RESEARCH ARTICLE

Prevention of polymicrobial biofilms composed of *Pseudomonas aeruginosa* and pathogenic fungi by essential oils from selected Citrus species

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One sentence summary: Pompia and grapefruit essential oils inhibit formation of monomicrobial and mixed polymicrobial biofilms, and may have the potential for prophylactic application against polymicrobial infections.

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ABSTRACT

Mixed microbial infections caused by *Pseudomonas aeruginosa* and pathogenic fungi are commonly found in patients with chronic infections and constitute a significant health care burden. The aim of this study was to address the potential polymicrobial antibiofilm activity of pompia and grapefruit essential oils (EOs). The mechanism of antimicrobial activity of EOs was analysed. EOs of pompia and grapefruit inhibited fungal growth with MIC concentrations between 50 and 250 mg L^{-1}, whereas no effect on *P. aeruginosa* growth was observed. Both citrus EOs inhibited formation of bacterial and fungal monomicrobial biofilms in concentrations of 50 mg L^{-1} and were efficient in potentiating the activity of clinically used antimicrobials *in vitro*. The concentration of 10 mg L^{-1} EOs inhibited mixed biofilm formation composed of *P. aeruginosa* and *Aspergillus fumigatus* or *Scedosporium apiospermum*. Citrus EOs affected quorum sensing in *P. aeruginosa* and caused fast permeabilisation of *Candida albicans* membrane. Pompia and grapefruit EOs potently inhibited biofilm formation and could be used for the control of common polymicrobial infections.

Keywords: polymicrobial biofilms; quorum sensing; *Pseudomonas aeruginosa*; antifungal; essential oils

INTRODUCTION

Biofilms are thin layers of microorganisms embedded in an extracellular polymeric matrix that strongly adhere to the surfaces of both organic and inorganic structures. Biofilms play an important role in virulence of many pathogenic bacteria including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *S. epidermidis* and common *Enterobacteriaceae* (Costerton, Stewart and Greenberg 1999; Singh *et al.* 2000; Bryers 2008). In a biofilm, microorganisms can be up to 1000-fold more resistant to antibiotics than planktonic (free-living) bacteria and can efficiently evade immune system (Oppenheimer-Shaanan, Steinberg and Kolodkin-Gal 2013). Biofilms can form in any place in the human body

where the host defense is compromised, such as in the lungs of patients with cystic fibrosis (CF), or in chronic wounds that have an impaired blood supply (Singh *et al.* 2000). Biofilm-related infections have also been found in patients with chronic otitis media and in patients with ventilator-associated pneumonia and tuberculosis (Burmolle *et al.* 2010). Biofilms associated with the cases of these diseases are polymicrobial in nature with bacteria coexisting with pathogenic yeasts or filamentous fungi (Peters *et al.* 2012). Polymicrobial infections and associated biofilms are harder to treat because of increased resistance to antimicrobial therapy, and usually have increased mortality outcomes compared with their monomicrobial counterparts (Harriott and Noverr 2011; Pammi *et al.* 2014).

Pseudomonas aeruginosa is a clinically important opportunistic human pathogen often associated with multidrug-resistant infections. Beside *P. aeruginosa*, which is the major coloniser of the CF lungs, other bacteria such as *Burkholderia cepacia* and *S. aureus* and fungi including *Candida albicans*, *Aspergillus fumigatus* and *Scedosporium* species are frequently coexisting (Peters *et al.* 2012). Among the numerous *Aspergillus* isolates recovered from the respiratory tracts of patients with CF, *A. fumigatus* is the most predominant species, followed by *A. terreus* (Bakare *et al.* 2003).

Pseudomonas aeruginosa virulence and biofilm formation are under control of quorum-sensing system (QS). In *C. albicans*, growth, stress resistance, morphogenesis and biofilm formation are also regulated by QS (Kruppa 2009). QS generally allows bacteria and fungi to monitor their cell density through the release of specific signaling molecules called autoinducers. At a high cell density, autoinducers' concentration reaches threshold initiating the signaling cascade that regulates expression of different genes, many of them being required for microbial pathogenicity (Schuster *et al.* 2003).

Essential oils (EOs) are complex mixtures of volatile compounds extracted from plants. Monoterpene and sesquiterpene hydrocarbons as well as their oxygenated derivatives are among the most abundant volatile constituents of citrus EOs (Jing *et al.* 2014). Numerous EOs have been reported to have different biological activities including antibacterial, antifungal, anti-insecticidal, anti-inflammatory, anticancer, antioxidant and other properties (Edris 2007). Several EOs have shown the ability to interfere with bacterial QS signaling and to inhibit biofilm formation (Vikram *et al.* 2010; Kerekes *et al.* 2013; Kalia *et al.* 2015; Alves *et al.* 2016). Specific combinations of the major components of EOs have been reported to potentiate activity of conventional antibiotics improving their effectiveness (Liu *et al.* 2015). The marked antimicrobial activity of EOs, their broad-spectrum activity and affordability make EOs a valuable alternative for antibiotics used in disinfectants, topical preparations against multidrug-resistant species or dental care products (Edris 2007). Although citrus EOs are generally recognized as safe, the active concentrations of some EOs do show detrimental effect on intestinal cells, so the toxicity of these compounds should be addressed and clearly documented before any medical application (Fisher and Phillips 2008).

This study addressed the ability of selected citrus EOs to inhibit biofilm formation in common polymicrobial infections. We have shown that pompia (*Citrus* × *monstrousa*) and grapefruit (*Citrus* × *paradisi*) EOs (EO$_P$ and EO$_G$, respectively) efficiently inhibited the formation of monomicrobial and polymicrobial biofilms composed of *P. aeruginosa* and pathogenic fungi. EO$_P$ and EO$_G$ showed the ability to interfere with bacterial QS and to cause *Candida* membrane damage.

MATERIALS AND METHODS

Microbial strains and growth conditions

Pseudomonas aeruginosa PAO1 NCTC 10332, *P. aeruginosa* PA14, *Chromobacterium violaceum* CV026 (McClean *et al.* 1997), *P. aeruginosa* PAOJP2 (pKD-rhlA) (Duan and Surette 2007) and *P. aeruginosa* PA14-R3 (ΔlasI Prsal::lux) (Massai *et al.* 2011) were used in this study. Bacterial strains were grown in Luria Bertani (LB) broth at 37°C on a rotary shaker at 180 rpm.

