

Evaluation of Enzyme Inhibition and Anti-Quorum Sensing Potentials of *Melaleuca alternifolia* and *Citrus sinensis* Essential Oils

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Abstract

In this study, *Melaleuca alternifolia* (Maiden and Betche) Cheel (tea tree) oil and *C. sinensis* (L.) Osbeck (orange) oil was procured, and their chemical compositions were characterized by gas chromatography coupled with mass spectrometry, with co-injection using authentic samples. The oils were rich in monoterpene hydrocarbons making up 84.5% of *C. sinensis* and 48.9% of *M. alternifolia* oils. High amounts of oxygenated monoterpenoids were also identified in *C. sinensis* (9.6%) and *M. alternifolia* (49.3%) oils. The three most abundant compounds were limonene (71.2%), linalool (5.5%), and β -myrcene (5.1%) in *C. sinensis*, and terpinen-4-ol (45.6%), γ -terpinene (19.4%) and α -terpinene (9.3%) in *M. alternifolia*. Enzyme inhibitions (anticholinesterase, antiurease, antityrosinase) of both essential oils were evaluated. In acetylcholinesterase assay, *M. alternifolia* and *C. sinensis* had inhibition concentration (IC_{50}) values of 153.7 ± 1.25 and 96.4 ± 1.0 $\mu\text{g}/\text{mL}$, respectively as compared to 5.42 ± 0.11 $\mu\text{g}/\text{mL}$ for galantamine, while in butyrylcholinesterase assay, *M. alternifolia* ($IC_{50} = 85.6 \pm 0.7$ $\mu\text{g}/\text{mL}$) and *C. sinensis* ($IC_{50} = 127.8 \pm 0.6$ $\mu\text{g}/\text{mL}$) exhibited moderate activities compared to galantamine ($IC_{50} = 45.8 \pm 0.8$ $\mu\text{g}/\text{mL}$). In the urease inhibitory activity, essential oils of *C. sinensis* and *M. alternifolia* showed $48.4\% \pm 0.9\%$ and $30.6\% \pm 0.7\%$ inhibitions at 200 $\mu\text{g}/\text{mL}$ concentration, respectively. Quorum sensing (QS) mediated violacein production in *Chromobacterium violaceum* CV12472 was inhibited by 100% at minimum inhibitory concentration (MIC) values for both oils while showing QS inhibition diameter zones in *C. violaceum* of 22.5 ± 0.4 mm and 14.3 ± 0.5 mm for *C. sinensis* and *M. alternifolia* oils, respectively at MIC concentration. The good quorum-sensing potential indicates that these oils can suppress microbial resistance and severity of infections.

Keywords

essential oils, *Melaleuca alternifolia*, *Citrus sinensis*, co-injection gas chromatography coupled with mass spectrometry, anticholinesterase, antiurease, antityrosinase, anti-quorum sensing

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Introduction

Essential oils are produced by plants to ensure their adaptation to the environment and also to fight attacks by predators. They contain mixtures of volatile and aromatic compounds that man has exploited for applications in traditional medicines and as food additives with proven biological activities such as anticholinesterase, antityrosinase, antimicrobial, antioxidant, antibiofilm, motility inhibition, cytotoxic, and anti-quorum sensing activities.^{1–5} Plants that are rich in volatile oils find many therapeutic applications with an intensive use in medicine. Medicinal properties are attributed to these volatile oils, which are composed of a complex mixture of secondary metabolic and aromatic compounds produced in different plant parts of the plant.^{6,7} Volatile constituents from plants possess a wide range of medicinal properties such as antioxidant, antiviral,

antifungal, antibacterial, and insecticidal activities, which is the reason for which they are being used in natural therapies, alternative medicine, pharmaceuticals, and food preservation.⁸

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Orange tree and tea tree oils are widely used as nutraceuticals. Tea tree oil is the essential oil derived from *Melaleuca alternifolia*, mostly by steam distillation, and is used traditionally to treat cuts, burns, abrasions, and acne in a range of cosmetic products such as antiseptics, deodorants, shampoos, and soaps.⁹ Tea tree oil is used largely for its antimicrobial properties and as an ingredient in many topical formulations to treat skin infections and, additionally, has other biological activities such as antiviral, insecticidal, anti-inflammatory, antitumor, biocidal, antiparasitic, and anthelmintic properties.^{10–12} *Citrus sinensis* produces an attractive essential oil with interesting bioactivities, including anticarcinogenic, antioxidant, antiangiogenic, antifungal, anticholinesterase, antianxiety, antimicrobial, larvicidal, and insecticide activities.^{8,13,14} Because of their high economic importance, orange oil (EO) is the most vital byproduct of Citrus processing obtained from orange peel through several extraction methods such as hydro-distillation, solvent extraction, cold pressing, and supercritical CO₂; many studies have reported its chemical composition and bioactivities.^{15,16}

