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Mandarin essential oils inhibit quorum sensing and virulence factors of *Pseudomonas aeruginosa*

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ABSTRACT

Pseudomonas aeruginosa produces biofilm and several virulence factors coordinated by quorum sensing (QS) in food. The interruption of QS is a target to control the bacterial virulence. Chemical preservatives used to control biofilm give rise to several food safety problems. For this reason, essential oils (EOs), generally recognized as safe products, are a hopeful alternative. The aim of this work was to determine the chemical composition of mandarin EOs obtained by cold-pressing (EOP) and cold-pressing followed by steam distillation (EOPD) and their antipathogenic properties against *P. aeruginosa*. Both EOs contained the highest quantities of monoterpene hydrocarbons, mainly limonene, followed by γ-terpinene, myrcene and α-pinene. Although the EOs were not able to inhibit the bacterial growth at 4 mg/mL, EOP and EOPD significantly inhibited the *P. aeruginosa* biofilm formation at 0.1 mg/mL. In addition to the EOs inhibited biofilm cell viability (41%), AHL production (33%) and resulted in higher than 75% reduction in elastase enzyme activity. In conclusion, *Citrus reticulata* EOs are suitable alternatives to chemical additives for general use in the food industry.

1. Introduction

Bacterial biofilm is a problem in the food industry due to the resistance to antimicrobial and cleaning agents. Several foodborne diseases outbreaks have been associated with biofilm (Srey, Jahid, & Ha, 2013) and it has become a significant challenge to food production (Shikongo-Nambabi, 2011; Sofos & Geornaras, 2010). *Pseudomonas* spp. is one of the main biofilms producing bacteria found in food (Rajkowski, 2009) and this bacterium could produce several virulence factors such as the enzyme elastase, B (LasB) which is produced by *Pseudomonas aeruginosa* (Stewart & Costerton, 2001). This secreted protease degrades a broad range of host tissue proteins and important biomolecules involved in innate immunity (Cathcart, Gilmore, Greer, Harriott, & Walker, 2009). In addition, LasB acts within the bacterial cell as a key regulator in the generation of the secreted polysaccharides that constitute the bacterial biofilm (Cathcart et al., 2009), thus LasB inhibition could be important for attenuation of *Pseudomonas* virulence (Sokol, Kooi, Hodges, Cachia, & Woods, 2000). *P. aeruginosa* employs quorum sensing (QS) to coordinate the communal behavior and it consists in the regulation and coordinated expression of genes in response to cell density. The bacterial virulence factors (such as biofilms and elastase enzyme) have been shown to specifically involve the recognition and response to self-generated secreted small molecules called autoinducers (AI-1). Gram negative bacteria produce *N*-acyl homoserine lactones (AHLs) as quorum signal molecules (Bassler & Losick, 2006) and several AHLs have been found in different foods (Gram et al., 2002). Moreover, the bacterial signal molecules *per se* may influence the outcome of an infection by modulating the host immune response (Telford et al., 1998). In the same way, the growth of microorganisms in food products may cause intestinal disorders, including vomiting and diarrhea (Friedman, Henika, & Mandrell, 2002).

Currently, chemical preservatives are used to prevent the growth of most foodborne bacteria; however, several food safety problems are related to chemical preservatives (Deba, Xuan,

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Yasuda, & Tawata, 2008). The design of antipathogenic substances from plant sources could be an important strategy to prevent food spoilage and foodborne diseases. Antipathogenic compounds do not kill bacteria or stop their growth; rather, they control bacterial virulence factors like biofilm, elastase activity, and prevent the development of resistant strains (Otto, 2004). The interruption of QS, or bacterial cell-to-cell communication, is one example of antipathogenic effects.

Essential oils (EOs) could be used as antimicrobial additives (Holley & Patel, 2005). In nature, EOs play an important role in the protection of plants, particularly in antibacterial ones. The selective toxicity towards several pathogens and the synergism of individual components with different mechanisms of action preventing the development of resistance are the main remarkable properties of the EOs (Bakkali, Averbeck, Averbeck, & Idaomar, 2008). In the food industry this antibacterial activity has been targeted against many food pathogens and food spoiling bacteria, including virulence factor inhibition, making these substances attractive as food preservatives. In addition, some of them belong to the class of natural compounds that is generally recognized as safe (GRAS) by the Food and Drug Administration (Hemaiswarya & Doble, 2009). Also, EOs have therapeutic uses in human medicine due to its anticancer, antinociceptive, antidiabetic, antiviral, antibacterial and antioxidant properties (Bhalla, Gupta, & Jaitak, 2013; Buchbauer, 2010; Joo et al., 2013; Pagonopoulou, Koutroumanidou, & Charalabopoulos, 2012; Tang, Rajarajeswaran, Fung, & Kanthimathi, 2013).

In the food industry, biofilms cause serious engineering problems such as impeding the flow of heat across a surface, increases in fluid frictional resistance of surfaces and increases in the corrosion rate of surfaces leading to energy and production losses. Pathogenic microorganisms grown on food surfaces and in processing environments can cross-contaminate and cause post processing contamination (Ganesh & Anand, 1998). According to Chorianopoulos, Giaouris, Skandamis, Haroutounian, and Nychas (2008), the information available on the use of EOs as disinfectants is still limited, pointing to the need for further studies. In fact, the antipathogenic activities of EOs could be exploited to develop bio-preservatives useful for sanitizing industrial surfaces against bacterial biofilms.