Fungal strains used in this study were obtained from the collection of National Reference Medical Mycology Laboratory (University of Belgrade, Serbia) including *Candida albicans* (ATCC 10231) and human clinical isolates (*C. parapsilosis* and *Scedosporium apiospermum* (blood isolates), *Aspergillus fumigatus* and *A. terreus* (sputum isolates)). Strains were grown on Sabouraud dextrose agar (HiMedia Laboratories, Mumbai, India) at 37°C for 24 h (yeasts) or 28°C for 2–5 days (molds).

Analysis of EOs

The composition of EO$_P$ was investigated by LC-MS/MS according to the previously reported method (Orcic *et al.* 2014). Standards of the compounds were purchased from Sigma-Aldrich Chem (Steinheim, Germany), Fluka Chemie GmbH (Buchs, Switzerland) or from ChromaDex (Santa Ana, CA, USA).

The Agilent 1200 series liquid chromatograph coupled with Agilent series 6410B electrospray ionization triple-quadruple mass spectrometer and controlled by MassHunter Workstation-B.03.01. software was used for analysis. Components were separated using a Zorbax Eclipse XDB-C18 4.6 mm × 50 mm × 1.8 μm (Agilent Technologies, Santa Clara, CA, USA) reversed-phase column. Compound-specific optimised MS/MS parameters are given in Table S1 (Supporting Information).

Antimicrobial susceptibility tests for planktonic cells

The minimum inhibitory concentration (MIC) of EO$_P$, EO$_G$ (CityCHEM, Belgrade, Serbia), (R)-(+)-limonene (97% purity; Sigma, Munich, Germany), gentamycin (Sigma) and itraconazole (Sigma) were determined according to standard broth microdilution assays recommended by the National Committee for Clinical Laboratory Standards (M07-A8) for bacteria and Standards of European Committee on Antimicrobial Susceptibility Testing (EDef 9.2 and EDef 7.2) for fungi (Casey *et al.* 2004).

Stock solutions of limonene, EO$_P$ and EO$_G$ (100 g L^{-1}, w/v) and itraconazole (4 g L^{-1}, w/v) were prepared in DMSO and stored in dark at 4°C. Gentamycin stock solution (50 g L^{-1}, w/v) was prepared in dH$_2$O and stored in dark at −20°C. The highest concentration of EOs used was 500 mg L^{-1}. The inoculums were 10^5 colony-forming units (cfu) mL^{-1} for bacteria and 2–5 × 10^5 cfu mL^{-1} for fungi. The MIC value corresponds to the lowest concentration that inhibited the growth after 24 h at 37°C for bacteria and yeasts and 48 h at 28°C for molds. DMSO was used for the growth control at the same concentration (the highest concentration 0.5%, v/v) as in the treatments.

Determination of synergistic effect of EO$_P$ and clinically used drugs on microbial growth: Combined effects of EO$_P$ and clinical drugs against *P. aeruginosa* PAO1 or *C. albicans* planktonic growth were determined by the broth microdilution method in 96-well microtiter plates, and the fractional inhibitory concentrations (FICs) were evaluated by the checkerboard method as earlier described with some modifications (Buyck, Tulkens and Van Bambeke 2015). Two-fold serial dilutions of gentamycin (starting at

50 mg L^{-1}) or itraconazole (starting at 5 mg L^{-1}) from horizontal rows of microtiter plate were subsequently cross-diluted vertically by 2-fold serial dilutions of EO$_P$ (starting at 100 mg L^{-1}) and inoculated with bacterial or fungal suspension (final density 10^5 cfu ml^{-1} for bacteria and 2–5 × 10^5 cfu mL^{-1} for fungi). The combined effects were determined based on ΣFIC. For combination of compound A (EO$_P$) and compound B (drug), the ΣFIC is calculated according to the following equation: ΣFIC = FIC$_A$ + FIC$_B$, where FIC$_A$ = MIC$_{A(in the presence of B)}$/MIC$_{A(alone)}$, and FIC$_B$ = MIC$_{B(in the presence of A)}$/MIC$_{B(alone)}$. The data were evaluated according to The European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2000) as follows: synergistic effect if ΣFIC ≤0.5; additive if ΣFIC >0.5 and ≤1; indifferent if ΣFIC >1 and <4; and antagonistic if ≥4. The experiments were repeated two times.

Antimicrobial susceptibility tests for monomicrobial biofilms

Biofilm quantification assays were performed in 96-well microtiter plate format (Sarstedt, Nuembrecht, Germany) using a crystal violet (CV; Serva, Heidelberg, Germany) staining of adherent cells for biomass measurements (Merritt, Kadouri and O'Toole 2005). Overnight culture of *P. aeruginosa* was diluted to 10^6 cfu mL^{-1} in LB and 100 μL was added to the wells in the presence of limonene, EOs or DMSO (0.1%, v/v). Biofilms were formed for 24 h and after washing adherent cells were stained with 0.1% (v/v) CV. The biofilm quantification assays for fungi were done in the same way, using inocula 10^6 cfu mL^{-1} and the RPMI 1640 medium (with glutamine and phenol red, without bicarbonate) buffered with MOPS (Sigma) supplemented with 2% (w/v) glucose.

The effect of EOs on *C. albicans* biofilm formation was also examined by introducing 3 h adhesion phase after inoculation. Following adhesion, supernatant was removed and, after two washing steps with PBS, citrus EOs, limonene or DMSO were applied as described above. Biofilms were quantified using CV or XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; Sigma) assay for cell metabolic activity detection. The extent of XTT reduction to a water-soluble orange formazan compounds was measured in the supernatant of treated cells spectrophotometrically as previously described (Peeters, Nelis and Coenye 2008).

Candida albicans biofilms were also formed using inocula 10^7 cfu mL^{-1} cells in RPMI 1640 medium (with glutamine and phenol red, without bicarbonate) buffered with MOPS supplemented with 0.2% (w/v) glucose. The biofilm formation assays were performed in six wells and repeated at least two times.

To determine combined effect of EO$_P$ with gentamycin or itraconazole on *P. aeruginosa* PAO1 or *C. albicans* biofilm formation, respectively, 2-fold EO$_P$ and the drug dilutions were prepared as described above and inoculated with 10^6 cfu mL^{-1} for bacteria or 10^6 cfu mL^{-1} for fungi. Biofilms were formed 24 h and measured using CV staining. The experiments were repeated two times.

