.21±1.84	55.29	6.37	46.28±0.84	36.82	7.24
PAO1	60.12±1.24	0	6.72	59.12±1.10	0	7.10

Values in the same column with different superscripts are significantly different ($p<0.05$).

clove EOs inhibited the swarming and swimming motility of PAO1 or different strains (D. Zhang et al., 2020) (34). Our results are in agreement with these results, indicating significant reduction of the motilities (Bai A & Vittal, 2014; Husain et al., 2015; Merghni et al., 2018; Zhang et al., 2020).

In the results of PAO1 antibiofilm activity, all AEOs (0.0125% v/v) showed a statistically significant ($p<0.05$) inhibition in the range of 38.11% to 77.36%. Eucalyptus, orange and lemon AEOs also showed the best activity on the biofilm formation of PAO1 (Table 4). PC-I and PC-II, lavender, thyme and clove AEOs also exhibited noteworthy antibiofilm effects on PAO1. Husain et. al (2015) showed that *M. piperita* EO at 0.375% (v/v) inhibited biofilm formation by 42.8% (23). Our data is considered to be more promising than their findings (Table 4). Additionally, spice and clove EOs were also reported to have a significant antibiofilm effect on PAO1 strain (Eris & Ulusoy, 2013; D. Zhang et al., 2020). These findings are in line with our results.

Antibiofilm effects of AEOs and their pharmaceutical combinations on human pathogens

In the antibiofilm results of Gram-negative bacteria, PC-I exhibited the most powerful antibiofilm effect on *E. coli, P. aeruginosa, A. baumannii, K. oxytoca* and *P. mirabilis*, which were found to be as effective as amoxicillin (Amx). No statistically significant difference was observed among the means of antibiotic and bacteria ($p>0.05$). The antibiofilm effect of thyme AEO was also as powerful as Amp for E. coli ($p>0.05$) (Table 4). Antibiofilm activity of lemon, clove and rosemary AEOs also exhibited a remarkable inhibition on *P. aeruginosa* ($p<0.01$) (Table 4). As for the Gram-negative bacteria, PC-I presented the best biofilm inhibition on *S. aureus* and *M. luteus*. Compared to Amp, PC-I showed significantly higher ($p<0.001$) antibiofilm effect on *S. aureus*, and as powerful as Amp on *M. luteus* ($p>0.05$). Moreover, PC-I significantly ($p < 0.05$) decreased the biofilm formation of *S epidermidis wt* and E. faecalis ATCC 29212. While PC-I exhibited high biofilm inhibition (max. 80.09%) on the majority of the Gram-positive bacteria, PC-II inhibited biofilm formation up to 50.70% (Table 5). One of the major and most commonly used AEO, thyme, also exhibited a significant ($p < 0.05$) antibiofilm effect on *S. epidermidis wt.* Actually, its biofilm inhibitions on *S. epidermidis wt.* and *S. aureus* were as powerful as Amp ($p>0.05$) (Table 5). Furthermore, the antibiofilm effect of eucalyptus, bergamot, cedrus, lavender and orange AEOs were also found to be as powerful as Amp ($p>0.05$) on *S. aureus* (Table 5).

In the antibiofilm results of all human pathogens, PC-I containing six aromatherapeutic EOs, thyme+bergamot+lemon+teatree+lavender+mint (1:2:4:5:5:5), exhibited excellent antibiofilm effect on *S. aureus, M. luteus, P. aeruginosa, A. baumannii, S. typhimurium, K. oxytoca, P. mirabilis* and *S. marcescens* than the individual effect of each oil in the combination, signifying its synergistic activity. PC-II also displayed synergistic antibiofilm effect on *S. marcescens* (Table 4 and Table 5). These results confirm previous reports indicating the significant increase of biological activities by the combinations of essential oils (Yap et al., 2014; Vieira et al., 2017). Additionally, the antibiofilm effects of PC-I on *M. luteus, P. aeruginosa, E. coli, K. oxytoca* and *P. mirabilis* were found to be as powerful as amoxicillin (Amx) and, its activity on *S. aureus* was more powerful than Amp. These findings are compatible with an earlier study (Kavanaugh & Ribbeck, 2012) in which cassia, Peru balsam, and red thyme EOs eradicated *Pseudomonas sp.* and *S. aureus* biofilms with higher efficiency than selected antibiotics.

On the other hand, PC-I exhibited no significantly different antibiofilm effect ($p >0.05$) from thyme and/or lemon EOs on *E. coli*.

Table 4. Antibiofilm activity of aromatherapeutic essential oils against some pathogenic Gram negative bacteria tested with micro-dilution assay .