Among the great variety of EOs, citrus fruit EOs and their major components have gained acceptance in the food industry and many foods tolerate their presence (Espina et al., 2011; Fisher & Phillips, 2006). Particularly, citrus EOs are complex mixtures of natural compounds whose content depends on the specific citrus cultivar, extraction and separation methods (Nannapaneni et al., 2009). Several factors influence the chemical composition of EOs, including the species, part of the plant, soil composition, age and vegetative cycle stage, geographical origin, and also the extraction method, and consequently their bioactive properties (Angioni, Barra, Coroneo, Dessi, & Cabras, 2006; Bakkali et al., 2008; Mejri, Abderrabba, & Mejri, 2010; Viljoen, Subramoney, Vuuren, Başer, & Demirci, 2005). Therefore it is important to know the whole chemical composition because the activity of the main components may be modulated by other minor compounds (Abdollah, Iraj, & Hamze-Ali, 2013; Hoet, Stévigny, Hérent, & Quetin-Leclercq, 2006). In that sense, for biological purposes, it is necessary to analyze the chemical composition of EOs tested rather than some of its components because the concept of synergism or antagonism appears to be more meaningful.

The aim of this work was to determine the chemical composition of different mandarin essential oils obtained industrially. The cold-pressing EO (EOP) that represent the 99.9% of the commercial EO produced industrially and the cold-pressing followed by steam distillation (EOPD), which is the liquid discharged from cold-pressing oil centrifuge that usually contains approximately 0.1 per cent oil, which may be recovered by steam distillation Moreover, we attempted to determine the inhibitory effects of both essential oils and the main compound (limonene) against planktonic cells and biofilms of *P. aeruginosa* strains, as well as the efficacy of sub-inhibitory bacterial growth concentrations in inhibiting biofilm, virulence factors, and productions of QS autoinducers.

2. Materials and methods

2.1. Mandarin essential oils

Mandarins (*Citrus reticulata*) were cultivated in Argentine, and their essential oils (commercial samples, mandarin essential oils) were obtained by cold-pressing (mandarin EOP) and cold-pressing followed by steam distillation (mandarin EOPD) respectively, during 2012 from Litoral Citrus Company.

2.2. Gas chromatography-mass spectrometry

Gas Chromatography-Mass Spectrometry analysis was carried out with a 5973N Agilent apparatus, equipped with a capillary column (95 dimethylpolysiloxane-5% diphenyl), HP-5MS UI (30 m long and 0.25 mm i.d. with 0.25 μm film thickness). The column temperature program was 60 °C during 5 min, with 3 °C min^{-1} increases to 180 °C, then 20 °C min increases to 280 °C, which was maintained for 10 min. The carrier gas was helium at a flow-rate of 1 mL/min. Split mode injection (ratio 1:30) was employed. Mass spectra were taken over the *m/z* 30–500 range with an ionizing voltage of 70 eV. Kovat's retention index was calculated using co-chromatographed standard hydrocarbons. The individual compounds were identified by MS and their identity was confirmed by comparison of their RIs, relative to C_8–C_{32} *n*-alkanes, and mass spectra with authentic samples or with data already available in the NIST 2005 Mass Spectral Library and in the literature (Adams, 2007).

2.3. Bacterial growth

Two strains of *P. aeruginosa* were used, *P. aeruginosa* ATCC 27853 as reference and a strain isolated from a patient with food poisoning, denominated Hospital Tucumán 5 (HT5). The strain HT5 is resistant to several antibiotics: aztreonam (30 μg), ceftazidime (30 μg); cefepime (30 μg), ciprofloxacin (5 μg); gentamicin (10 μg); imipenem (10 μg), meropenem (10 μg) as well as piperacilin-tazobactam (110 μg). However, it is sensitive to amikacin (30 μg).

Overnight cultures of two strains of *P. aeruginosa* were diluted to reach an OD (0.125 ± 0.005) at 560 nm in Luria–Bertani (LB) medium.

The diluted culture (180 μL) was placed in one of the 96 wells of a microtitre polystyrene plate. Solutions of mandarin cold-pressing EO, cold-pressing and distillated EO and pure limonene, the main component of both EOs, in DMSO/distilled water (1:1) were prepared separately, and 20 μL of each one was pipetted to the plastic microtitre plate wells individually (8 replicates) in order to reach final concentrations of 4, 2, 1, 0.5, 0.2, and 0.1 mg/mL, respectively.

Control wells (8 replicates) contained the diluted culture (180 μL) and 20 μL of a solution of DMSO/water (1:1) in which the final concentration of DMSO was 2.5%. Bacteria grew in LB medium at 37 °C and growth was detected as turbidity (600 nm) using a microtitre plate reader (Power Wave XS2) Biotek, Vermont, USA.

2.4. Biofilm formation assay

For biofilm quantification, a micro method based on a protocol previously reported was employed (O'Toole & Kolter, 1998).

