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Citrus paradisi and *Citrus reticulata* essential oils interfere with *Pseudomonas aeruginosa* quorum sensing *in vivo* on *Caenorhabditis elegans*

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A R T I C L E I N F O

Keywords:
Pseudomonas aeruginosa
Caenorhabditis elegans
Citrus paradisi
Citrus reticulata
Essential oils
Quorum sensing

A B S T R A C T

Background: *Pseudomonas aeruginosa* is a Gram-negative opportunistic pathogen, considered a leading cause of acute and chronic infections in immunocompromised patients. *P. aeruginosa* uses quorum sensing to control virulence and biofilm formation. To combat this human-resistant pathogen increasing attention has been paid to anti-QS compounds from natural products as potential therapeutic agents.

Purpose: To assess the efficacy of *C. paradisi* (Grapefruit) and *C. reticulata* (Mandarin) commercial EOs (obtained by cold-pressing and cold-pressing followed by steam distillation, named EOP and EOPD, respectively) and their majority component, limonene, in inhibiting the effect of QS-controlled virulence factors of *P. aeruginosa* PAO1 and PA14 in a *C. elegans* infection model.

Results: *C. paradisi* and *C. reticulata* EOs at 0.125% (v/v) significantly inhibited the *in vitro* biofilm formation of PAO1 and PA14 strains between 40 and 50%. This EOs concentration, safe for *C. elegans* according to the survival and brood size assays, rescued the nematodes from *P. aeruginosa* infections, reducing their death rate by 20–30% in the paralysis assay, and increasing worm lifespan with median survivals of 6 (Grapefruit) and 5 (Mandarin) in the slow killing assay (3 for non-treated worms). Limonene protected nematodes from death in these assays although less effectively than both EOs species. Grapefruit EOs rescued the nematodes in 45–50% from phenazine-death, while mandarin EOs and limonene rescued them in 30%. In the food choice assay, worms preferred the PA14 grown with 0.125% of mandarin and grapefruit EOs or limonene after 3-4 hours (CI=0.11 to 0.18).

Conclusion: Our results suggest that *C. paradisi* and *C. reticulata* EOPDs, with lower commercial value but similar *in vivo* effects than EOPs, are sources of anti-QS agents for controlling *P. aeruginosa* infections, therefore should be considered as potential candidates for the development of novel therapeutics against persistent microorganisms.

P. aeruginosa	*Pseudomonas aeruginosa*
C. elegans	*Caenorhabditis elegans*
C. reticulata	*Citrus reticulata*
C. paradisi	*Citrus paradisi*
E. coli OP50	*Escherichia coli* OP50
EOs	Essential Oils
EOP**M/G**	**M**andarin/**G**rapefruit Essential Oil obtained by cold-pressing
EOPD**M/G**	**M**andarin/**G**rapefruit Essential Oil obtained by cold-pressing followed by steam distillation
QS	*Quorum sensing*
LB	Luria Bertani
BHI	Brain Heart Infusion
OD_{560}	Optical Density at 560 nm
DMSO	Dimethyl Sulfoxide
w/v	weight/volume
v/v	volume/volume
NGM	Nematode Growth Medium

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PGS Peptone Glucose Sorbitol
HCN Hydrogen Cyanide.

Introduction

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen able to colonize a wide variety of environments and hosts. It is considered a leading cause of acute and chronic infections affecting mainly mechanically ventilated and immunocompromised individuals (Gogoi et al., 2015). To adapt to and colonize various ecological niches, *P. aeruginosa* forms biofilms and produces several extracellular virulence factors regulated by QS, a density-dependent cell-cell communication mechanism (Fuqua et al., 2001). In addition, *P. aeruginosa* is intrinsically resistant to many antibiotics due to low outer membrane permeability and adaptive resistance mechanisms, representing a problem from a treatment perspective (Hancock and Speert, 2000). To overcome this problem, increasing attention has been paid to anti-biofilm and anti-QS compounds from natural products as potential therapeutic agents. Several plant-derived compounds, such as EOs, have shown the ability to disrupt the bacterial communication systems leading to an attenuation of microbial virulence (Lu et al., 2021).

Citrus EOs are complex mixtures of volatile organic compounds, rich in monoterpene and sesquiterpene hydrocarbons, as well as their oxygenated derivatives (González-Mas et al., 2019). These EOs have been reported with several biological activities, including broad-spectrum antimicrobial, anti-viral, anti-insecticidal, anti-inflammatory, anti-cancer, immunomodulatory, among others (Dosoky and Setzer, 2018). These properties together with the reasonable price they offer in the market, make EOs a potential and valuable alternative for pharmaceutic and food industries. But even though the benefits EOs offer and their GRAS status, the information available of their toxicity is not extensively studied, thus should be addressed (Tisserand R., 2014).