Antimicrobial susceptibility tests for polymicrobial biofilms

Polymicrobial biofilm quantification assays were performed using the CV staining. Both *P. aeruginosa* PAO1 and fungal inocula were adjusted to 10^6 cfu mL^{-1} in Brain Hearth Infusion (BHI) medium (BD Difco, Oxford,, UK). Equal volumes (50 μL each) of *P. aeruginosa* PAO1 and every fungal strain were added to a well and incubated with different concentrations of limonene, EOs

or DMSO (0.1%, v/v) at 37°C. After 24 h, CV staining and biofilm quantification were performed as described above. The experiment was performed in four wells and repeated three times.

Biofilm microscopy

To study the effect of EO$_P$ on mixed biofilms containing *P. aeruginosa* PAO1 and molds, polymicrobial biofilms were developed on glass cover slips using modified method by Manavathu, Vager and Vazquez (2014) and examined under the microscope. Mold inocula were adjusted to 10^6 cfu mL^{-1} in BHI and incubated in plastic petri dish containing glass cover slips. After 24 h, non-adherent fungal cells were removed and the fresh media containing 10^6 cfu mL^{-1} of *P. aeruginosa* PAO1 and EO$_P$ (100 mg L^{-1}) or DMSO (0.1%, v/v) were added. After 48 h, biofilms were washed with 0.9% NaCl and stained with 2.5 μM SYTO9 green fluorescent dye and 2.5 μM propidium iodide (PI) red fluorescent dye of Live/Dead staining kit (LIVE/DEAD® BacLight Bacterial Viability Kit, Thermo Fisher Scientific, Waltham, MA, USA). Cells were observed under a fluorescence microscope (Olympus BX51, Applied Imaging Corp., San Jose, CA, USA) under ×60 magnification.

Quorum sensing inhibition assays

Chromobacterium violaceum disc assay
The assay was performed according to Borges *et al.* (2014) with some modifications. N-(hexanoyl)-l-homoserine lactone (HHL; Sigma) (5 μM) was used as inducer of violacein production in molten semi-solid LB agar (0.3% w/v). Cellulose discs containing limonene, EOs (250 μg/disc) or DMSO (5 μL) were placed on solidified agar and incubated for 24 h at 30°C. Inhibition of violacein synthesis was defined by the presence of white haloes in a purple background.

Pyocyanin assay
Pseudomonas aeruginosa PA14 indicator strain was cultured overnight in the presence of limonene, EOs or 0.1% DMSO, and pyocyanin in the supernatant was quantified using UV–vis spectrophotometer Ultrospec 3300pro (Amersham Biosciences, Piscataway, NJ, USA) at 695 nm as reported previously (O'Loughlin *et al.* 2013). All experiments were performed in triplicate and repeated at least three times.

AHL production assays
AHL production assays were performed as previously described with some modifications (Duan and Surette 2007; Massai *et al.* 2011). For the determination of 3OC12-HSL and C4-HSL levels in culture supernatants, *P. aeruginosa* PAO1 was grown overnight at 37°C in LB. Cultures were diluted 1:1000 in 10 mL LB in the presence of limonene, EOs or DMSO and grown at 37°C. Aliquot of 6 h old culture supernatants (20 μL) was added to 180 μL of LB inoculated with *P. aeruginosa* PA14-R3 (measurements of 3OC12-HSL) or *P. aeruginosa* PAOJP2 (measurements of C4-HSL) (final OD$_{600}$ 0.045). Plates were incubated at 37°C with shaking, and cell density and bioluminescence were simultaneously measured after 4 h of incubation using Tekan Infinite200 multiplate-reader (Tecan Group Ltd, Männedorf, Switzerland). Luminescence values were normalised per cell density.

Fungal membrane damage assay

The effect of EOs on fungal membrane integrity was addressed using PI uptake assay (Cox *et al.* 2000). *Candida albicans* cells (10 mL culture) were grown overnight in SAB medium, washed

Table 1. Antimicrobial activity of citrus EOs and limonene determined after 1 day incubation for *P. aeruginosa* and 2 days incubation for fungi.

Pathogen	MIC (mg L^{-1})[a]		
	EO$_P$	EO$_G$	Limonene
Pseudomonas aeruginosa	>500	>500	>500
Candida albicans	50	50	100
Candida parapsilosis	100	50	100
Aspergillus fumigatus	100	100	>250
Aspergillus terreus	250	250	>250
Scedosporium apiospermum	100	100	>250

The MIC determined as the lowest concentration of compound at which no evident growth was observed. Values are average of two independent experiments performed in four wells.

and resuspended in PBS buffer. Aliquots (0.25 mL) were added to stirred conical flasks containing 4.75 mL buffer and the required amount of limonene, EOs or DMSO. Following 30 min and 2 h incubation at room temperature, 50 μL aliquots were transferred into FACS tubes containing 950 μL phosphate buffer (Becton Dickinson, Immunocytometry Systems, Mountain View, CA). Cells were stained with 10 mg L^{-1} PI (Molecular Probes, Eugene, Oregon), and the percentage of PI stained cells was determined using flow cytometer (CyFlow Space, Partec, Münster, Germany).

Statistical analysis

The results were analysed by one-way ANOVA and by the Bonferonni test using SPSS 20 (SPSS Inc., Chicago, IL, USA) software. A P value < 0.05 was considered significant.

RESULTS

Antimicrobial activity of citrus EOs against planktonic cells

EOs used in this study were mostly comprised of limonene, 92% (w/v) in EO$_P$ (Table S1, Supporting Information) and 97% (w/v) in EO$_G$ (Ou *et al.* 2015). Less abundant components in EO$_P$ were myrcene and geranial, comprising 1.5% and 1.1% (w/v), respectively. All the other components comprised <1% (w/v). Therefore, limonene as the main component of both EOs was also included in bioactivity analysis.

Antimicrobial activity of EO$_P$, EO$_G$ and limonene was examined on *Pseudomonas aeruginosa* PAO1 and different fungal clinical isolates including yeasts and molds (Table 1). Neither limonene nor citrus EOs affected the growth of planktonic bacteria even at high concentrations of 500 mg L^{-1} (Fig. S1, Supporting Information). The fungicidal effect of EO$_P$ or EO$_G$ was species specific, with lower MIC concentrations for yeasts than for molds. *Candida* species were the most susceptible, whereas *Aspergillus terreus* showed the lowest sensitivity to citrus EOs treatments. EO$_P$ or EO$_G$ showed stronger antifungal activity than pure limonene indicating synergistic effect with other components in the mixture.