The objective of this present study was to report an up-to-date phytochemical profile using gas chromatography coupled with mass spectrometry (GC-MS) of tea tree (*M. alternifolia*) and orange (*C. sinensis*) oils, as well as evaluating the enzyme inhibition (acetylcholinesterase (AChE), butyrylcholinesterase (BChE), urease, tyrosinase) and quorum sensing activity of the oils.

Materials and Methods

Essential Oils

Tea tree (*Melaleuca alternifolia*) oil and orange (*Citrus sinensis*) oil were procured from a pharmacy in Mugla, Turkey. They were stored in dark bottles at 4 °C in a refrigerator until GC-MS analysis and bioassays.

GC-flame ionization detector (FID) and GC-MS Analyses

The essential oils were analyzed by GC-FID and GC-MS systems. For GC analyses, a Rxi-5Sil MS fused silica capillary non-polar column (30 m × 0.25 I.D., film thickness 0.25 μm) and FID was used. Temperatures for the injector and detector were set to 250 °C–270 °C, respectively. Helium was used as carrier gas with a 1.4 mL/min flow rate. The sample injection volume was 0.2 μL with a split ratio of 20:1. A Class GC10 GC computer program was used to determine the percentage composition of the essential oils. The initial oven temperature was held at 60 °C for 5 min, then increased up to 240 °C with 4 °C/min increments and held at this temperature for 10 min.

For GC-MS analyses, an ion trap MS and a Rxi-5Sil MS fused silica non-polar capillary column (30 m × 0.25 mm I.D., film thickness 0.25 μm) were used. The carrier gas was helium with a 1.4 mL/min flow rate. The injector and MS transfer line temperatures were 220 °C–290 °C, respectively. The

temperature of the ion source was 200 °C, and the injection volume was 0.2 μL with a split ratio of 20:1. EI-MS measurements were taken at 70 eV ionization energy. Mass range was from m/z 28 to 650 amu. Scan time was 0.5 s with 0.1 s inter-scan delays. The oven temperature was held at 60 °C for 5 min, then increased up to 240 °C with 4 °C/min increments and kept at this temperature for 10 min.

For identification of essential oil components, co-injection with standards (whenever possible) was used, together GC retention indices determined using a homologous series of C₇–C₃₀ alkanes (Supelco), and computer matching with the Wiley, TRILIB, and NIST08 libraries, as well as a comparison of fragmentation patterns documented in the literature.¹⁷ Quantification of each of the essential oil's individual constituents was based on internal normalization for the components.

Anticholinesterase Activity

AChE and BChE inhibitory activity was evaluated spectrophotometrically according to Ellman's method, with slight modifications.^{18,19} AChE from electric eel and BChE from horse serum were utilized, with the substrates acetylthiocholine iodide and butyrylthiocholine chloride. The cholinesterase activity was measured using DTNB (5,5'-dithio-bis(2-nitrobenzoic acid)). Galantamine was used as a reference compound. The tests were performed in a 96-well plate microplate reader (SpectraMax, Molecular Devices). The results are expressed as 50% inhibition concentration (IC₅₀).

Anti-Urease Activity

The indophenol method was used to determine urease inhibition activity by measuring ammonia production.^{19,20} Sodium phosphate buffer (100 mM, pH 8.2), urease enzyme (Jack bean source) solution (25 μL) and urea (100 mM, 50 μL) were mixed and incubated at 30 °C for 15 min after the addition of test samples (10 μL). Following incubation, 45 μL of 1% (w/v) phenol reagent and 70 μL of 0.005% (w/v) alkali reagent were added to each well. After 50 min of incubation, absorbance was measured with the microplate reader at 630 nm. Thiourea was used as the standard compound. The results are given as inhibition percentage (%) at 200 μg/mL concentration of the essential oils.