Caenorhabditis elegans is one of the simplest invertebrates used as a model organism to study host-pathogen interactions (Mahajan-Miklos et al., 1999), to search for new antimicrobials (Kong et al., 2016), and to predict compound toxicity (Hunt et al., 2020). In the laboratory, *C. elegans* is maintained in Petri dishes and fed with *E. coli* OP50 (Stiernagle, 2006). Changing the food source for pathogenic bacteria, it is possible to infect and kill the worms by different mechanisms. The *P. aeruginosa*-mediated killing of *C. elegans* mainly depends on QS-controlled virulence factors (Tan and Ausubel, 2000), which could be attenuated by several plant extracts and EOs, retarding the death of the nematode (Adonizio et al., 2008; Ganesh and Rai, 2016; Husain et al., 2015).

This work was carried out to assess the efficacy of *C. paradisi* (Grapefruit) and *C. reticulata* (Mandarin) commercial EOs (obtained by cold-pressing and cold-pressing followed by steam distillation) in inhibiting the effect of QS-controlled virulence factors of *P. aeruginosa* PAO1 and PA14 in a *C. elegans* infection model.

Material and methods

Essential oils

EOs used in this study were commercial samples of food-grade quality derived from mandarin (*Citrus reticulata*) (N°: 006/8012BC) and grapefruit (*Citrus paradisi*) (N°: 010/8015CD). Oils were extracted by cold-pressing (EOP) and cold-pressing followed by steam distillation (EOPD) from "Litoral" Citrus Company, Argentine. (+)-Limonene (97%) was purchased from Sigma-Aldrich (Sigma-Aldrich Chemie, Steinheim, Germany)

Bacterial and Caenorhabditis elegans stock and maintenance

P. aeruginosa PA14 and PAO1 strains were used for determining the QS inhibitory potential of the citrus EOs. *Escherichia coli* OP50 and *P. aeruginosa* were grown in LB broth medium and incubated at 37 °C for 24 h, at 250 rpm.

The wild-type *C. elegans* Bristol strain N2, and *glp-4(bn2)* strain were used for the *in vivo* assays and maintained under standard culture conditions with *E. coli* OP50 as a food source on NGM (Brenner, 1974). *glp-4 (bn2)* is a temperature-sensitive sterile strain at 25 °C. Worms were synchronized using the bleaching method (Stiernagle, 2006). L1 larvae were poured onto the lawn of *E. coli* OP50 (Brenner, 1974), and plates were incubated at 20 °C until the L4-stage was reached (Stiernagle, 2006).

Bacterial growth and biofilm

The growth curves for *P. aeruginosa* PA14 and PAO1 cultured in the presence of citrus EOs were analyzed. Briefly, an overnight culture of *P. aeruginosa* PA14 or PAO1 was diluted in LB broth to reach OD_{560}: 0.1 ± 0.05. A 96-well microtiter plate was full-filled with the diluted culture and 0.125, 0.25, 0.5, 1, 2 and 4% (v/v) of each citrus EOs or DMSO (1% v/v- control) (8 replicates). Plates were incubated at 37 °C and OD_{560} readings were taken every 1 h for up to 24 h using a microtiter plate reader (Power Wave XS2, Biotek, Vermont, USA).

The anti-biofilm activity of the citrus EOs was tested as previously described (O'Toole and Kolter, 1998). Briefly, after the 24 h incubation of bacterial cultures prepared as described above, the resulting biofilm was stained with 0.1% (w/v) aqueous crystal violet solution. Absolute ethanol was added to solubilize the dye and absorbance was measured at 595 nm using a microtiter plate reader (Power Wave XS2, Biotek, Vermont, USA).

Toxicity assay

The acute toxicity assay was performed in 24-well plates with NGM and EOs, limonene (0.125, 0.25, 0.5, and 1% v/v) or 1% DMSO (added to the agar while it was still in liquid form but <40 °C, and mixed thoroughly) (6 replicates). L4-stage N2 worms (20–30) were placed in each well, and plates were incubated at 25 °C for 24 h (n = 120–180). Worm viability was determined under a stereo-microscope. Worms that failed to respond to the platinum pick touch were marked as dead.

The reproduction assay was performed on NGM plates mixed with 0.125% (v/v) of each mandarin and grapefruit EOs (EOP and EOPD), seeded with *E. coli* OP50 and, incubated at 37 °C for 18 h. One L4-stage nematode was placed per plate and transferred each day for 5 days (8 per treatment). The total number of eggs laid from each *C. elegans* treated and non-treated was counted every day under a stereo-microscope.