Biofilms formed after 24 h incubation of bacterial cultures prepared as described in the previous paragraph, were stained with 20 μL of an aqueous solution of crystal violet, (0.1% w/v) for 20 min. After washing with water, the liquid was discarded from the wells and the material that remained fixed to the polystyrene (containing biofilm) was washed with PBS (thrice). Crystal violet bound to biofilm was removed from each well employing 200 μL absolute ethanol during 30 min at 37 °C with shaking. Absorbance (540 nm) of ethanol solutions of crystal violet was determined using a microtitre plate reader (Power Wave XS2. Biotek, Vermont, USA). Ciprofloxacin, a known biofilm inhibitor, was incorporated in the same bioassay as a positive control at 5 μg/mL. At this concentration, ciprofloxacin inhibited the biofilm formation but did not significantly modify the bacterial growth (Sandasi, Leonard, VanVuuren, & Viljoen, 2011).

2.5. Biofilm metabolic activity assay

The metabolic activity of the biofilm formed by the bacteria was assessed using a 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) reduction assay with the same modifications (Jadhav, Shah, Bhave, & Palombo, 2013; Schillaci, Arizza, Dayton, Camarda, & Stefano, 2008). The wells were incubated with 200 μL of each *P. aeruginosa* strain (DO 0.09 ± 0.02) during 24 h. The preformed biofilm after 24 h incubation was gently removed and the plates were air-dried. After that, 20 μL of the above mentioned concentration of both EOs and limonene were incorporated into each well containing 180 μL of PBS (pH 6.5), and incubated by 24 h at 37 °C, and then the microplate was washed again. As a final step, 100 μL of MTT solution (0.5 mg/mL) was pipetted into each well and incubated for 3 or 6 h at 37 °C under sterile conditions. The insoluble purple formazan (obtained by enzymatic hydrolysis of MTT by the dehydrogenase enzyme found in living cells) was further dissolved in dimethyl sulphoxide (DMSO, Sigma–Aldrich). Finally, the absorbance was then measured at 570 nm using the microplate reader.

2.6. Quantification of N-acyl homoserine lactones (AHLs)

AHLs were measured to determinate the QS inhibition by the β-galactosidase activity assay. In this assay a reporter strain was employed. *P. aeruginosa* qsc 119 is a mutant donated by P. Greenberg (Whiteley, Lee, & Greenberg, 1999) that cannot produce its own AHLs (QS signal molecules). The reporter strain responds by producing β-galactosidase, to exogenous active signal molecules generated by wild types *P. aeruginosa* strains. *P. aeruginosa* qsc 119 was constructed using a chromosomal promoter under the control of AHLs linked to lacZ. In consequence, β-galactosidase activity is under QS-control and in direct relationship with the autoinducers (AHLs) activity (Whiteley et al., 1999).

An overnight culture of the reporter strain grown at 37 °C in LB was diluted ten times in the same medium, reaching values of absorbance of 0.26 at OD_{560nm}. A 100 μL portion of this suspension was mixed in each microplate well with 100 μL cell-free culture supernatant obtained from *P. aeruginosa* (ATCC 27853 or HT5) cultured in LB media containing 4, 2, 1, 0.5, 0.2, and 0.1 mg/mL of mandarin EOs during 24 h. Azithromycin (5 μg/mL). This interferes with the QS process without modifying bacterial growth and was used as QS positive control (Tateda et al., 2001). Control wells (8 replicates) containing cell-free culture supernatant (100 μl) obtained from each strain of *P. aeruginosa* (ATCC 27853 or HT5) cultured in LB media (180 μL) plus 20 μl of DMSO-water (1:1). β-galactosidase activity was measured spectrophotometrically by Miller test (Miller, 1972).

2.7. Elastase B activity assay

Elastolytic activity was determined using a modification of the method described by Caballero et al. (2001). Elastin Congo red (100 μL) (Sigma) dissolved in Tris–HCl (pH 8.0) at a concentration of 5 mg/mL was mixed with 100 μL cell-free culture supernatant from each *P. aeruginosa* grown, during 24 h, in LB media containing 4, 2, 1, 0.5, 0.2, and 0.1 mg/mL of both mandarin EOs and limonene. The reaction mixture (200 μl) was incubated at 37 °C for 24 h and centrifuged at 13,000 rpm for 10 min. The absorbance (at 495 nm) of each supernatant is a measure of the enzyme activity.

2.8. Statistical data analysis

Differences in the mean values were evaluated by analysis of variance (ANOVA). The Tukey test was used for all pair-wise multiple comparisons of groups. In all analyses, values of $p < 0.05$ were considered statistically different (Statistix 7.1, 2002).

3. Results

The chemical composition of commercial mandarin essential oils was determined by GC/MS analysis. Fifty-nine compounds accounting of between 99.34 and 99.75% of the total essential oils were identified. Components were clustered (Table 1) in homologous series of monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes and others and then listed according to Kovat's retention index calculated in GC on apolar HP-5MS column.

3.1. Effects of both EOs and limonene on bacterial growth

The effects of mandarin EOs and limonene (main compound) on the *P. aeruginosa* growth are shown in Table 2 in comparison with the control experiment.

The natural products of mandarin did not show substantial inhibition on the *P. aeruginosa* ATCC 27853 growth. The cell growth inhibition of EOP was 17% 15% and 14% at 4, 2, and 1 mg/mL, respectively. EOPD and pure limonene did not diminish the bacterial growth (Table 2).

The growth inhibitory effect of EOP, EOPD and limonene against the *P. aeruginosa* HT5 was moderate. The higher concentration assayed (4 mg/mL) produced an inhibition of 33, 25, and 24%, respectively. At the lower concentration assayed (0.1 mg/mL) the growth reduction was 15, 10, and 9%, respectively (Table 2).