Anti-Tyrosinase Activity

A published spectrophotometric method was used to determine the inhibition of tyrosinase (mushroom source).²¹ L-DOPA was employed as the substrate of the reaction. Briefly, 150 μL of sodium phosphate buffer (pH 6.8, 100 mM), 10 μL of sample solution, and 20 μL of tyrosinase solution in buffer were mixed together and incubated for 10 min at 37 °C. After incubation, 20 μL of L-DOPA was added. Kojic acid was used as reference compound. Absorbances were read at 475 nm at

37 °C in a 96-well microplate. The results are given as 50% IC₅₀.

Microbial Strains and Determination of Minimum Inhibitory Concentration (MIC)

The used microorganisms in this study were *Pseudomonas aeruginosa* PA01, *Chromobacterium violaceum* CV12472, and *C. violaceum* (CV026). MICs were determined by the microtitre broth dilution method.^{22,23} The MIC was defined as the lowest EO concentration that yielded no visible growth. The test medium was Mueller-Hinton Broth and the density of bacteria was 5×10^5 colony-forming units/mL. Cell suspensions (100 µL) were inoculated into wells (96-well microtitre plates) in the presence of EO with different final concentrations (2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125 mg/mL). The inoculated microplates were incubated at 37 °C for 24 h before being read.

Violacein Inhibition Assay Using CV12472

All EOs were subjected to qualitative analysis to find their quorum-sensing inhibition (QSI) potentials against *Chromobacterium violaceum* CV12472, as described previously.^{22,23} Overnight culture (10 µL) of CV (adjusted to 0.4 OD at 600 nm) was added into sterile microtiter plates containing 200 µL of LB broth and incubated in either the presence or absence of sub-MIC concentrations of EOs of *Melaleuca alternifolia* and *Citrus sinensis* broth containing CV12472 as a positive control. These plates were incubated at 30 °C for 24 h and observed for the reduction in violacein pigment production. The absorbance was read at 585 nm. Each experiment was made in triplicate and the percentage of violacein inhibition was calculated via the following formula:

$$\text{Violacein inhibition(\%)} = \frac{\text{OD 585 control} - \text{OD 585 sample}}{\text{OD 585 control}} \times 100$$

Bioassay for QSI Activity Using CV026

Quorum sensing (QS) inhibition was evaluated as described elsewhere.^{23,24} Five mL of warm molten Soft Top Agar (1.3 g agar, 2.0 g Tryptone, 1.0 g sodium chloride, 200 mL deionized water) was seeded with 100 µL of overnight CV026 culture and 20 µL of 100 µg/mL C₆HSL was added as exogenous acyl homoserine lactone (AHL) source. This preparation was softly mixed and poured immediately over the surface of a solidified Luria Bertani Agar plate as an overlay. Wells 5 mm in diameter were made on each plate after solidification of the overlay. Each well was filled with 50 µL of MIC and sub-MIC concentrations of filter-sterilized EO. A white or cream-colored halo around this well against a purple lawn of activated CV026 bacteria indicated QSI. Each experiment was repeated three times. The assay plates were incubated at 30 °C for three days, and then the diameters of the QS inhibition zones were measured.

Swarming Motility Inhibition on *P. aeruginosa* PA01

The swarming motility inhibition assay was used as described previously.^{23,25} Briefly, overnight cultures of *P. aeruginosa* PA01 strain were point inoculated at the center of swarming plates consisting of 1% peptone, 0.5% NaCl, 0.5% agar, and 0.5% filter-sterilized D-glucose with various concentrations of EO (50, 75, and 100 µg/mL). The plate without the EO was maintained as control and the plates were incubated at an appropriate temperature in an upright position for 18 h. The swarming migration was recorded by following swarm fronts of the bacterial cells. The reduction in swarming migration was recorded by measuring the swarm zones of the bacterial cells after 18 h and calculating the percentage inhibitions.