Chemotaxis assay

The chemotaxis assay (Beale et al., 2006) was performed on NGM with bacterial food seeded in two equidistant spots from the center of the plate and incubated for 18–24 h at 37 °C. One spot corresponded to *P. aeruginosa* PA14 previously grown with 0.125% (v/v) of each mandarin and grapefruit EOs (EOP and EOPD) or limonene, for 15 h at 37 °C and 160 rpm. The other spot corresponded to *P. aeruginosa* PA14 grown with 1% DMSO in the same conditions mentioned above or *E. coli* OP50. L4-stage N2 nematodes (20–30) were transferred to the center of the plate and every hour the nematodes were quantified in each spot under a stereo-microscope (n = 60–90 per treatment). For each experiment, a chemotaxis index (CI) was calculated as CI = ((number of animals in test bacteria –number of animals in control) / total number of scored animals. Score of + 1.0: maximal attraction, −1.0: total repulsion, and 0: lack of response.

Slow killing assay

The slow-killing assay (Tan et al., 1999) was performed spreading an overnight culture of *P. aeruginosa* PA14 on modified NGM and 0.125% of each mandarin and grapefruit EOs (EOP and EOPD), limonene, or 1% DMSO (added to the agar while it was still in liquid form but <40 °CC, and mixed thoroughly). Plates were incubated at 37 °C for 24 h. 20–30 L4-stage *glp-4* worms were placed on each plate and incubated at 25 °C (60 - 90 worms per trial). Under a stereo-microscope, worms were scored every 1 day until all the infection control nematodes were dead. Plates with *E. coli* OP50 were used as the negative control. A worm was considered dead when it no longer responded to touch.

Fast killing assay

The fast-killing assay (Mahajan-Miklos et al., 1999) was performed on PGS plates with 0.125% of each citrus EOs, limonene, or 1% DMSO (added to the agar while it was still in liquid form but <40 °C, and mixed thoroughly). Plates were seeded with an overnight culture of *E. coli* OP50 or *P. aeruginosa* PA14, incubated for 24 h at 37 °C. L4-stage N2 nematodes (20–30) were transferred to each PGS plate (n = 60–90 per treatment). Worms were evaluated for viability under a stereo-microscope every hour for a total of 5 h. Worms were considered dead when they no longer responded to physical stimuli.

Paralytic killing assay

The paralytic killing assay (Darby et al., 1999; Mahajan-Miklos et al., 1999) was carried out on BHI agar plates with 0.125% of each mandarin and grapefruit EOs (EOP and EOPD), limonene, or 1% DMSO (added to the agar while it was still in liquid form but <40 °C, and mixed thoroughly), and seeded with *P. aeruginosa* PAO1 previously grown in BHI broth (OD_{660}= 0.1). Plates were incubated for 24 h at 37 °C. N2-adult nematodes (20–30) were placed onto the PAO1 lawns and plates were incubated for 4 h at 25 °C (60 - 90 worms per treatment). Nematodes were scored every hour under a dissecting microscope. Plates with *E. coli* OP50 were used as a control for estimating the natural death of the nematodes. Worms were considered dead if they did not move spontaneously and did not respond to touch with a platinum wire pick and the absence of pharyngeal movement.

Statistical analysis

Experiments were repeated at least two-three times. The statistical significance of bacterial growth and biofilm formation in presence of EOs was evaluated by one-way ANOVA followed by Dunnett or Tukey's tests (GraphPad Prism 6.0 and/or InfoStat for Windows). Differences were considered significant at the 95% level of confidence. Data of fast, slow and, paralysis killing experiments were analyzed using Kaplan-Meier survival analysis and log-rank test (software GraphPad Prism 6.0). Values of $p<0.05$ or less indicated significant differences between tested populations.

Results and discussion

In this work, we used the *P. aeruginosa* - *C. elegans* infection model to test the capacity of the commercially available essential oils obtained by two extraction methods from *Citrus reticulata* (Mandarin: EOPM and EOPDM) and *Citrus paradisi* (Grapefruit: EOPG and EOPDG) to find new anti-infectives that rescue worms from infection. With this approach, we aimed to identify potential samples that attenuate the *P. aeruginosa* virulence factors *in vivo*. We compared the potential of these anti-infectives against limonene, the main component of the studied oils (Luciardi et al., 2016, 2020).