To examine clinical relevance of citrus EOs, we have studied whether EO$_P$ show synergistic activity with clinically used drugs such as aminoglycoside antibiotic gentamycin, used to treat *P. aeruginosa* infections and itraconazole and used to treat various fungal infections. EO$_P$ exhibited no effect on planktonic growth of *P. aeruginosa* PAO1 and did not affect gentamycin activity against planktonic bacteria (Table S2, Supporting Information). However, EO$_P$ showed synergistic activity with itraconazole against planktonic *Candida albicans* (Table S2). Itraconazole at subinhibitory concentration (MIC/4) applied together with 12.5 mg L^{-1} of EO$_P$ inhibited fungal growth by 80%.

Inhibition of monomicrobial biofilm formation by citrus EOs

To test whether EO$_P$, EO$_G$ and limonene can affect cell adhesion and formation of microbial biofilms, we first examined their effect on monomicrobial biofilms (Fig. 1a–c). In the case of *P. aeruginosa* PAO1, both EOs and limonene inhibited biofilm formation comparably and up to 50% at concentrations of 100 mg L^{-1}. *Candida albicans* was the most susceptible among tested organisms with up to 70% biofilms inhibition occurring at 10 mg L^{-1} of EO$_G$ treatment (Fig. 1b). Generally, *A. terreus* and *Scedosporium apiospermum* were the least affected, except in a case of EO$_P$ that was able to inhibit *S. apiospermum* biofilms up to 30% at 100 mg L^{-1} (Fig. 1a). *Aspergillus fumigatus* biofilm formation was significantly affected only by EO$_G$. In accordance to antifungal effect, both citrus EOs were more active against yeast biofilms than pure limonene. Concentrations higher than 100 mg L^{-1} were not tested due to cytotoxicity of citrus EOs observed on human cells (Fig. S2, Supporting Information).

Biofilm formation of *C. albicans* is the best-understood process comparing to biofilm formation by other medically important fungi. This allowed us to examine the effect of EOs on *C. albicans* biofilm formation to greater extent (Fig. 2). The process of *C. albicans* biofilm development consists of two phases, including the phase of adherence to the surface and the phase of biofilm formation, which involves extracellular matrix production and the phase of biofilm maturation (Chandra *et al.* 2001). To distinguish which phase of *C. albicans* biofilm development has been affected by citrus EOs, upon inoculation cells were left 3 h to attach to the bottom of microtiter wells and then exposed to EOs or limonene for 24 h (Fig. 2). Treatments with EO$_P$, EO$_G$ or limonene dose dependently reduced biomass of *C. albicans* biofilms (Fig. 2a) and inhibited metabolic activity of yeast cells within biofilms (Fig. S3, Supporting Information). The treatments with EO$_P$, EO$_G$ or limonene were two times more effective when applied after the process of adhesion than simultaneously with inoculation (Fig. 1a–c), suggesting that the process of biofilm formation and not the adhesion phase is the main target of citrus EOs.

The invasive properties of *C. albicans* are modulated by different environmental factors such as glucose level in the media, which influences hyphae development and the yeast-to-hypha transition process (Buu and Chen 2014). With the aim to obtain denser *C. albicans* biofilms, we increased the cell density using 10-fold higher inocula for cell adhesion and induced hyphae development by reducing glucose concentration in the media to 0.2%. The changed growth conditions did not markedly affect the efficiency of citrus EOs treatments on *C. albicans* biofilm formation, exhibiting 70% inhibition in the presence of 100 mg mL^{-1} EOs or limonene (Fig. 2b).

We next addressed the combined effect of EO$_P$ and clinically used drugs on the formation of microbial biofilms. Low concentration of EO$_P$ (<12.5 mg L^{-1}) potentiated activity of subinhibitory concentration of gentamycin (1.5 mg L^{-1}) against biofilm formation by *P. aeruginosa* PAO1 resulting in biofilm inhibition by 75% (Fig. 3a). Combination of 0.075 mg L^{-1} of itraconazole with <3.1 mg L^{-1} EO$_P$ inhibited biofilm formation in *C. albicans* by 60% (Fig. 3b).
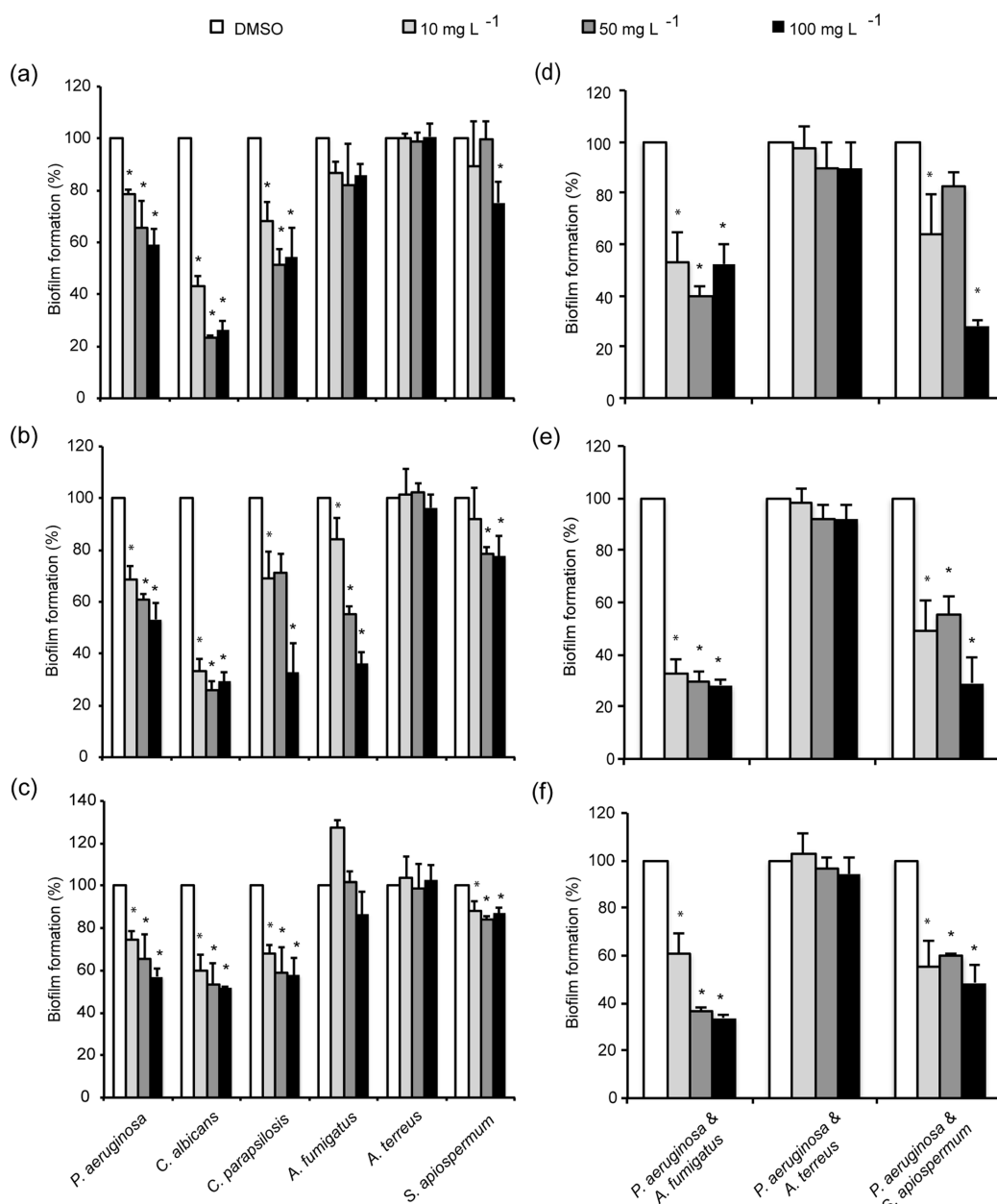
Figure 1. Effects of citrus EOs and limonene on cell adhesion and biofilm formation. Biofilms were formed 24 h in the presence of 0.1% DMSO (white) or 10 (light grey), 50 (dark grey) and 100 (black) mg L^{-1} of (**a**, **d**) pompia, (**b**, **e**) grapefruit EOs (**c**, **f**) or limonene. Values are average of three independent experiments $\pm$ SD and *$P < 0.05$ refers to DMSO treatment.