Results

Chemical Composition of *M. alternifolia* and *C. sinensis* Oils

GC-MS was used to determine the chemical composition of the essential oils from *M. alternifolia* and *C. sinensis* and the results are reported in Table 1. The total number of identified compounds was 35 (99.8%) and 16 (99.8%) for *C. sinensis* and *M. alternifolia* oils, respectively. The oils were rich in monoterpene hydrocarbons, which comprised 84.5% of the detected components in *C. sinensis* and 48.9% in *M. alternifolia*. High amounts of oxygenated monoterpenoids were also identified in *C. sinensis* (9.6%) and *M. alternifolia* (49.3%) oils. Sesquiterpene hydrocarbons formed 3.6% and 1.4% of *C. sinensis* and *M. alternifolia* oils, respectively, and oxygenated sesquiterpenoids 1.0% and 0.2%, respectively. The three most abundant compounds in *C. sinensis* were limonene (71.2%), linalool (5.5%), and β-myrcene (5.1%), while in *M. alternifolia* oil they were terpinen-4-ol (45.6%), γ-terpinene (19.4%) and α-terpinene (9.3%).

Enzyme Inhibition Potential of *M. alternifolia* and *C. sinensis* Oils

The potential to alleviate Alzheimer's disease (AD) was made by evaluating the AChE and BChE inhibitory activities of *M. alternifolia* and *C. sinensis* oils, and the results representing the IC₅₀ values are given in Table 2. In the AChE assay, *M. alternifolia* and *C. sinensis* had IC₅₀ values of 153.7 ± 1.25 and 96.41 ± 0.98 , respectively, as compared to 5.42 ± 0.11 for galantamine used as standard. The inhibition of BChE for *M. alternifolia* (IC₅₀ = 85.6 ± 0.7) and *C. sinensis* (IC₅₀ = 127.8 ± 0.6) were moderate compared to galantamine (IC₅₀ = 45.8 ± 0.8). *C. sinensis* and *M. alternifolia* showed moderate inhibitions with values of $48.4\% \pm 0.9\%$ and $30.6\% \pm 0.7\%$ against urease at 200 µg/mL concentration, respectively. Kojic acid (IC₅₀ = 23.5 ± 0.3) was used as standard in the antityrosinase assay and showed better activity than the oils of *M. alternifolia* (IC₅₀ = 82.3 ± 0.5) and *C. sinensis* (IC₅₀ = 145.8 ± 0.8).

Table 1. Chemical Composition of the Essential Oils of *Citrus sinensis* and *Melaleuca alternifolia*.

No	RI ^a	LRI ^b	Compounds	<i>Citrus sinensis</i> (%) ^c	<i>Melaleuca alternifolia</i> (%) ^c	Identification methods
1	914	922	α -Pinene	0.8	2.1	Co-GC, MS, RI
2	954	965	Sabinene	1.3	0.3	Co-GC, MS, RI
3	962	974	β -Pinene	0.5	0.4	Co-GC, MS, RI
4	986	988	β -Myrcene	5.1	0.2	Co-GC, MS, RI
5	1006	998	Octanal	0.2	—	MS, RI
6	1010	1014	α -Terpinene	—	9.3	Co-GC, MS, RI
7	1023	1020	<i>p</i> -Cymene	0.2	7.6	Co-GC, MS, RI
8	1026	1024	Limonene	71.2	1.3	Co-GC, MS, RI
9	1030	1026	1,8-Cineole	—	5.2	Co-GC, MS, RI
10	1057	1054	γ -Terpinene	—	19.4	Co-GC, MS, RI
11	1080	1086	Terpinolene	0.2	3.2	Co-GC, MS, RI
12	1107	1098	Linalool	5.5	—	Co-GC, MS, RI
13	1114	1121	Limonene oxide	0.7	—	MS, RI
14	1120	1127	<i>cis</i> - <i>p</i> -Mentha-2,8-dien-1-ol	1.0	—	MS, RI
15	1131	1137	<i>cis</i> -Verbenol	0.9	—	Co-GC, MS, RI
16	1143	1145	Citronellal	0.2	—	Co-GC, MS, RI
17	1148	1150	Neral	0.4	—	Co-GC, MS, RI
18	1151	1156	Borneol	—	0.2	Co-GC, MS, RI
19	1163	1171	Terpinene-4-ol	1.6	45.6	Co-GC, MS, RI
20	1177	1181	Myrtenol	0.7	—	Co-GC, MS, RI
21	1188	1186	α -Terpineol	2.2	3.5	Co-GC, MS, RI
22	1192	1201	Verbenone	0.4	—	Co-GC, MS, RI
23	1196	1204	Decanal	0.8	—	MS, RI
24	1202	1215	Carveol	0.6	—	Co-GC, MS, RI
25	1212	1222	Nerol	0.2	—	Co-GC, MS, RI
26	1250	1239	Carvone	0.7	—	Co-GC, MS, RI
27	1385	1387	β -Cubebene	0.4	—	Co-GC, MS, RI
28	1412	1399	β -Elemene	0.2	—	MS, RI
29	1452	1447	β -Caryophyllene	0.3	—	Co-GC, MS, RI
30	1455	1454	<i>trans</i> - β -Farnesene	0.6	—	Co-GC, MS, RI
31	1457	1460	Alloaromadendrene	—	0.6	Co-GC, MS, RI
32	1464	1473	γ -Muurolene	0.5	—	MS, RI
33	1474	1484	Germacrene D	0.5	—	Co-GC, MS, RI
34	1492	1496	Valencene	0.6	—	Co-GC, MS, RI
35	1497	1500	α -Muurolene	0.1	—	MS, RI
36	1541	1532	δ -Cadinene	0.4	0.7	Co-GC, MS, RI
37	1554	1557	Spathulenol	0.1	—	Co-GC, MS, RI
38	1563	1582	Caryophyllene oxide	0.4	—	Co-GC, MS, RI
39	1578	1592	Viridiflorol	—	0.2	MS, RI
40	1695	1699	β -Sinensal	0.2	—	MS, RI
41	1785	1806	Nootkatone	0.4	—	MS, RI
			Monoterpene hydrocarbons	84.5	48.9	
			Oxygenated monoterpenoids	9.6	49.3	
			Sesquiterpene hydrocarbons	3.6	1.4	
			Oxygenated sesquiterpenoids	1.0	0.2	
			Others	1.1	—	
			Total identified (%)	99.8	99.8	
			Total number of components	35	16	