AEOs / Biofilm inhibition (%)	*P. aeruginosa* ATCC 27853	*P. aeruginosa* PAO1	*A. baumannii* ATCC 19606	*K. pneumoniae* ATCC 700603	*S. thyphimurium* ATCC 14074	*K.oxytoca* ATCC 43165	*P. mirabilis* ATCC 7002	*S. marcescens* ATCC 27117	*E. coli* ATCC 25922
PC-I	75.08±4.03^{a,g}	61.08±0.03	61.38±5.35^{a}	50.36±9.30^{a,c}	47.25±1.30^{a}	63.07±1.15^{a}	66.64±4.16^{a,h}	57.37±4.00^{a}	58.73±7.12^{a,c}
PC-II	50.51±2.24^{b}	60.52±0.10	25.08±0.61^{b}	15.68±4.10^{b}	18.20±6.03^{b}	23.08±2.80^{b,e}	55.66±9.11^{b,e}	57.78±2.43^{a}	-
Eucalyptus	41.00±1.02^{c}	74.20±0.07	30.32±5.21^{b,c}	15.28±1.60^{b}	16.58±3.72^{b,c}	35.13±7.40^{c,g}	51.00±2.00^{b,f}	22.08±4.02^{b}	-
Bergamot	22.05±4.05^{d}	38.11±0.01	22.26±0.30^{b}	16.22±0.56^{b}	11.25±0.28^{c}	4.36±0.20^{d}	21.54±2.11^{c,d}	8.00±0.80^{c}	-
Cedrus	20.78±5.45^{d}	55.76±0.10	22.25±0.30^{b}	16.20±0.50^{b}	11.28±0.30^{c}	15.73±5.45^{e}	15.11±0.80^{c,d}	11.60±0.33^{c,d}	-
Lavander	21.80±2.44^{d}	65.50±0.04	33.00±7.04^{b,c}	40.00±6.18^{a}	4.70±1.04^{d}	30.73±7.40^{b,c}	63.24±6.40^{a,e}	23.55±1.60^{b,e}	-
Orange	22.00±1.60^{d}	77.36±0.02	23.30±3.20^{b}	23.57±8.70^{b}	40.02±0.02^{e}	6.64±0.40^{d}	20.50±1.30^{c,d}	17.17±5.14^{b,d}	-
Mint	23.28±4.22^{d}	46.70±0.01	23.00±0.65^{b}	16.44±0.56^{b}	17.83±4.02^{b,c}	28.40±2.42^{b,c}	51.61±1.22^{b}	30.34±2.43^{e}	-
Tea Tree	20.80±3.00^{d}	58.10±0.03	28.02±7.06^{b}	20.03±6.15^{b}	11.32±0.66^{b}	33.00±1.50^{c,f}	55.66±2.60^{b,e}	38.41±1.11^{f}	-
Thyme	67.67±2.20^{e}	63.40±0.06	40.00±1.00^{c,e}	47.04±1.18^{a,c}	3.82±0.04^{d}	41.08±1.71^{g}	41.17±0.33^{f}	40.25±1.30^{f}	55.20±5.11^{a,c}
Lemon	63.60±2.10^{e}	73.30±0.10	10.50±0.26^{d}	54.45±0.60^{c}	1.60±0.03^{d}	25.54±1.45^{b,c}	27.17±0.04^{c}	18.53±0.91^{b,d}	48.53±5.71^{a,b}
Clove	60.17±3.75^{e}	60.36±0.01	18.35±0.06^{d}	43.17±1. 00^{a,c}	46.12±0.50^{a,e}	36.80±1.82^{f,g}	35.75±0.78^{g}	40.76±0.70^{f}	44.68±0.52^{b}
Rosemary	56.68±0.57^{b}	45.31±0.02	45.68±1.45^{e}	26.63±1.17^{b}	-	33.25±1.44^{b,c}	15,86±0.52^{d}	20.80±0.50^{b}	50.02±6.54^{a,b}
Ampicillin (Amp)	80.25±0.32^{g}	N.D.	75.85±0.10^{f}	71.42±0.20^{d}	75.26±0.08^{f}	63.85±0.80^{a}	74.64±0.30^{h}	68.07±0.31^{g}	60.87±0.67^{c}

Biofilm inhibition of the aromatherapeutic essential oils (AEOs) and combinations (PC-I and PC-II) at the sub-MIC concentration of 0.1% v/v and 0.0125% v/v against Gram-negative human pathogens and PAO1 as a biosensor strain, respectively. Amoxicillin at 2 µg/ml was used as a positive control. The values of biofilm inhibition (%) represent averages ± standard deviations (SD) for triplicate experiments. Values in the same column with different superscripts are significantly different ($p < 0.05$). ND: Not detected.

Table 5. Antibiofilm activity of aromatherapeutic essential oils against some pathogenic Gram positive bacteria tested with micro-dilution assay.