Citrus EOs on bacterial growth and biofilm formation

Several authors have informed the broad-spectrum antimicrobial capacity of mandarin and/or grapefruit EOs on bacteria and fungi, although with elevated MIC values (Raspo et al., 2020; Yi et al., 2018). In our study, MICs against PA14 and PAO1 strains could not be reached until the highest concentration of EOs evaluated (4%). Although inhibition of ~15% in the PA14 and PAO1 planktonic cell growth was observed at 1% ($p<0.05$) (Sup. Fig. 1).

Luciardi et al. reported the anti-biofilm and anti-pathogenic *in vitro* activities of grapefruit and mandarin EOs on a clinical foodborne *P. aeruginosa*, resistant to antibiotics, and a collection *P. aeruginosa* strain (ATCC27853) (Luciardi et al., 2016, 2020). These EOs inhibited biofilm formation, metabolic activity in the biofilm cells, autoinducer production, as well as elastase activity, without affecting the growth of both *P. aeruginosa* strains. In our work, we observed an inhibition of PAO1 and PA14 biofilm formation of 40–50% with 0.125% of mandarin and grapefruit EOs (Table 1). This effect was statistically comparable between the EOP and EOPD of each citrus species. Limonene also inhibited both *P. aeruginosa* strains biofilm formation, although less potently than the analyzed EOs ($p<0.05$), suggesting that the inhibition resulted from the combined or synergistic effect of the whole mixture of compounds present in *C. paradisi* and *C. reticulata* EOs. This result is similar to others on mono and/or polymicrobial biofilms where limonene is the EO main component (Pekmezovic et al., 2016).

Citrus EOS toxicity

C. elegans is an interesting alternative model to predict compound toxicity using a whole animal with conserved processes with mammals (Adonizio et al., 2008; Hunt et al., 2020). We studied the possible toxicity of mandarin, and grapefruit EOs on *C. elegans* and observed that the survival of the nematodes in the presence of 0.125% of EOs was up to 95% (Fig. 1). From 0.25% to 1% of each citrus EO, nematode death increased with concentration. Their major component, limonene, showed higher toxicity than the EOs assayed, reaching almost 50% of mortality at 1%. Besides the lethality assay, which determines the acute toxicity of the EOs on *C. elegans*, studying their effect on nematode reproduction represents an important parameter in chemical hazard analysis (Lanzerstorfer et al., 2021). In presence of 0.125% of each EO, *C. elegans* brood size was similar to the control, indicating the lack of toxicity of *C. paradisi* and *C. reticulata* citrus EOs.

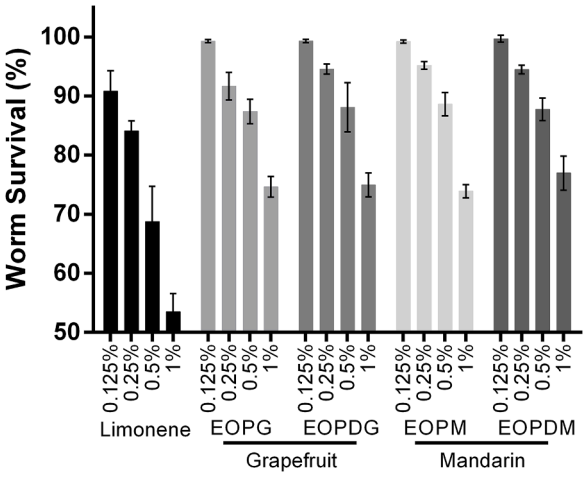

Fig. 1. *C. elegans* survival (%) in presence of different concentrations of *C. reticulata* (Mandarin) and *C. paradisi* (Grapefruit) EOs. EOP: Essential Oil obtained by cold-pressing, EOPD: Essential Oil obtained by cold-pressing followed by steam distillation.

Table 1
P. aeruginosa biofilm formation in presence of different concentrations of limonene and citrus EOs: *C. paradise* (EOPG and EOPDG), *C. reticulata* (EOPM and EOPDM).