Effects of citrus EOs on polymicrobial biofilm formation

The effect of citrus EOs on polymicrobial biofilms formed by *P. aeruginosa* and different pathogenic fungi was then assessed. The formation of polymicrobial biofilms in the absence of any treatment was detected by microscopy after staining with fluorescent DNA dyes Syto9 and PI (Fig. 4). Syto9 can diffuse through the plasma membrane and stains all cells, while PI is a membrane impermeabile and labels only cells with damaged membranes. In the absence of treatments, mixed biofilms composed of *P. aeruginosa* and molds were successfully formed. When *P. aeruginosa* was incubated with *Candida* only bacterial biofilms were observed (data not shown) possibly due to the known

antagonistic effect of *P. aeruginosa* and *C. albicans* (Holcombe *et al.* 2010).

Biofilms consisting of *P. aeruginosa* and *A. fumigatus* were the most susceptible to all three treatments followed by biofilms formed by *P. aeruginosa* and *S. apiospermum* (Fig. 1d–f). EO$_G$ was the most efficient inhibiting *P. aeruginosa* and *A. fumigatus* mixed biofilm up to 70% at 10 mg L^{-1} (Fig. 1d). Mixed biofilms formed by *P. aruginosa* and *A. terreus* stayed resistant to citrus EOs or limonene treatments in concentrations up to 100 mg L^{-1}.

Fluorescent microscopy of polymicrobial biofilms (Fig. 4) showed that in the presence of EO$_P$ the formation of mixed biofilms by *P. aeruginosa* and *A. fumigatus* was inhibited and only planktonic bacteria could be detected. The same treatment of
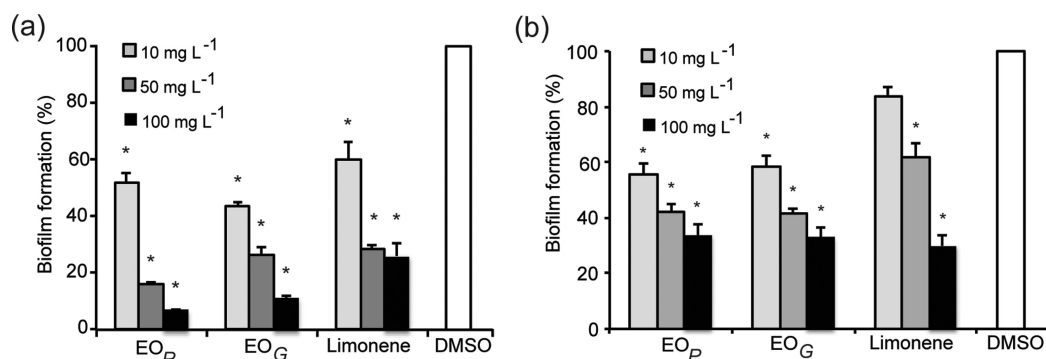
Figure 2. Effects of citrus EOs and limonene on biofilm formation in *C. albicans*. Cell were left to attach to the bottom of microtiter plate for 3 h and the biofilms were formed in the presence of different concentrations of pompia (EO$_P$), grapefruit (EO$_G$) EOs, limonene or 0.1% DMSO. After 24 h biomass was quantified using CV staining. Biofilms were formed using (**a**) 10^6 inoculum in RPMI 1640 medium containing 2% glucose or (**b**) 10^7 inoculum in RPMI 1640 medium containing 0.2% glucose. Values are average of two independent experiments ± SD and *$P < 0.05$ refers to DMSO treatment.
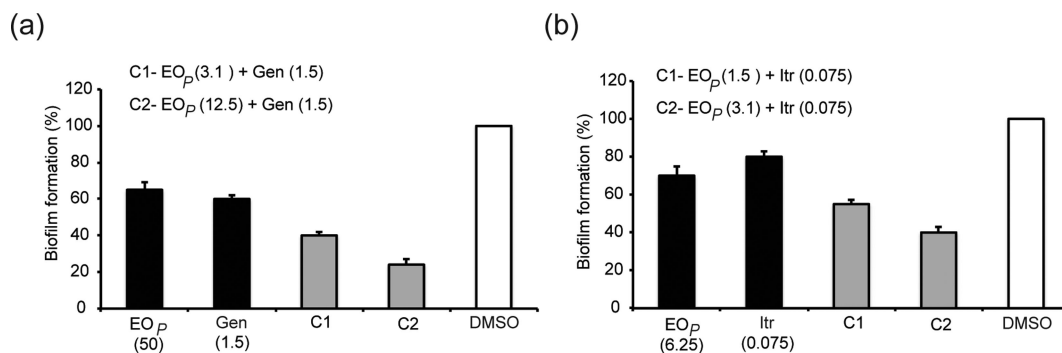
Figure 3. Combined effects of EO$_P$ and clinical drugs on microbial biofilm formation. (**a**) *Pseudomonas aeruginosa* and (**b**) *C. albicans* biofilms were formed 24 h in the presence of subinhibitory concentrations (mg L^{-1}) of EO$_P$ and gentamycin (Gen) or itraconazole (Itr), respectively. Values are average of two independent experiments ± SD.