For both strains the bacterial growth inhibition was correlated with the concentration assayed and was higher due the presence of EOP.

3.2. Biofilm biomass

Mandarin EOP inhibited the biofilm formation of *P. aeruginosa* ATCC 27853. The inhibition was 82, 69, 43, 31, 24, and 15% in presence of 4, 2, 1, 0.5, 0.2, and 0.1 mg/mL, respectively. The mandarin EOPD inhibited 88, 82, 81, 74, 68 and 46% the amount of biofilm at concentration of 4, 2, 1, 0.5, 0.2, and 0.1 mg/mL. In contrast, the pure limonene, main constituent of EOs at 4, 2, 1, 0.5, 0.2, and 0.1 mg/mL, inhibited 49, 49, 45, 43, 36, and 36%, respectively (Table 3).

EOP inhibited the *P. aeruginosa* HT5 biofilm development. The diminution was 73, 62, 59, 53, 51, and 46% in presence of 4, 2, 1, 0.5, 0.2, and 0.1 mg/mL, respectively (Table 3). With respect to the inhibitory properties of EOPD against *P. aeruginosa* clinical strain the decrease of biofilm mass was 92, 85, 84, 81, 75, and 49%, whereas pure limonene inhibited 58, 42, 39, 38, 33, and 33%,

Table 1
Chemical composition of commercial mandarin essential oils.

RI	Compound	Peak area (%) Mandarin EOP	Peak area (%) Mandarin EOPD
Monoterpene hydrocarbons		**97.657**	**96.584**
926	α-Thujene	0.106	0.107
933	α-Pinene	1.151	1.195
972	Sabinene	0.185	0.124
974	β-Pinene	0.866	1.263
990	Myrcene	2.508	3.423
1035	Limonene	89.819	86.873
1056	*trans*-Ocimene	0.080	0.101
1066	γ-Terpinene	2.763	3.263
1087	Terpinolene	0.179	0.231
1112	1,3,8-*p*-Menthatriene	–	0.004
Oxygenated monoterpenes		**0.655**	**1.296**
1098	Linalool	0.244	0.706
1134	*cis*-Limonene oxide	–	0.014
1135	*cis*-*p*-Mentha-2,8-dien-1-ol	–	0.007
1138	*trans*-Limonene oxide	–	0.011
1152	Citronellal	0.076	0.040
1174	Terpinen-4-ol	0.025	0.148
1186	α-Terpineol	0.055	0.190
1238	Neral	0.049	0.052
1240	Carvone	0.022	0.030
1267	Geranial	0.059	–
1269	Perilla aldehyde	0.021	–
1350	Citronellyl acetate	0.019	0.006
1361	Neryl acetate	0.059	0.029
1380	Geranyl acetate	0.026	0.054
1405	Limonen-10-yl-acetate	–	0.009
Sesquiterpene hydrocarbons		**0.424**	**1.237**
1345	δ-Elemene	0.004	0.067
1369	α-Copaene	0.049	0.082
1383	β-Cubebene	0.033	–
1385	β-Elemene	0.017	0.267
1411	β-Caryophyllene	0.024	0.044
1422	β-Copaene	–	0.023
1427	γ-Elemene	–	0.004
1430	α-*trans*-Bergamotene	0.007	0.010
1432	α-Guaiene	–	0.003
1446	α-Humulene	–	0.056
1452	*trans*-β-Farnesene	0.054	0.018
1474	Germacrene D	0.100	0.161
1482	Valencene	–	0.019
1488	Bicyclogermacrene	0.016	0.027
1492	α-Muurolene	0.004	0.009
1496	Germacrene A	0.006	0.068
1501	α-Farnesene	0.064	0.265
1516	δ-Cadinene	0.046	0.096
1549	Germacrene B	–	0.018
Oxygenated sesquiterpenes		**0.005**	**0.122**
1690	β-Sinensal	0.005	0.027
1747	α-Sinensal	–	0.095
Others		**0.601**	**0.635**
999	Octanal	0.138	–
1079	Octanol	–	0.030
1101	Nonanal	0.067	0.057
1201	Decanal	0.315	0.430
1210	Octanol acetate	–	0.007
1259	2*E* Decenal	–	0.004
1301	Undecanal	0.016	0.016
1311	2*E*,2*Z* Decadienal	–	0.005
1399	dimethyl Anthranilate	0.006	–
1402	Dodecanal	0.053	0.073
1442	2*Z*,6*E* Dodecadien-1-al	–	0.006
1461	2*E* Dodecenal	–	0.007
1972	Hexadecanoic acid	0.006	–
Total		**99.342**	**99.752**

RI, retention index relative to C_8–C_{32} *n*-alkane on HP-5MS column.

respectively (Table 3).

The inhibition of biofilm formation by the ATTC and HT5 strains at 1000 and 200 μg/mL of EOPD, respectively was similar to the inhibition of ciprofloxacin (5 μg/mL).

Biofilm inhibition by EOs was higher than the growth inhibition. The relation between biofilm production (measured at DO540 nm)/bacterial growth (measured at DO560 nm) was defined as specific biofilm produced, i.e., the amount of biofilm each bacterium forms. The specific biofilm production by *P. aeruginosa* ATCC 27853 for the control media was 1.17. When it is lower than control, it indicates an

Table 2
Effect of Mandarin EOs on *Pseudomonas aeruginosa* growth.