^aRetention index experimentally determined using homologous series of C₇-C₃₀ alkanes on Rxi-5Sil MS fused silica column.

^bLinear retention index taken from Adams¹⁷ and/or NIST 08.

^cPercentage concentration, Identification methods: **Co-GC**: Co-injection with authentic compounds, **RI**: based on comparison of calculated with those reported in Adams¹⁷ and NIST 08: based on comparison with Adams¹⁷ and NIST 08 databases. The bold indicates the constituents present in relatively high amounts.

Violacein Inhibition, Anti-QS and Swarming Motility Inhibition of M. alternifolia and C. sinensis Oils

QS inhibition was evaluated by measuring the percentage of inhibition of violacein production in CV12472 and QS

inhibition zones in CV026 mutant strains at MIC and sub-MIC concentrations. Prior to the determination of anti-quorum sensing activities, the MIC values were measured at 1 and 2 mg/mL *C. sinensis* oil and *M. alternifolia* oil on CV12472,

Table 2. Cholinesterase, Urease, and Tyrosinase Inhibitory Activities of the Essential Oils From *Citrus sinensis* and *Melaleuca alternifolia*.

Extract/standard	Cholinesterase inhibitory activity		Urease inhibitory activity Inhibition (%) ^b	Tyrosinase inhibitory activity IC ₅₀ (µg/mL)
	AChE assay IC ₅₀ (µg/mL) ^a	BChE assay IC ₅₀ (µg/mL)		
<i>C. sinensis</i>	153.7 ± 1.2	127.8 ± 0.6	48.4 ± 0.9	145.8 ± 0.8
<i>M. alternifolia</i>	96.4 ± 0.98	85.6 ± 0.7	30.6 ± 0.7	82.3 ± 0.5
Galantamine ^c	5.42 ± 0.11	45.8 ± 0.8	NT	NT
Thiourea ^c	NT	NT	82.5 ± 0.2	NT
Kojic acid ^c	NT	NT	NT	23.5 ± 0.3

^aIC₅₀ values represent the means ± SEM of three parallel measurements ($P < .05$).

^bInhibition (%) at 200 µg/mL concentration of samples.

^cReference compounds.