AEOs / Biofilm inhibition (%)	*B. cereus* ATCC 6633	*S. epidermitis* wt	*S. aureus* ATCC 29213	*E. faecalis* ATCC 29212	*M. luteus* ATCC 7468
PC-I	-	46.84±6.04^{a}	80.09±3.00^{a}	26.64±0.31^{a}	78.00±4.07^{a}
PC-II	-	21.40±5.06^{b}	50.70±0.50^{b}	24.34±4.12^{a}	31.23±5.42^{b}
Eucalyptus	-	20.28±4.28^{b}	55.11±7.74^{c,e}	24.60±4.20^{a}	31.70±1.33^{b}
Bergamot	-	20.03±4.25^{b}	55.38±6.41^{c,e}	20.01±1.77^{a}	53.37±0.70^{c}
Cedrus	-	17.83±5.62^{b}	55.22±0.55^{c,e}	21.11±0.70^{a}	10.44±2.80^{d}
Lavander	-	27.72±5.07^{b}	61.30±5.84^{c,e}	35.58±3.30^{b}	64.72±2.04^{e}
Orange	-	23.40±7.22^{b}	57.53±7.48^{c,e}	12.73±2.05^{c}	51.48±1.45^{c}
Mint	-	18.00±2.80^{b}	52.70±1.00^{c}	20.47±2.15^{a}	57.55±0.52^{c}
Tea Tree	-	26.62±5.57^{b}	40.32±3.18^{d}	36.08±1.01^{b}	30.23±1.00^{b}
Thyme	33.11±1.70^{a}	70.74±0.07^{c,e}	58.55±1.30^{c,e}	50.37±2.00^{d}	65.36±0.53^{e}
Lemon	15.04±1.60^{b}	62.36±1.21^{c,d}	35.56±0.65^{d}	48.34±1.26^{d}	57.52±0.50^{c}
Clove	12.08±3.44^{b}	61.24±0.72^{c,d}	33.50±2.57^{d}	11.50±1.60^{c}	57.37±0.42^{c}
Rosemary	11.50±2.06^{b}	56.83±1.54^{a,d}	51.03±0.80^{b,c}	2.67±0.04^{e}	53.22±0.56^{c}
Ampicillin	60.76±0.72^{c}	80.04±0.32^{e}	67.00±1.09^{e}	66.02±0.75^{f}	77.33±0.31^{a}

Biofilm inhibition of the aromatherapeutic essential oils (AEOs) and combinations (PC-I and PC-II) at the sub-MIC concentration of 0.1% v/v against Gram-positive human pathogens. Amoxicillin at 2 µg/ml was used as a positive control. The values of biofilm inhibition (%) represent averages ± standard deviations (SD) for triplicate experiments. Values in the same column with different superscripts are significantly different ($p<0.05$).

Also, its inhibitory effect on *K. pneumoniae* was not higher than each EO, signifying an indifferent effect defined as the absence of interaction between Eos (Table 5). Unlike PC-I, PC-II including thyme and tea tree Eos (1:1) displayed an antagonistic antibiofilm effect on *E. faecalis* and *K. oxytoca*; a lower antibiofilm effect of PC-II than each oil was observed. It also exhibited an indifferent antibiofilm effect on *S. typhimurium* and *P. mirabilis* (Table 4 and Table 5). These findings showed that thyme EO displayed a better effect on biofilm formation of many pathogens than PC-II and, the antibiofilm effect of tea tree EO on most of the pathogens was no different from PC-II. These results confirmed the study (Oh et al., 2017), where single essential oil also had a better result on antibiofilm formation than blended essential oil.

Our findings also revealed that PC-I exhibited remarkable synergistic effect on most of Gram-negative and some Gram-positive human pathogens; however, PC-II displayed indifferent and antagonistic effects on most of the pathogens. The biofilm inhibitory actions of EOs combinations could be related to the content of EOs, interaction between EOs in the mixture, type of pathogen, or evaluation methods of biofilm inhibition. Thus, these findings are also in line with previous reports where various interactions of EOs were explained (Yap et al., 2014; Luís et al., 2016; Tariq et al., 2019).