EOs	Limonene		*C. paradisi*				*C. reticulata*			
			EOPG		EOPDG		EOPM		EOPDM	
%	PA14	PAO1	PA14	PAO1	PA14	PAO1	PA14	PAO1	PA14	PAO1
0	2.00±0.1	1.56±0.1								
0.125	1.44±0.05^{a}	1,11±0.09^{A}	1.05±0.05^{b}	0.82±0.05^{B}	1.04±0.09^{b}	0.79±0.1^{B}	1.06±0.07^{b}	0.91±0.06^{B}	1.02±0.02^{b}	0.89±0.04^{B}
0.25	1.09±0.1^{a}	0.83±0.09^{A}	0.85±0.06^{b}	0.62±0.05^{B}	0.71±0.08^{b}	0.55±0.07^{B}	0.90±0.09^{b}	0.65±0.05^{B}	0.87±0.1^{b}	0.66±0.06^{B}
0.5	0.76±0.05^{a}	0.50±0.07^{A}	0.49±0.04^{b}	0.33±0.07^{B}	0.45±0.04^{b}	0.29±0.03^{B}	0.57±0.04^{b}	0.35±0.06^{B}	0.56±0.08^{b}	0.31±0.08^{B}
1	0.4 ± 0.04^{a}	0.26±0.05^{A}	0.16±0.08^{b}	0.09±0.06^{B}	0.17±0.08^{b}	0.15±0.06^{B}	0.21±0.03^{b}	0.11±0.07^{B}	0.25±0.08^{b}	0.11±0.06^{B}

Values are average of independent experiments ± Standard Deviation. $^{a,b/A,B}$ Values with different letters are significantly different between EOs with equal concentrations for each strain. EOP: Essential Oil obtained by cold-pressing, EOPD: Essential Oil obtained by cold-pressing followed by steam distillation.

In vivo anti-pathogenic activities

For the *in vivo* anti-pathogenic assays, we decided to use 0.125% of each citrus EO, because this concentration was safe for the worms according to the toxicity assays, and effective inhibiting *P. aeruginosa* PA14 and PAO1 biofilm formation up to 50%.

Food choice assay

C. elegans is a free-living nematode that feeds on bacteria in its terrestrial environment through a chemotaxis mechanism that allows them to locate food sources or avoid danger by feeling attracted to certain bacterial-derived compounds or repelling noxious bacteria. The pathogenic bacteria *P. aeruginosa* PA14 is infectious and mortal to *C. elegans*. However, *C. elegans* is highly attracted to the smells of this pathogen, specifically to the odor of the acylated homoserine lactones (AHSLs), autoinducers produced by *P. aeruginosa,* and mediators of QS (Beale et al., 2006). Although this attraction, *C. elegans* uses behavioral strategies to defend itself from bacterial pathogens, like olfactory learning. The animal learns to avoid the smell of noxious bacteria after exposure if the ingestion causes a harmful infection (Niu et al., 2010).

We used a binary feeding assay to determine whether the inhibition observed *in vitro* in the production of *P. aeruginosa* autoinducers (Luciardi et al., 2016, 2020) affected the behavior of *C. elegans* in the choice of their food. When the nematodes were given to choose between PA14 grown alone or in presence of 0.125% of mandarin and grapefruit EOs, there was a significant difference in the election of food (Fig. 2). At the beginning of the assay, worms chose indistinguishably between both food spots, but after three to four hours most of the nematodes were attracted to PA14-EOs/limonene food spots (with CI=0.11 to 0.18). In the control plate, PA14 repelled nematodes, finding them mainly in the OP50 spot (CI=−0.3). Previously, we exposed nematodes to EOs without food and they showed no preference for the oil odors, as well as limonene, which was already reported as a neutral odor for *C. elegans* in a chemotaxis assay (Bargmann et al., 1993). Therefore, the effect of food choice observed could be a result of PA14 pathogenicity inhibition.

Paralytic killing assay

The hydrogen cyanide is a typical pseudomonad secondary metabolite necessary for the virulence of *P. aeruginosa* and depends directly on the quorum sensor regulators LasR and RhlR (Pessi and Haas, 2000).