P. aeruginosa and *S. apiospermum* biofilms caused bacterial and fungal cell death within biofilms. The biofilm matrix could still be observed due to the positive Syto9 and PI staining of DNA released around the cells. When *P. aeruginosa* and *A. terreus* mixed biofilms were incubated with EO$_P$, bacterial biofilms were significantly reduced while *A. terreus* hyphae stayed intact.

Effect of citrus EOs on bacterial quorum sensing

Formation of *P. aeruginosa* biofilms is mostly regulated by QS (Fazli *et al.* 2014). To understand the mechanism of *P. aeruginosa* biofilms inhibition by citrus EOs, we performed AHL-based *in vitro* QS competition assay using suitable biosensor strain (*Chromobacterium violaceum* CV026). *Chromobacterium violaceum* produces water insoluble purple pigment violacein in response to interaction of its AHL receptor CviR with exogenously provided AHLs with a short carbon chain length including HHL. A change in violacein production occurs due to the competitive binding of the HHL from the media with specific inhibitor to the AHL receptor. QS inhibition was demonstrated by the zones of violacein inhibition in the presence of EO$_P$ or EO$_G$ confirming that both citrus EOs interfered with the interaction between HHL and their receptor (Fig. 5a). Under these conditions, limonene did not affect violacein production.

The interference of citrus EOs and limonene with short-chain AHL production in *P. aeruginosa* was then examined. Instead of providing exogenously HHL in the media, violacein production in *C. violaceum* CV026 was stimulated by applying AHLs produced by *P. aeruginosa* PAO1 incubated with limonene, EO$_P$ or EO$_G$. Vio-

lacein production zones were smaller when *C. violaceum* CV026 was stimulated with AHLs obtained from *P. aeruginosa* incubated with citrus EOs comparing to DMSO. These results suggested that citrus EOs affected QS in *P. aeruginosa* by inhibiting short-chain AHL production (Fig. 5b).

Further confirmation of QS inhibiting activity of tested substances was obtained from the measurements of pyocyanin production in treated *P. aeruginosa* PA14 (Table 2). All treatments reduced pyocyanin concentration in the culture supernatants between 25% and 50% in comparison to DMSO controls. EO$_P$ exhibited the most effective pyocyanin inhibition among the tested compounds.

The inhibition of AHL production by EO$_P$ or EO$_G$ was quantified in assays with two biosensors, *P. aeruginosa* PA14-R3 used to measure 3OC12-HSL levels and *P. aeruginosa* PAOJP2 used for measurements of C4-HSL. EO$_P$ inhibited both C12- and C4-AHL synthesis with the reduction of 45% and 65% of AHLs, respectively (Fig. 6). Limonene and EO$_G$ inhibited long-chain AHL production by 20%, while no reduction of C4-AHLs was detected.