Aromatherapeutic EOs with the advantages of possessing low mammalian toxicity, relative accessibility, and quick degradation in water and soil are used in the medicinal industry. EOs obtained from plants belonging to the families, especially, Lamiaceae, Myrtaceae and Rutaceae are known to have important potentials in terms of medicinal practices (Kavanaugh & Ribbeck, 2012; Raut & Karuppayil, 2014). In our study, cedrus EO, from the family Pinaceace, at sub-MIC of 0.4% (v/v) displayed a low antibiofilm effect on most of the pathogens; nevertheless, thyme EO, except on *S. typhimurium*, Lamiaceae, and also clove EO, Myrtaceae, at 0.4% (v/v) exhibited higher biofilm inhibition on most of the pathogens (Table 1-2). These results are mostly in harmony with a previous study (Alibi et al., 2020) in which thyme and clove EO, at sub-inhibitory concentrations, showed remarkable antibiofilm effect on all the tested multidrug-resistant clinical strains (1). In another research, it was found that EOs, also derived from thyme, orange and rosemary, significantly ($p<0.05$) inhibited the biofilm formation of *S. epidermidis* ATCC 12228, *E. coli* O33 and O157:H7 strains (Cáceres et al., 2020). Our results also exhibited that thyme and rosemary EOs hampered the biofilm formations of *S. epidermidis wt* and *E. coli* ATCC 25922 significantly ($p<0.05$); however, orange EO did not inhibit the biofilm formation of *E. coli* strain. This discrepancy might have occurred due to the methods used to obtain the EOs and different strains, changeable experimental conditions and the variable volatile content of EOs.

The current studies also indicated that mint, tea tree, lavender, lemon, eucalyptus, and rosemary EOs prevented the biofilm formation of different clinical and/or standard strains of methicillin-resistant *S. aureus* (MRSA), *B. cereus, P. aeruginosa, P. putida, S. aureus, E. coli* and mixed-culture biofilms, which were compatible with our antibiofilm results revealing higher antibiofilm potentials of many EOs on especially *S. aureus* 29212 and *P. aeruginosa* 27853 (Kerekes et al., 2013; Vieira et al., 2017; Merghni et al., 2018). Also, in our results, the biofilm formation of *E. coli* and *B. cereus* strains was only hampered by thyme, lemon, clove and rosemary EOs. It was reported that biofilm inhibitory differences between the strains indicate species-

specific activity of the oils and the specific mechanisms of resistance to the oils might be at work (Kavanaugh & Ribbeck, 2012). For instance, certain EOs can work on the bacterial cell wall or cell membrane. Therefore, the composition of these cellular components could be key to specifying susceptibility to EOs.

Essential oils are an excellent alternative to use as antibiotics against resistant strains of bacteria. Most antibiotics on the market, which are based on inhibiting growth and killing bacteria, are out of use due to the development of microbial resistance and there is a need for alternatives that can be used instead (Chatterjee & Vittal, 2021; Hong, Wang, Chen, & Zhu, 2021). In this context, in order to include essential oils in therapeutic treatments, they should be included in various cell culture studies and the results should be evaluated. In the study by Alibi et al. to determine the cytotoxicity of EOs in the Vero cell line, essential oils were found to have a higher affinity for the bacterial species evaluated (Alibi et al., 2020). Most of the resistance and virulence traits in bacteria occur through quorum sensing mechanisms involving bacterial cell-cell communication. Therefore, breaking the quorum sensing system would be a good strategy. Overall, the high antibiofilm and anti-QS activities detected for essential oils position them as promising natural products for the development of new and better therapeutic strategies for emerging clinical problems (Saeki, Kobayashi, & Nakazato, 2020; Boudiba et al., 2021). Consequently, essential oils can be used as an alternative to synthetic antioxidants, as natural products may be more compatible with living systems and safer than synthetic ones. Clinical trials are needed to confirm the place of EOs in clinical medicine.

CONCLUSIONS

This is the first detailed study that confirms the anti-quorum sensing and antibiofilm potentials of essential oils and their pharmaceutical combinations applied in aromatherapy. The research results clearly demonstrate that all aromatherapeutic essential oils (AEOs), especially PC-I and thyme AEOs, hamper the biofilm formation of most of the pathogens, particularly on *P. aeruginosa* and *S. aureus* in relation with respiratory infections. All AEOs, especially thyme, lavender, eucalyptus and orange AEOs, also significantly ($p<0.05$) inhibited the swarming and swimming motility of PAO1 strain, which could be considered as antipseudomonal agents. PC-I, PC-II, bergamot, lavender, mint, tea tree, thyme, clove and rosemary AEOs displayed highly active QS inhibition (>95%). This study also proved that each AEO, specifically PC-I and thyme EO, could have a potential to use an alternative therapy for bacterial infections in particular for those caused by biofilm formation, and all AEOs could be a candidate of anti-QS agents. Moreover, the remarkable synergistic action of PC-I, demonstrating more powerful antibiofilm effect than amoxicillin on *S. aureus*, suggests that the combined use of AEOs could enhance their therapeutic actions by eradicating bacterial biofilm. Consequently, new aromatherapeutic formulations should be produced for the cure of especially respiratory infections associated with the bacterial QS and biofilm formation, and the mechanism of actions of QS and bacterial biofilm formation for AEOs are needed to be investigated to discover new complementary and alternative therapies against infectious diseases, moreover, to reduce the tragic effects of antibiotic resistance.

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