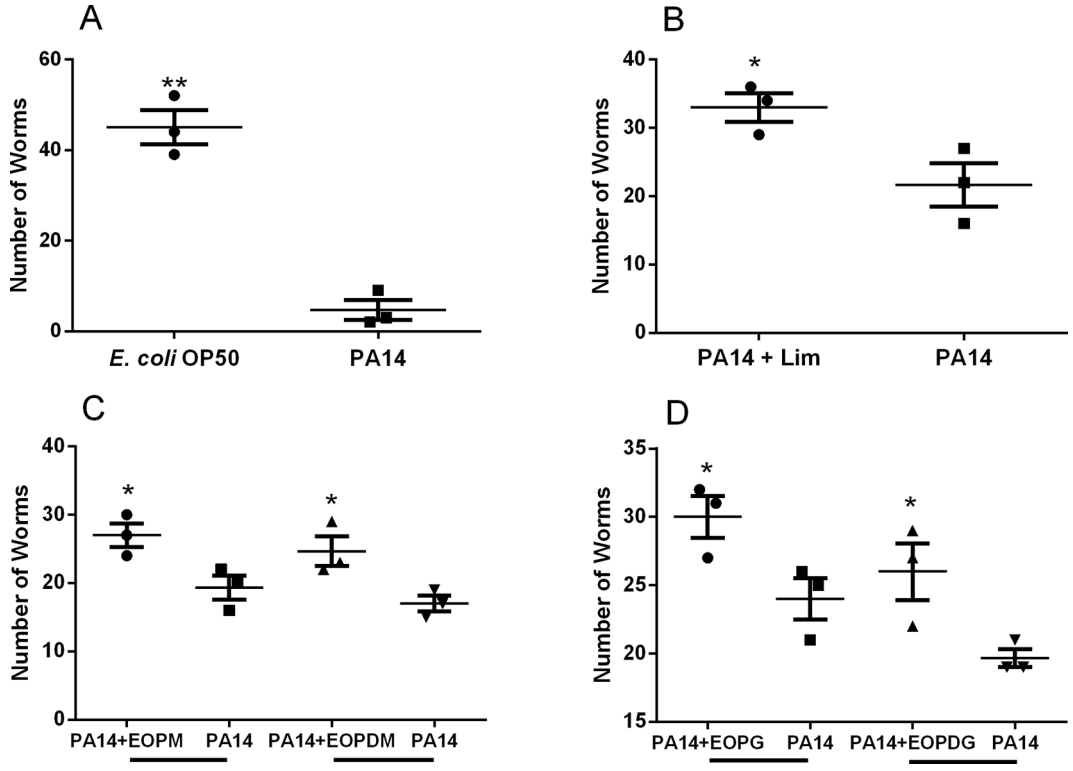

Fig. 2. *C. elegans* food choice assay. **(A)** PA14 *vs.* OP50, **(B)** PA14 - 0.125% limonene *vs*. PA14, **(C)** *C. reticulata* (Mandarin) EOs 0.125% -PA14 *vs*. PA14 and **(D)** *C. paradisi* (Grapefruit) EOs 0.125% - PA14 *vs*. PA14. Asterisks indicate a significant difference between the PA14 grown alone, and in presence of the EOs (*$p < 0.05$; **$p < 0.001$). EOP: Essential Oil obtained by cold-pressing, EOPD: Essential Oil obtained by cold-pressing followed by steam distillation.

HCN is the primary toxin responsible for the rapid killing of nematodes (Gallagher and Manoil, 2001). We observed that in the presence of 0.125% of mandarin and grapefruit EOs, worms were rescued from paralysis and death caused by PAO1 between 20 and 25% (Fig. 3). While limonene rescued the worms only in 8%. 100% of non-treated worms died after 4 h, and no deaths were observed in *C. elegans* fed with *E. coli* OP50. Although the role of HCN in *Pseudomonas* pathogenesis in humans is largely unexplored, this poison was found in several *P. aeruginosa* infections, such as burns and sputa of cystic fibrosis patients (Singh et al., 2000). The results of the paralytic assay suggest that the addition of citrus EOs decreased the expression of *P. aeruginosa* PAO1 virulence factor HCN, possibly, by interfering with the QS system.

Fast killing assay

In a rich, high-salt medium, *P. aeruginosa* PA14 kills the nematodes within hours. This "fast killing" is mediated by the production of phenazines, toxins under the QS control, and toxics for worms (Mahajan-Miklos et al., 1999). In this assay, we observed that 60% of the infected nematodes without EOs treatment died within 3 h and the remaining 40% were completely dead after 5 h. However, limonene and citrus EOs prevented the death of the nematode significantly different from non-treated worms at 0.125%. Grapefruit EOP and EOPD rescued 40% of the nematodes at the end of the 5 h. Whilst in the presence of limonene, mandarin EOP and, EOPD, 20, 24 and, 30% remained alive, respectively. Nematode death was not observed on *E. coli* OP50 plates at the end of the incubation time (Fig. 4).

Slow killing assay

When *P. aeruginosa* PA14 is grown on a low-nutrient medium, it is capable of killing *C. elegans* over several days (Tan et al., 1999). This assay, called "Slow killing", depends exclusively upon the accumulation and replication of *P. aeruginosa* in the worm gut. Moreover, although its mechanism is not clear yet, QS is required (Tan et al., 1999). Using this assay, we evaluated the effect of 0.125% of mandarin and grapefruit EOs on the survival of *C. elegans* infected with *P. aeruginosa* PA14. Fig. 5 shows that the death of infected nematodes in the presence of mandarin, grapefruit EOs, and limonene was delayed, with median survivals of 5, 6, and 4, respectively, in comparison with a median survival of 3 for nematodes without treatment. There was no significant difference in the killing curves between the two EOs (EOP and EOPD) assayed of each citrus species. In all cases, mandarin and grapefruit EOs rescued the nematode from death at a higher level than limonene, suggesting a marked effect of the citrus EOs on *P. aeruginosa* virulence on *C. elegans*.