Effect of citrus EOs on *Candida albicans* membrane integrity

To address whether citrus EOs can affect integrity of fungal membranes, we chose yeast cells as a model for membrane damage analysis due to their suitability for FACS measurements. *Candida albicans* cells were exposed to citrus EOs or limonene and were analysed by flow cytometry over time. After 30 min of treatment EO$_G$ caused membrane permeabilisation in 10% of fungal
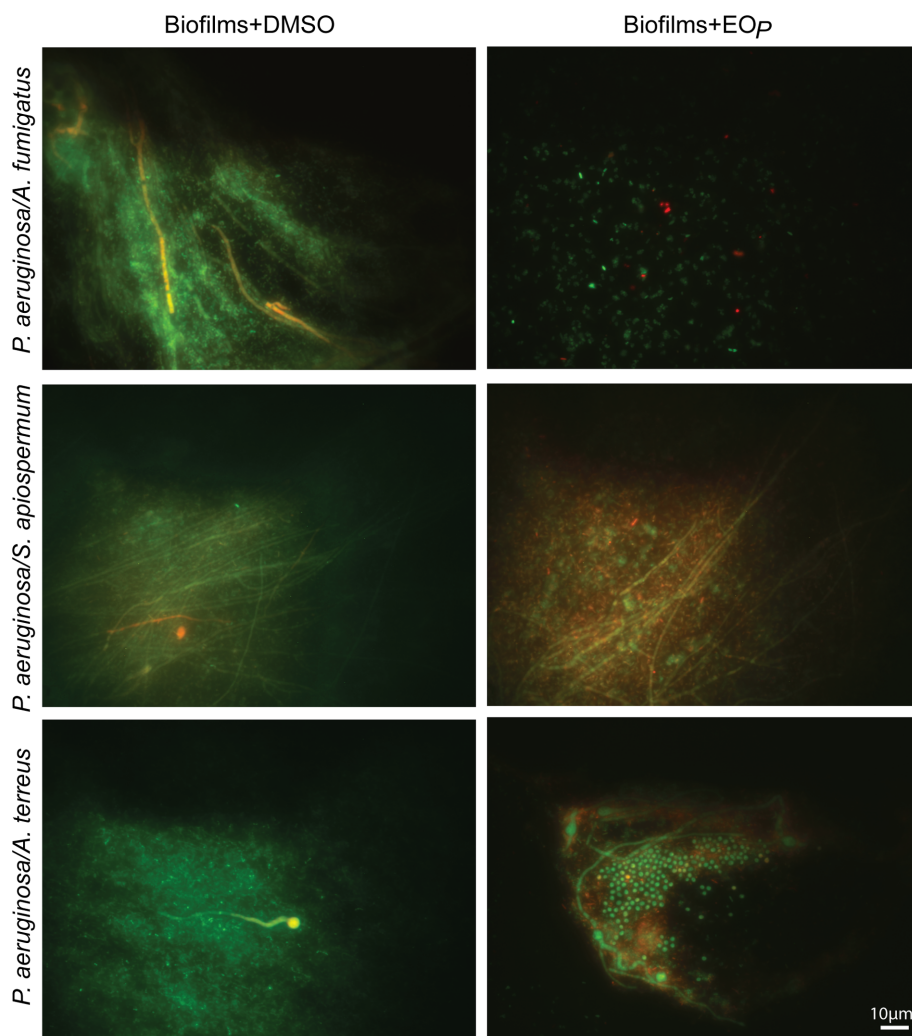
Figure 4. Fluorescent microscopy images of mixed biofilms formed by *P. aeruginosa* with *A. fumigatus*, *P. aeruginosa* with *S. apiospermum* or *P. aeruginosa* with *A. terreus* in the presence of 0.1% DMSO or 100 mg L^{-1} EO$_P$. Biofilms were grown for 48 h and afterwards stained with Syto9 (green) and PI (red). Scale bar represents 10 μm.
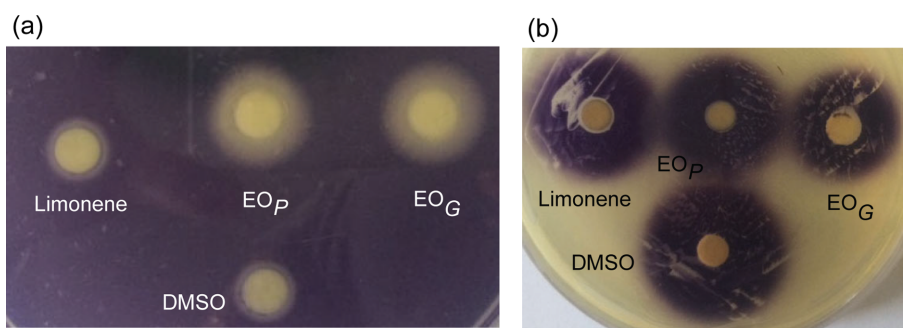
Figure 5. Anti-quorum-sensing activity of citrus EOs and limonene detected by *C. violaceum* CV026. (**a**) Direct effect of limonene, EO$_P$ or EO$_G$ (250 μg/disc) on violacein production. (**b**) Inhibition of short-chain AHL production after the treatment of *P. aeruginosa* PAO1 with 100 mg L^{-1} of limonene, citrus EOs or DMSO. AHLs extracted from 10^{10} *P. aeruginosa* PAO1 cells were applied per disc.

cells as compared to PI uptake in DMSO-treated cells (Fig. 7). No PI staining was detected in limonene or EO$_P$-treated cells. After 2 h incubation of *C. albicans* with limonene or citrus EOs membrane, permeabilisation was observed in 82%–88% of cell population. These results showed that EO$_P$ and EO$_G$ induce fungal membrane damage employing the same mechanism of activity-like limonene.

DISCUSSION

Polymicrobial interactions are important in a variety of diseases including infections of the respiratory system, formation of dental plaque, skin and mucosal infections and bloodstream infections. The aim of this study was to address the antimicrobial potential of selected EOs to prevent and treat polymicrobial

Table 2. Inhibition of *P. aeruginosa* PA14 pyocyanin production in the presence of citrus EOs or limonene.

Treatment (100 mg L^{-1})	Pyocyanin production (%)[a]
EO$_P$	57 ± 8
EO$_G$	65 ± 6
Limonene	75 ± 7

Values are given relative to a control where cells are grown with DMSO and are average of two independent experiments ± SD performed in triplicate.

infections and biofilms formed by *Pseudomonas aeruginosa* and the most common pathogenic fungal isolates. Antimicrobial activities of many citrus EOs have been extensively studied; however, this is the first report on the activity against mixed polymicrobial biofilms consisting of bacteria and pathogenic fungi. In addition, this is the first report of antibiofilm formation activity of EO extracted from *Citrus × monstruosa* (pompia).

Many EOs exhibit potent antimicrobial activity inhibiting growth of both Gram-positive and Gram-negative bacteria, as well as fungi (Edris 2007; Frassinetti *et al.* 2011; Jing *et al.* 2014). Citrus EOs such as grapefruit, bergamot, orange, lime and lemon
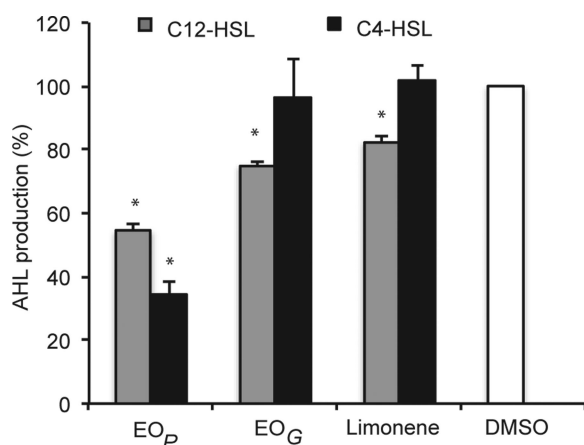

Figure 6. Inhibition of 3OC12-AHL and C4-AHL in *P. aeruginosa* PAO1 after the treatment with 100 mg L^{-1} citrus EOs or limonene. Values are given relative to a control where cells are grown with DMSO and are average of three independent experiments ± SD. *$P < 0.05$ refers to DMSO control.
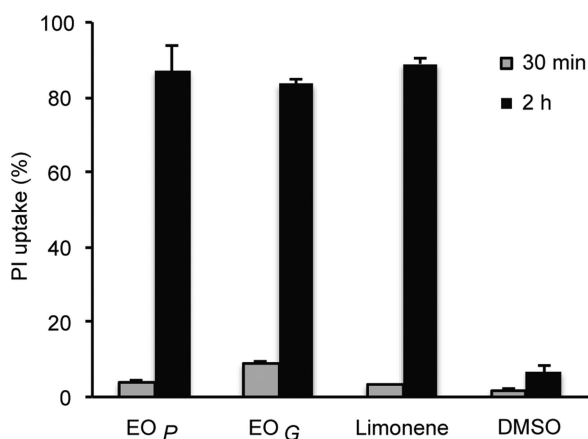