NT, not tested; IC₅₀, inhibition concentration; AChE, acetylcholinesterase; BChE, butyrylcholinesterase.

respectively, and on CV026 the MIC values were 0.5 and 0.25 mg/mL for *C. sinensis* oil and *M. alternifolia* oil, respectively. The violacein inhibition varied from 100% ± 0.0% at MIC to 21.8% ± 0.6% at MIC/16 for *M. alternifolia* oil and from 100% ± 0.0% at MIC to 16.1% ± 1.4% at MIC/4 for *C. sinensis* oil, as shown in Table 3. Antiquorum sensing zones varied from 22.5 ± 0.4 mm at MIC to 11.0 ± 0.2 mm at MIC/8 for *C. sinensis* oil and 14.3 ± 0.5 mm at MIC to 08.0 ± 1.0 mm at MIC/2 for *M. alternifolia* oil, as indicated in Table 4. Swarming motility was inhibited at 100 µg/mL by *C. sinensis* oil (32.5% ± 0.2%) and *M. alternifolia* oil (19.1% ± 1.0%), and, also at 75 µg/mL, *C. sinensis* oil (17.9% ± 0.1%) and *M. alternifolia* oil (6.0% ± 0.0%), but no inhibition was observed at 50 µg/mL, as shown In Table 5.

Discussion

The GC-MS results obtained showed high amounts of terpinene-4-ol, γ-terpinene, α-terpinene, 1,8-cineole, *p*-cymene, and α-terpineol in *M. alternifolia* oil, which is usually rich in monoterpenes and oxygenated monoterpenes, with the major compound being terpinen-4-ol, as in previous studies.^{26,27} The primary components of *M. alternifolia* oil and

the percentage compositions were certified to be terpinen-4-ol (37.1%), γ-terpinene (20.7%), α-terpinene (10.1%), 1,8-cineole (4.9%), terpinolene (3.6%), *p*-cymene (2.1%) and α-terpineol (3.8%) as specified.²⁸ These very characteristic components of tea tree oil are equally reported in this study as terpinen-4-ol (45.6%), γ-terpinene (19.4%), α-terpinene (9.3%), 1,8-cineole (5.2%), terpinolene (3.2%), *p*-cymene (7.6%) and α-terpineol (3.5%). The difference in their respective amounts was notably terpinen-4-ol and *p*-cymene, whose compositions were significantly higher in our study. Orange oil showed high amounts of limonene and β-myrcene, as would be expected, as this is characteristic of its composition.^{29–31} The chemical composition of *C. sinensis* oil reported here shows key components as limonene (71.2%), β-myrcene (5.1%), linalool (5.5%), and α-pinene (2.1%). These results corroborate, though with some little qualitative and quantitative differences, those reported in one previous study, which reported limonene (71.8%), β-myrcene (4.6%), linalool (3.9%) and α-pinene (1.2%),³² and with some other additional components that have been reported in this plant's oil.³³ These differences in the chemical composition of essential oils can be genetic or caused by differences in environmental, climatic, and seasonal factors. The chemical constituents present in the oils confer

Table 3. Inhibition of Violacein Production in *C. violaceum* CV12472 by Test Samples.

	MIC (mg/mL)	Violacein inhibition (%)				
		MIC	MIC/2	MIC/4	MIC/8	MIC/16
<i>C. sinensis</i>	1	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	28.9 ± 3.6	16.1 ± 1.4
<i>M. alternifolia</i>	2	100.0 ± 0.0	48.2 ± 1.1	21.8 ± 0.6	—	—

Table 4. Quorum Sensing Inhibition Zones in *C. violaceum* CV026 by Test Samples.

	MIC (mg/mL)	Antiquorum sensing inhibition zones (mm)			
		MIC	MIC/2	MIC/4	MIC/8
<i>C. sinensis</i>	0.5	22.5 ± 0.4	19.0 ± 1.0	14.5 ± 0.5	11.0 ± 0.2
<i>M. alternifolia</i>	0.25	14.3 ± 0.5	08.0 ± 1.0	—	—