Chemical composition and bioactivity

The bioactivity of EOs depends on their chemical composition, which depends on several factors, such as variety, geographical and climatic conditions of cultivation, and/or collection (Barra, 2009). The chemical profile of EOs also depends on the extraction method used. In our case, although mandarin and grapefruit EOs obtained by cold-pressing followed by steam distillation (EOPD) displayed a difference in the chemical profile when compared to the EOs obtained by cold-pressing (EOP) (Table 2) (Luciardi et al., 2016, 2020), we could not observe any significant differences between their anti-pathogenic effect on *C. elegans*.

Grapefruit EOP has furanocoumarins in its chemical composition (Table 2) (Luciardi et al., 2020), and it is known that these compounds present in grapefruit juice and other sources of this citrus can irreversibly inhibit the activity of the cytochrome P450 3A4 (CYP3A4) enzyme in the gastrointestinal tract, enhancing systemic drug concentration through impaired drug metabolism and causing serious adverse effects (Bailey et al., 2013). Taking into account the negative interaction of grapefruit EOP, the lower commercial value, and the antipathogenic *in vivo* effect of grapefruit and mandarin EOPD, we consider these oils are promising alternatives as anti-QS agents.

Many EOs can modulate the response of the host immune system to bacterial infections contributing to the whole antimicrobial properties of the oils (Sandner et al., 2020). Hence, the effects of mandarin and grapefruit EOs observed in this work could be a combination of their anti-pathogenic effect against *P. aeruginosa* as well as their immunomodulatory properties on *C. elegans*.

Conclusion

Targeting bacterial QS systems is an interesting strategy to combat human infections, mainly those caused by antibiotic-resistant bacteria. Here we showed that *C. reticulata* and *C. paradisi* EOs could attenuate QS-controlled virulence factors of *P. aeruginosa* by significantly inhibiting biofilm formation and reducing the death rate of *C. elegans*. Our results suggest that commercial mandarin and grapefruit EOPDs, with lower commercial value than EOP, are sources of anti-QS agents for controlling *P. aeruginosa* infections. Further studies should be addressed to understand the mechanisms of QS interference.

Credit author statement

D'Almeida, R: Conceptualization, Methodology, Supervision, Writing- Original draft preparation. **Sued, N.**: Visualization, Investigation. **Arena, M**: Resources, Writing- Reviewing, Funding acquisition.

Declaration of conflicting interests

The authors have no potential conflict of interest to disclose.

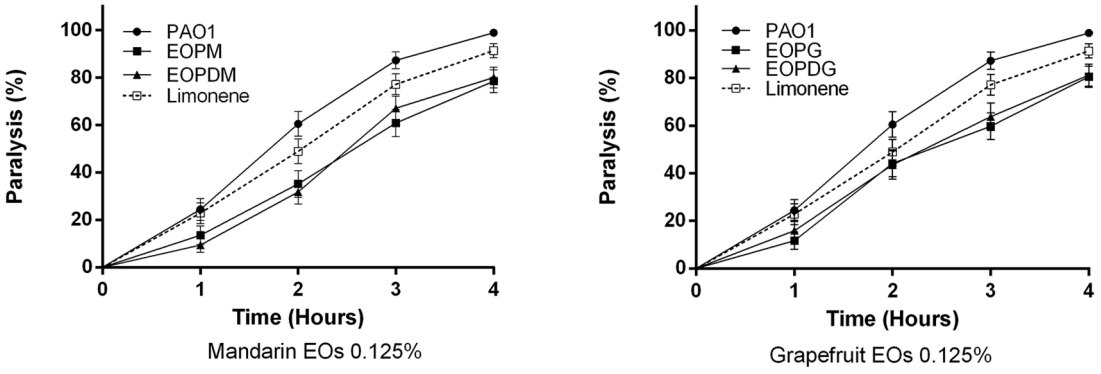

Fig. 3. Paralysis assay. Essential oils (0.125%) of *C. reticulata* (Mandarin) **(A)** and *C. paradisi* (Grapefruit) **(B)** protect *C. elegans* from paralysis caused by *P. aeruginosa* PAO1. EOP: Essential Oil obtained by cold-pressing, EOPD: Essential Oil obtained by cold-pressing followed by steam distillation.