Figure 7. Uptake of PI by *C. albicans* cells after the treatments with citrus EOs or limonene (100 mg L^{-1}). PI staining was quantified by flow cytometry. Values are average of two independent experiments ± SD, with 100 000 events measured in each experiment.

inhibit growth of common foodborne and medically important bacterial pathogens, mostly having high MIC concentration values (Fisher and Phillips 2006; Prabuseenivasan, Jayakumar and Ignacimuthu 2006; Uysal *et al.* 2011). Depending on the chemical composition, citrus EOs obtained from different species show stronger bactericidal effect against Gram-positive than Gram-negative bacteria (Fisher and Phillips 2008). Antifungal activity of citrus EOs has also been well established particularly on food spoilage fungi such as *Aspergillus*, *Penicillium*, *Fusarium* or *Alternaria*. Lemon, mandarin, grapefruit and orange EOs efficiently inhibited mycelial growth of these molds (Viuda-Martos *et al.* 2008). Consistent with previous study (Uysal *et al.* 2011), our results show that EO$_P$, EO$_G$ and limonene do not affect growth of planktonic *P. aeruginosa*. Both EOs and limonene efficiently inhibited growth of medically important fungi with the higher activity against yeasts comparing to mold clinical isolates. Only *A. terreus* stayed resistant to citrus EOs treatments. This was expected result since *A. terreus* causes infections difficult to treat even with conventional antifungal drugs such as amphotericin B, which is the 'gold standard' in the treatment of invasive aspergillosis in the immunocompromised patients (Sutton *et al.* 1999). EO$_P$ or EO$_G$ showed stronger antifungal activity than limonene, exhibiting two times lower MIC concentrations. Since limonene is the major compound in pompia (92%) and grapefruit (97%) EOs (Jing *et al.* 2014), our results suggest that the antimicrobial activities of EO$_P$ or EO$_G$ depend on synergistic effect of different components found in these EOs.

Previous studies have shown that citrus EOs can be effective against bacterial biofilms. Grapefruit EO has been shown to inhibit formation of Enterohemorrhagic *Escherichia coli* biofilms, while lemon EO inhibited both monomicrobial and mixed biofilms formed by *E. coli* and *Bacillus cereus* (Vikram *et al.* 2010; Kerekes *et al.* 2013). Although not effective against planktonic cells, EO$_P$ or EO$_G$ reduced *P. aeruginosa* biofilm formation by 40% and also affected fungal biofilms formation, showing stronger activity against yeasts than molds. Monomicrobial biofilms are rare and medically more relevant are mixed species infections that form polymicrobial biofilms. The *in vitro* examination of certain mixed infections has proven difficult due to interspecies competition under laboratory conditions. *Pseudomonas aeruginosa* and *C. albicans* commonly coexist in chronic infections, however, under *in vitro* conditions bacterial virulence factors such as pyocyanin inhibit fungal growth and mixed biofilm formation (Kerr *et al.* 1999; Kaleli *et al.* 2007; Gibson, Sood and Hogan 2009). In physiological conditions, virulence factors production could be reduced through signaling molecules exchange or could be less effective or inactivated by the host immune system. By sequential biofilm development, we were able to detect mixed biofilms involving *P. aeruginosa* and *A. fumigatus*, *A. terreus* or *Scedosporium apiospermum,* but not *P. aeruginosa* and *C. albicans*. According to our microscopy images, *P. aeruginosa* was the predominant species in these mixed biofilms. Both EOs and limonene inhibited formation of *P. aeruginosa* and *A. fumigatus* or *P. aeruginosa* and *S. apiospermum* biofilms, but once formed, *P. aeruginosa* or fungal biofilms could not be disrupted (data not shown). Interestingly, the addition of *P. aeruginosa* together with citrus EOs for 24 h of fungal biofilm development, mixed biofilms composed of *P. aeruginosa* and *A. fumigatus* were completely inhibited, while biofilms composed of *P. aeruginosa* and *S. apiospermum* were significantly reduced. The applicability of these *in vitro* results needs to be confirmed in the future, using *in vivo* mice models of infection and wounds.

Different phytochemicals including EOs showed synergistic antimicrobial activity when applied with subinhibitory

concentrations of clinically used drugs (Monte *et al.* 2014; Liu *et al.* 2015). We demonstrated synergistic effect of EO$_P$ against planktonic *C. albicans* cells when used with subinhibitory concentration (MIC/4) of antimycotic itraconazole. Combined application of subinhibitory concentrations of antibiotic gentamycin or itraconazole with EO$_P$ improved both drugs activity against *P. aeruginosa* or *C. albicans* biofilm formation, respectively. Thus, these potentiating activities of citrus EOs could be used to decrease active concentrations of the drugs reducing their side effects and could help recycle old antibiotics or antifungals that became ineffective due to resistance problems.

Limonene can cause inhibition of intracellular or extracellular enzymes, such as pectin methyl esterase or cellulose (Marei, Rasoul and Abdelgaleil 2012). However, due to its hydrophobic nature it gets partitioned into lipid bilayers of the cell membrane causing its permeabilisation and the cell death. This study demonstrated that EO$_P$, EO$_G$ and limonene induce yeast membrane permeabilisation within 2 h of treatments suggesting that membrane damage is the primary cause of the cell death.

EOs can affect bacterial virulence through interference with QS. It has been reported that rose, geranium, lavender and rosemary EOs potently inhibited QS; eucalyptus and citrus EOs moderately reduced violacein production; and the chamomile, orange and juniper oils were ineffective (Szabo *et al.* 2010). In this study, QS inhibition in *P. aeruginosa* occurred through interference with AHL pathways with EO$_P$ being the most effective suggesting that some other component within EO$_P$ in addition to limonene attributed to its activity. Identification of the component that contributes to the strong QS inhibition observed for EO$_P$ needs to be addressed in the near future.

In conclusion, EO$_P$ or EO$_G$ shows a potent activity against monomicrobial and polymicrobial biofilm formation. Their anti-QS activity, instead of bactericidal activity, suggests that these citrus EOs have a good potential to be used for the control of common polymicrobial infections with resistance unlikely to occur. Thus, these two EOs should be further examined in the prophylactic application against polymicrobial infections.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSPD online.

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