Growth	*Pseudomonas aeruginosa* ATCC 27853			*Pseudomonas aeruginosa* HT5		
Concentration mg/mL	EOP	EOPD	Limonene	EOP	EOPD	Limonene
0.1	0.923 ± 0.042[a]	0.995 ± 0.014[a]	1.061 ± 0.015[b]	1.176 ± 0.026[*,a]	1.210 ± 0.062[*,a]	1.264 ± 0.031[*,a]
0.2	0.889 ± 0.055[*,a]	0.982 ± 0.022[b]	1.054 ± 0.023[c]	1.172 ± 0.032[*,a]	1.212 ± 0.020[*,b]	1.253 ± 0.046[*,b]
0.5	0.871 ± 0.049[*,a]	0.991 ± 0.055[b]	1.016 ± 0.015[b]	1.154 ± 0.083[*,a]	1.215 ± 0.046[*,b]	1.229 ± 0.060[*,b]
1	0.872 ± 0.027[*,a]	0.981 ± 0.037[b]	1.022 ± 0.021[b]	1.071 ± 0.081[*,a]	1.188 ± 0.043[*,b]	1.215 ± 0.041[*,b]
2	0.849 ± 0.049[*,a]	0.978 ± 0.040[b]	1.019 ± 0.018[b]	0.971 ± 0.044[*,a]	1.098 ± 0.050[*,b]	1.176 ± 0.044[*,c]
4	0.828 ± 0.051[*,a]	0.915 ± 0.075[*,b]	0.987 ± 0.019[b]	0.925 ± 0.034[*,a]	1.038 ± 0.088[*,b]	1.046 ± 0.012[*,b]
Control	1.004 ± 0.050			1.383 ± 0.041		
Ciprofloxacin	0.184 ± 0.012			0.350 ± 0.016		

Effects of mandarin EOS (after 24 h incubation) on *P. aeruginosa* ATCC 27853 and *P. aeruginosa* HT5 growth (Absorbance at 560 nm), were determined. Ciprofloxacin concentration: 5 μg/ml. Data are expressed as means ± standard deviation ($n = 9$).
*Significant difference compared to each control ($P < 0.05$).
[a,b,c] Values with different superscripts are significantly different between EOs with equal concentrations.

Table 3
Effect of Mandarin EOs on *Pseudomonas aeruginosa* biofilm formation.

	Pseudomonas aeruginosa ATCC 27853			*Pseudomonas aeruginosa* HT5		
Concentration mg/mL	EOP	EOPD	Limonene	EOP	EOPD	Limonene
0.1	1.000 ± 0.039[*,a]	0.639 ± 0.004[b]	0.752 ± 0.001[b]	0.747 ± 0.010[*,a]	0.704 ± 0.047[a]	0.933 ± 0.017[b]
0.2	0.898 ± 0.020[a]	0.372 ± 0.013[b]	0.752 ± 0.001[c]	0.679 ± 0.011[a]	0.349 ± 0.047[b]	0.927 ± 0.027[c]
0.5	0.810 ± 0.061[a]	0.307 ± 0.011[b]	0.679 ± 0.003[c]	0.652 ± 0.032[a]	0.261 ± 0.046[b]	0.860 ± 0.043[c]
1	0.667 ± 0.082[a]	0.212 ± 0.052[b]	0.643 ± 0.003[a]	0.574 ± 0.032[a]	0.219 ± 0.034[b]	0.848 ± 0.010[c]
2	0.367 ± 0.089[a]	0.219 ± 0.028[b]	0.603 ± 0.005[c]	0.522 ± 0.031[a]	0.205 ± 0.010[b]	0.812 ± 0.034[c]
4	0.215 ± 0.049[a]	0.146 ± 0.016[b]	0.598 ± 0.008[c]	0.368 ± 0.010[a]	0.110 ± 0.016[b]	0.586 ± 0.020[c]
Control	1.175 ± 0.011			1.389 ± 0.069		
Ciprofloxacin	0.286 ± 0.004			0.327 ± 0.016		

Effects of mandarin EOS (after 24 h incubation) on *P. aeruginosa* ATCC 27853 and *P. aeruginosa* HT5 biofilm (determined with crystal violet at 0.1%) were determined. Ciprofloxacin concentration: 5 μg/ml. Data are expressed as means ± standard deviation ($n = 9$).
*All the results obtained have significant difference compared to respective control ($P < 0{\cdot}05$).
[a,b,c] Values with different superscripts are significantly different between EOs with equal concentrations($P < 0.05$).

inhibitory effect on the specific production of biofilm (Amaya et al., 2012). Under the conditions studied the EOs and limonene tend to reduce biofilm specific production, decreasing from 1.08 to 0.26 for EOP; from 0.64 to 0.16 for EOPD, and from 0.71 to 0.61 for limonene, respectively, when the concentration increased from 0.1 to 4 mg/mL.

The specific biofilm production by *P. aeruginosa* HT5 for the control media was 1.00. The specific biofilm formation decreased from 0.63 to 0.40 for EOP; from 0.58 to 0.1 for EOPD and from 0.74 to 0.56 for limonene, respectively, when the concentration increased from 0.1 to 4 mg/mL.