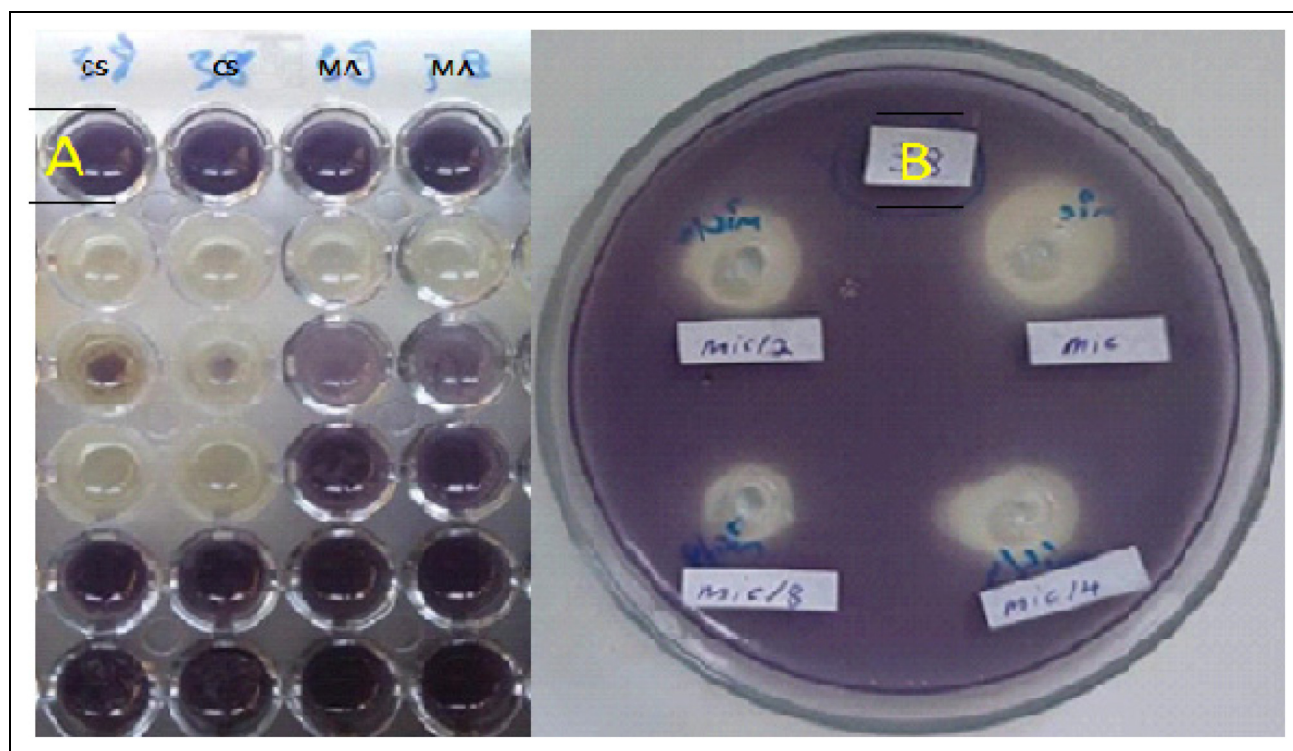
Table 5. Swarming Motility Inhibition of *P. aeruginosa* PA01 by Test Samples.

	Swarming inhibition (%)		
	100 µg/mL	75 µg/mL	50 µg/mL
<i>C. sinensis</i>	32.50 ± 0.25	17.95 ± 0.11	—
<i>M. alternifolia</i>	19.14 ± 1.00	06.00 ± 0.00	—

the biological activities and since they vary qualitatively and quantitatively, their biological activities will also vary.

In the enzyme inhibition assays, *M. alternifolia* was more active than *C. sinensis*. Studies on anticholinesterase activities of *M. alternifolia* oil are scarce. In one such study, *M. alternifolia* oil and two of its major constituents, 1,8-cineole and terpinen-4-ol, were shown to inhibit AChE.³⁴ No reports for the BChE inhibition were found for *M. alternifolia* oil. Equally, *C. sinensis* oil and its major constituent limonene showed good AChE inhibition and the results were almost the same for the oil and limonene,³⁵ suggesting that limonene could account substantially for the activity. Inhibition of AChE and BChE has much significance because they are key enzymes for hydrolysis of acetylcholine, which is important in the transmission of nerve impulses. It is desirable to maintain acetylcholine levels high by inhibiting AChE and this effect can be applied in the treatment of diseases characterized by low acetylcholine levels, such as AD.³⁶ Essential oils are rich in bioactive terpenes and oxygenated terpenoids and these types of compounds are known to possess anticholinesterase activities. Monoterpenes