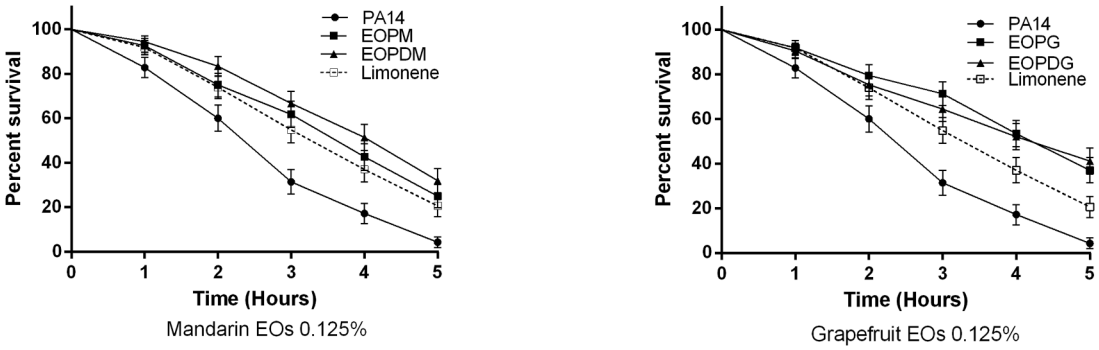

Fig. 4. Fast killing assay. Essential oils (0.125%) of *C. reticulata* (Mandarin) **(A)** and *C. paradisi* (Grapefruit) **(B)** protect *C. elegans* from death caused by *P. aeruginosa* PA14 phenazines. EOP: Essential Oil obtained by cold-pressing, EOPD: Essential Oil obtained by cold-pressing followed by steam distillation.

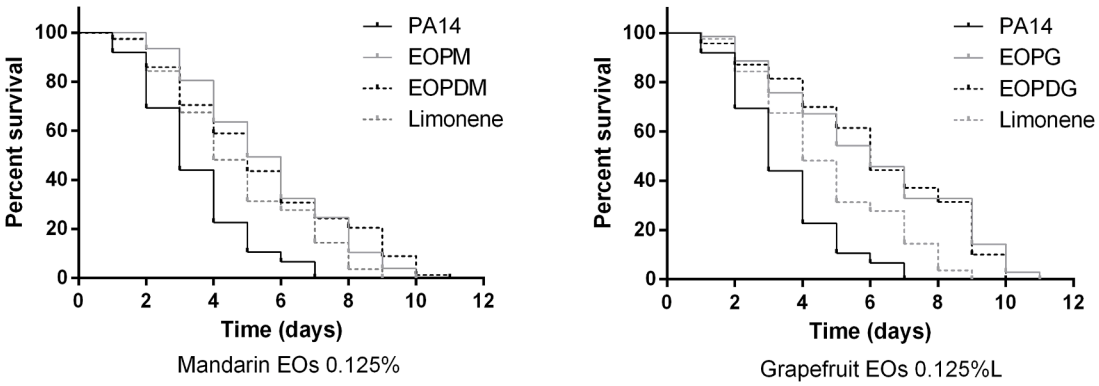

Fig. 5. Slow killing assay. Essential oils (0.125%) of *C. reticulata* (Mandarin) **(A)** and *C. paradisi* (Grapefruit) **(B)** protect *C. elegans* from death caused by *P. aeruginosa* PA14. EOP: Essential Oil obtained by cold-pressing, EOPD: Essential Oil obtained by cold-pressing followed by steam distillation.

Table 2
Comparison of the chemical composition of commercial *C. reticulata* (Mandarin)[1] and *C. paradisi* (Grapefruit)[2] essential oils obtained by two different methodologies.

Compounds	EOP *(Peak area -%)*		EOPD *(Peak area -%)*	
	Mandarin	Grapefruit	Mandarin	Grapefruit
Monoterpene hydrocarbons	**97.657**	**94.688**	**96.584**	**94.440**
Oxygenated monoterpenes	**0.655**	**0.743**	**1.296**	**1.91**
Sesquiterpene hydrocarbons	**0.424**	**1.629**	**1.237**	**1.33**
Oxygenated sesquiterpenes	**0.005**	**0.743**	**0.122**	**1.910**
Coumarins	–	**1.104**	–	–
Flavonoids	–	**0.079**	–	–

EOP: essential oils obtained by cold-pressed method; EOPD: essential oil obtained by cold-pressed method followed by steam distillation. Gas chromatography-mass spectrometry analysis reported in [1]Luciardi et al., 2016 and [2]Luciardi et al., 2020.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phyplu.2021.100160.

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