3.3. Bacterial viability into biofilm

The viable cells of the ATCC strain in biofilm showed a percentage of inhibition ranging from 33 to 25% to the concentration range from 4 to 0.1 mg/mL of EOP and EOPD, as well as between 34 and 11% in the same range of concentrations of limonene (Table 4). On the other hand, for HT5 strain after 24 h exposition of EOP the decrease was 59, 57, 52, 53, 49, and 22% of the concentration ranging of 4 to 0.1 mg/mL (Table 4). In presence of EOPD the inhibition of biofilm viability was 60, 61, 61, 53, 45, and 41% at 4, 2, 1, 0.5, 0.2, and 0.1 mg/mL, respectively. Limonene alone had lower cell viability inhibition (43, 33, 31, 21, 15, and 13%, respectively) at the same concentrations.

3.4. Effects of mandarin EOs on bacterial signal molecules

As shown in Table 5, mandarin EOP, EOPD, as well as limonene against *P. aeruginosa* strains inhibit AHLs production.

At 4 mg/mL EOP, EOPD and limonene reduced 49, 50, and 34% the AHLs production by *P. aeruginosa* ATCC. At the same concentration, the autoinducers' inhibition was 38, 37, and 30% by the HT5 strain.

It is important to highlight that comparable results correlated with the concentration used were obtained at lower concentrations of mandarin EOs assayed against both bacterial strains.

The inhibition of AHLs by EOs was higher than that of growth inhibition. The relationship between AHL production (measured at β-galactosidase units)/bacterial growth (measured at DO560 nm) was defined as specific AHLs produced, the AHLs that each bacterium forms. The specific production of autoinducers for the control media by *P. aeruginosa* ATTC 27853 was 18.93. When it is lower than control, it shows that specific production is lower than that of control. Under the conditions studied the EOs and limonene tend to reduce autoinducer-specific production. The specific AHL production decreased from 14.41 to 11.69 for EOP; from 12.31 to 10.43 due to EOPD, and from 15.00 to 12.69 for limonene, respectively, when the concentration increased from 0.1 to 4 mg/mL.

The autoinducer-specific production for the control media by *P. aeruginosa* HT5 was 13.01. Under the conditions studied EOs and limonene tend to reduce autoinducer-specific production. However, in this case the specific AHL production increased from 10.47 to 12.07 for EOP; from 9.95 to 10.85 for EOPD and from 11.86 to 12.00 for limonene, respectively, when the concentration increased from 0.1 to 4 mg/mL.

For both strains the specific AHL production in presence of the antibiotic azithromycin was higher than the control media, 50.38 and 29.92 for ATCC and food poising isolated strain, respectively, suggesting that the stress due to growth inhibition appears to have increased autoinducer-specific production.

Table 4
Effect of Mandarin EOs on *Pseudomonas aeruginosa* biofilm cell viability.

MTT	*Pseudomonas aeruginosa* ATCC 27853			*Pseudomonas aeruginosa* HT5		
Concentration mg/mL	EOP	EOPD	Limonene	EOP	EOPD	Limonene
0.1	1.531 ± 0.003^{*,a}	1.529 ± 0.075^{a}	1.833 ± 0.055^{b}	1.108 ± 0.046*	0.831 ± 0.012^{b}	1.228 ± 0.004^{c}
0.2	1.519 ± 0.028^{a}	1.474 ± 0.081^{a}	1.832 ± 0.055^{b}	0.720 ± 0.020^{a}	0.774 ± 0.014^{a}	1.208 ± 0.004^{b}
0.5	1.510 ± 0.059^{a}	1.492 ± 0.038^{a}	1.580 ± 0.028^{b}	0.670 ± 0.011^{a}	0.666 ± 0.035^{a}	1.122 ± 0.004^{b}
1	1.507 ± 0.073^{a}	1.465 ± 0.021	1.350 ± 0.037	0.686 ± 0.018^{a}	0.550 ± 0.051^{b}	0.980 ± 0.006^{c}
2	1.460 ± 0.083^{a}	1.360 ± 0.028^{a}	1.329 ± 0.026^{a}	0.607 ± 0.010^{a}	0.547 ± 0.010^{b}	0.951 ± 0.005^{c}
4	1.381 ± 0.089^{a}	1.348 ± 0.047^{a}	1.319 ± 0.050^{a}	0.588 ± 0.049^{a}	0.555 ± 0.004^{b}	0.815 ± 0.010^{c}
Control	2.036 ± 0.020			1.419 ± 0.015		
Ciprofloxacin	0.477 ± 0.015			0.331 ± 0.005		

Effects of mandarin EOS (after 24 h incubation) on *P. aeruginosa* ATCC 27853 and *P. aeruginosa* HT5 cell viability (within microplate-established biofilm) were determined. Ciprofloxacin concentration: 5 μg/ml. Data are expressed as means ± standard deviation ($n = 9$).
*All the results obtained have significant difference compared to respective control.
^{a,b,c} Values with different superscripts are significantly different between EOs with equal concentrations($P < 0.05$).

Table 5
Effect of Mandarin EOs on *Pseudomonas aeruginosa* autoinducers (AHLs) production.