and oxygenated monoterpenoids were abundant in both oils and it is believed that they are able to interact with AChE and BChE and act as either competitive or noncompetitive inhibitors of cholinesterase due to their lipophilicity and small molecular sizes, which makes them more likely to cross the blood-brain barrier and exert their effect.^{2,37} The oils from both *M. alternifolia* and *C. sinensis* showed relatively moderate inhibition of urease, which can be a good indication for their use as food additives. Urease is a nickel-containing enzyme that carries out the catalysis and hydrolysis of urea into carbon dioxide and ammonia and is responsible for some pathological diseases, such as gastric and peptic ulcers, and, therefore, urease inhibition may be the main defense against *H. pylori* due to preventing it from adhering to the gastric mucosa.^{4,19,38} Tyrosinase is a copper-containing enzyme that plays a critical role in the biosynthesis of melanin pigments, and its inhibition can suppress hyperpigmentation disorders and skin melanoma. The results reported here showed that the essential oils possess greater activity than the standard compound used. Therefore, the potential of *M. alternifolia* and *C. sinensis* oils to inhibit this enzyme is a desirable effect as well. Plant oils consist of hydrophobic mixtures which act as competitive inhibitors on tyrosinase and thereby on melanin synthesis, which indicates that they can be applied in the development of skin whitening agents.³⁹ The mechanism of action of *M. alternifolia* oil and *C. sinensis* oil may possibly involve the chelation of either copper or nickel ions present in tyrosinase or urease metalloenzymes, respectively.

**Figure 1.** Violacein inhibition plate (a); antiquorum sensing plate (b).

CV produces a violet coloration while growing by a QS mediated process and this coloration can be measured with reliability.¹⁹ *C. sinensis* oil had a higher activity for violacein inhibition than *M. alternifolia* oil as *C. sinensis* oil could inhibit violacein production right down to MIC/16 concentration, while *M. alternifolia* oil could not inhibit violacein production beyond MIC/4. Violacein is an antioxidant protecting the bacterial membrane against oxidative stress, thus inhibition of violacein by *M. alternifolia* oil, as well as *C. sinensis* oil can suppress virulence during infections. Working at sub-MIC concentrations, the hypothesis of the bactericidal effect of honey that occurs at high concentrations is eliminated giving way to a quorum-sensing investigation.⁴⁰ The ability to disrupt QS in bacteria can eliminate virulence and resistance and this can be accounted for by the presence of synergistic and interdependent components found in the oils. It is very important to search for new therapies capable of inhibiting bacterial cell-to-cell communication networks, referred to as QS, which is responsible for the increase in virulence and resistance.^{19,22,23} Unlike CV12472, which produces violacein as it grows, CV026 is a biosensor mutant strain which only produces violacein when an acyl homoserine lactone hormone is supplied to it externally. *C. sinensis* oil showed larger QSI zones and hence higher activity of inhibition than *M. alternifolia* oil. The QSI zones correspond to the cream-colored zones around the wells, while violacein inhibition is revealed by the absence of violacein pigment in the wells (Figure 1). Considering violacein inhibition in CV12472 and CV026, both oils inhibited signal production as is the case with CV12472 and signal reception as is the case with CV026. *Pseudomonas aeruginosa* PA01 utilizes flagellum-mediated motility to approach a surface, attaches, and then further spreads via the surface through associated motilities, such as swarming and twitching.^{41,42} The swarming motility inhibition results indicate that *C. sinensis* oil is more active as it had a greater percentage inhibition than *M. alternifolia* oil at 100 and 75 µg/mL. Swarming motility is a favorable step toward the establishment of biofilms and colonization of surfaces by bacteria and therefore the ability of the oils to inhibit this swarming motility could reduce biofilm risks during infections.

Conclusion

AD, caused by progressive neurodegeneration, is usually attributed to low acetylcholine levels in cholinergic synapses in regions of the brain. AChE and BChE are key enzymes associated with the hydrolysis of acetylcholine and butyrylcholine, respectively, and hence their inhibition is a good therapeutic solution to AD. *M. alternifolia* oil and *C. sinensis* oil showed appreciable AChE and BChE inhibitions with *M. alternifolia* oil being more active than that of *C. sinensis*. This activity could be attributed to the rich chemical components detected and quantified in the oils by GC-MS analysis. Evaluation of inhibition of quorum-sensing effects in bacteria, which are the means of bacterial cell-to-cell communication used in the development of resistance to drugs and development of severity during infections, indicated promising results; *C. sinensis* oil was more

active than that of *M. alternifolia*. This results substantiate the application of tea tree oil and orange oil in the management of diseases and infections.


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