	Pseudomonas aeruginosa ATCC 27853			*Pseudomonas aeruginosa* HT5		
Concentration mg/mL	EOP	EOPD	Limonene	EOP	EOPD	Limonene
0.1	13.300 ± 0.005^{*,a}	12.242 ± 0.010^{b}	15.923 ± 0.004^{c}	12.322 ± 0.005^{*,a}	12.040 ± 0.006^{b}	14.991 ± 0.004^{c}
0.2	12.665 ± 0.004^{a}	12.183 ± 0.002^{b}	15.590 ± 0.004^{c}	12.233 ± 0.004^{a}	11.800 ± 0.003^{b}	14.745 ± 0.002^{c}
0.5	11.772 ± 0.008^{a}	10.482 ± 0.014^{b}	15.170 ± 0.024^{c}	12.233 ± 0.010^{a}	11.500 ± 0.003^{b}	14.490 ± 0.004^{c}
1	10.043 ± 0.012^{a}	10.091 ± 0.006^{b}	14.491 ± 0.005^{c}	11.700 ± 0.007^{a}	11.354 ± 0.003^{b}	13.610 ± 0.014^{c}
2	10.110 ± 0.007^{a}	10.210 ± 0.010^{b}	14.022 ± 0.004^{c}	11.400 ± 0.022^{a}	11.200 ± 0.002^{b}	13.051 ± 0.003^{c}
4	9.681 ± 0.007^{a}	9.550 ± 0.006^{b}	12.522 ± 0.005^{c}	11.171 ± 0.005^{a}	11.261 ± 0.010^{b}	12.552 ± 0.003^{c}
Control	19.000 ± 0.003			18.000 ± 0.003		
Ciprofloxacin	9.250 ± 0.010			10.461 ± 0.006		

Effects of mandarin EOS (after 24 h incubation) on *P. aeruginosa* ATCC 27853 and *P. aeruginosa* HT5 AHLs production (determined as β-galactosidase units using *P. aeruginosa* qsc 119 as reporter strain) were determined. Ciprofloxacin concentration: 5 μg/ml. Data are expressed as means ± standard deviation ($n = 9$) $P \leq 0.05$.
*All the results obtained have significant difference compared to respective control.
^{a,b,c} Values with different superscripts are significantly different between EOs with equal concentrations ($P < 0.05$).

3.5. Effects of mandarin EOs against elastase activity of *P. aeruginosa* strains

The mandarin EOP and EOPD diminished the elastase activity of *P. aeruginosa* ATCC 27853 in values higher than 77% at all the concentrations assayed. The presence of limonene decreased from 75 to 52% of the enzyme activity between the higher and lower concentrations used (Table 6).

The inhibition of elastase of the *P. aeruginosa* HT5 strain was ranged between 72 and 88% for the presence of EOP, and from 82 to 85% by the addition of EOPD, to the media. The major compounds found in essential oils reduced the range from 80 to 66% (Table 6). It is worth noting that the wild strain exhibits 72% higher elastase activity than the collection strain, which demonstrates its pathogenicity.

The relationship between elastase inhibition and AHL production was lower by all the EOs assayed, suggesting that the enzyme inhibition had not only decreased in the enzyme production coordinate by QS, but also because of enzyme activity inhibition.

4. Discussion

4.1. Essential oils composition

In mandarin essential oils, high quantities of monoterpene hydrocarbons (96.58–97.66%) were found. In both analyzed essential oils limonene (89.82 and 86.87%, respectively), followed by γ-terpinene (2.76–3.26%) myrcene (2.51–3.42%) and α-pinene

Table 6
Effect of Mandarin EOs on *Pseudomonas aeruginosa* elastase B activity.

	Pseudomonas aeruginosa ATCC 27853			*Pseudomonas aeruginosa* HT5		
Concentration mg/mL	EOP	EOPD	Limonene	EOP	EOPD	Limonene
0.1	0.027 ± 0.005^{*,a}	0.029 ± 0.003^{a}	0.078 ± 0.002^{b}	0.079 ± 0.002^{*,a}	0.052 ± 0.010^{b}	0.097 ± 0.001^{c}
0.2	0.027 ± 0.010^{a}	0.030 ± 0.003^{a}	0.078 ± 0.002^{b}	0.051 ± 0.010^{a}	0.048 ± 0.005^{a}	0.097 ± 0.001^{b}
0.5	0.031 ± 0.004^{a}	0.031 ± 0.004^{a}	0.057 ± 0.004^{b}	0.052 ± 0.004^{a}	0.048 ± 0.010^{a}	0.094 ± 0.004^{b}
1	0.033 ± 0.005^{a}	0.033 ± 0.001^{a}	0.050 ± 0.004^{b}	0.051 ± 0.010^{a}	0.049 ± 0.010^{a}	0.089 ± 0.002^{b}
2	0.036 ± 0.006^{a}	0.034 ± 0.002^{a}	0.043 ± 0.004^{a}	0.041 ± 0.010^{a}	0.050 ± 0.005^{a}	0.072 ± 0.004^{b}
4	0.038 ± 0.004^{a}	0.034 ± 0.001^{a}	0.040 ± 0.003^{a}	0.035 ± 0.010^{a}	0.034 ± 0.002^{a}	0.058 ± 0.004^{b}
Control	0.165 ± 0.004			0.284 ± 0.002		
Ciprofloxacin	0.023 ± 0.003			0.093 ± 0.005		

Effects of mandarin EOS (after 24 h incubation) on *P. aeruginosa* ATCC 27853 and *P. aeruginosa* HT5 elastase activity were determined. Ciprofloxacin concentration: 5 μg/ml. Data are expressed as means ± standard deviation ($n = 9$).
*All the results obtained have significant difference compared to respective control.
^{a,b,c} Values with different superscripts are significantly different between EOs with equal concentrations($P < 0.05$).

(1.15–1.19%) were the main compounds.

Among the oxygenated monoterpenes and sesquiterpene fractions, qualitative and quantitative differences were found. Mandarin EOPD contains more quantities in these fractions than mandarin EOP (0.65 *vs* 1.30%; 0.42 *vs* 1.24% and 0.005 *vs* 0.12%, respectively). So, *cis*-limonene oxide, *cis*-*p*-mentha-2,8-dien-1-ol, *trans*-limonene oxide and limonene-10-yl acetate were only found in mandarin EOPD whereas geranial and perilla aldehyde was only identified in mandarin EOP.

According with previous reported previous reports about commercial *Citrus* EOs, the steam-distilled citrus oils (EOPD), that represent the oils that remains in the peel and effluent after pressing and separation is removed by distillation and is obtained as result of processing this fruit for juice, are inferior to cold-pressing oil (EOP), both in flavor and keeping characteristics. In other words, there is a marked difference in the chemical and physical properties of the two (Sinclair, 1972). Maybe the EOPD with lower commercial value can be revalued if we consider their anti-pathogenic properties and their potential as food preservatives. Indeed biofilm inhibition at low concentrations by EOPD is higher than the EOP.

The results reflect those of previous works in which limonene is the major chemical component of citrus EOs, ranging from 32 to 98% (Svoboda & Greenaway, 2003). Moreover, in an EO from Spain similar compounds were reported: α-pinene, sabinene, β-pinene, myrcene, octanal, *p*-cymene, linalool, *cis*-limonene oxide, *cis*-*p*-mentha-2,8-dien-1-ol, citronellal, decanal, carvone, perilla aldehyde, β-cubenene, β-copaene, and valencene (Espina et al., 2011).

However, other compounds found in the EOs in Spain such as: *trans*-*p*-mentha-2,8-dienol, *cis*-pinocarveol, cryptone, *p*-mentha-1,8-dien-7-ol, *trans*-carveol, *cis*-carveol, (+)-*p*-mentha-1,8-dien-3-one, thymol, carvacrol, perilla alcohol, (*Z*)-patchenol, (*E*)-patchenol, and (*Z*)-trimenal were not found in the mandarin essential oils assayed here. These results indicated the impact of the region on the EO composition.

4.2. Antibacterial and antipathogenic properties

In the present study, the antibacterial activity of mandarin EOs was poor. This result is in concordance with a previous report suggesting that essential oils would have to be used in considerable concentrations to achieve an antibacterial potential (Gibriel, Hanan, Rady, & Abdelaleem, 2013; Hanamanthagouda et al., 2010). Moreover, neither of the mentioned components of *Citrus reticulata* EOs assayed individually is able to inhibit *P. aeruginosa* growth at the concentration present in the EOs (Ahmad, Viljoen, & Chenia, 2014).

However, the mandarin EOs could be considered as antipathogenic because they inhibit the production of biofilm and AHLs, as well as the cells' metabolism into biofilm and the elastase activity of *P. aeruginosa* strains. These results suggest that the inhibition effects displayed by the EOP and EOPD did not obey exclusively to their action on the growth and that the QS mechanism was involved, coherently, with previous studies (Amaya et al., 2012; Cartagena et al., 2014; Gilabert, Cartagena, Escobar, Bardón, & Arena, 2014; 2015).

The most significant decrease of the biofilm formation of EOPD with respect to EOP and limonene could be attributed to the different composition of the minor constituents. The concentration of linalool and α-terpineol in EOPD is 3 times the value of EOP, furthermore γ-terpinene and terpinolene have a higher concentration of EOPD.

The antibiofilm property of linalool against *Candida albicans* was described previously (Hsu, Lai, Chuang, Lee, & Tsai, 2013). In addition, α terpineol and linalool are informed as inhibitors of violacein production mediated by QS in *Chromobacterium violaceum* (Ahmad et al., 2014).

The presence of QS inhibitors in natural foods is interesting and indeed there are several vegetables and herbs that are non-toxic to humans and readily available. The essential oils of cinnamon (Niu, Alfre, & Gilbert, 2006) and clove (Khan, Zahin, Hasan, Husain, & Ahmad, 2009) are also known to possess QSI potentials. The exclusion or reduction of chemical additives used in a wide variety of foods is demanded, while natural or GRASS additives are seen as a benefit for both quality and safety. The use of essential oils in consumer goods is expected to increase in the future owing to the rise of green consumerism, which stimulates the use and development of plant-derived products (De Silva, 1995), because both consumers and regulatory agencies are more comfortable with the use of natural antimicrobials.

To conclude, the present study showed the ability of low concentration of mandarin EOs to interfere the QS, the biofilm formation, cell viability into biofilm, the AHLs production and the elastase enzyme of *P. aeruginosa*. For these reasons it proposes that *Citrus reticulata* essential oils could be considered as suitable alternatives to control *P. aeruginosa* pathogenicity. Moreover if is taken into account, the lower use and production of EOPD, its lower commercial values as well as its higher antipathogenic activity; this EOs is the best promising alternative as food preservative.

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