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ORIGINAL ARTICLE

The impact of plant volatiles on bacterial quorum sensing

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Significance and Impact of the Study: Antimicrobial and antiquorum sensing (QS) properties of 29 common essential oil compounds were evaluated. Interruption of QS may lead to the development of therapeutic, antivirulence agents to control disease-causing pathogens which are preferable over antimicrobial agents as the latter drives selection pressure on microbial communities to acquire resistance. Twenty-two compounds inhibited QS, while seven promoted the QS to a variable extent in *Chromobacterium violaceum* and *Pseudomonas aeruginosa*. Preliminary results suggest that QS-inhibitory compounds of natural origin may inspire the formulation of new generation of antimicrobial agents to control infectious pathogens.

Keywords
Chromobacterium violaceum, monoterpenes, natural compounds, *Pseudomonas aeruginosa*, pyocyanin, quorum sensing, violacein.

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Abstract

Studies describing the use of essential oil constituents as antimicrobial agents have steadily increased; however, some phyto-constituents are often overlooked due to unfavourable minimum inhibitory concentration (MIC) values. Virulence depends on transcriptional factors which are regulated by cell-to-cell communication called quorum sensing (QS). This study was undertaken to evaluate the antimicrobial and anti-QS properties of 29 compounds commonly found in essential oils using two bioreporter strains. QS-inhibitory activity was assessed qualitatively by agar diffusion and quantitatively by spectrophotometric assays. MICs of all the tested compounds ranged from 0·032 to >5 mg ml^{-1}. Twenty-two compounds displayed varying levels of QS inhibitory activity with zones of violacein inhibition ranging from 9 to 16 mm. Majority of tested molecules inhibited violacein and pyocyanin production in *Chromobacterium violaceum* and *Pseudomonas aeruginosa*, while seven compounds increased violacein and pyocyanin production. Interestingly, it was observed that the (+)-enantiomers of carvone, limonene and borneol increased violacein and pyocyanin production, while their levorotary analogues inhibited this production. α-Terpineol and *cis*-3-nonen-1-ol exhibited >90% violacein inhibition, suggesting their potential as QS inhibitors. This preliminary study indicates that plant volatiles have the potential to impede or promote bacterial communication and further studies need to be undertaken to explore the contribution of structural analogues and stereochemistry of molecules in this process.

Introduction

The development of antibiotics to control growth of pathogenic bacteria was hailed as one of the greatest achievements of the 20th century. However, with the frequent use (and abuse) of antibiotics, bacteria are under harsh selective pressures, which has led to the emergence of multidrug resistant pathogens. To overcome these problems, searching for alternative approaches which rely on the inhibition of the pathogenic traits rather than on killing or inhibiting the growth of the bacteria has gained popularity in the research community. The manifestations of bacterial virulence are often associated with quorum sensing (QS) or cell-to-cell communication. Therefore, inhibition of QS has emerged as a novel approach to target pathogenic bacteria as anti-QS drugs targeting bio-

chemical processes which are not essential for the survival of the micro-organism but are important for their pathogenesis (Rasmussen and Givskov 2006; Roy *et al.* 2011). Acyl homoserine lactones (AHLs) and oligopeptides are the most common QS molecules in Gram-negative and Gram-positive bacteria, respectively (Parsek *et al.* 1999; Alvarez *et al.* 2012), and have been targeted to discover the antipathogenic properties of natural products (Hentzer *et al.* 2002; Rasmussen *et al.* 2005; Ishida *et al.* 2007).

Plants are known to produce secondary metabolites as part of their defence arsenal (González-Lamoth *et al.* 2009; Mazid *et al.* 2011). These secondary metabolites have been used by ancient and modern civilization as antimicrobial agents (Cowan 1999; Ciocan and Bara 2007; Reichling 2010). A voluminous body of evidence exists describing the potent antimicrobial activities of plant essential oils and their individual constituents, without exploring their efficacy against bacterial virulence. This has resulted in numerous natural extracts (including essential oils) being overlooked and/or dismissed on the basis of their high minimum inhibitory concentration (MIC) values.

The clinical demand and the increase in resistant pathogens have prompted the search for phyto-constituents with anti-QS activities (Nazzaro *et al.* 2013; Tan *et al.* 2013). In this regard, quick and simple methods using biosensor strains for determining the effect of the test agents on QS and AHL molecules have gained interest (Zahin *et al.* 2010). Inhibition of QS-regulated violacein and pyocyanin production in *Chromobacterium violaceum* and *Pseudomonas aeruginosa* respectively, is a common research approach to screen for anti-QS and antibiofilm activity (Njoroge and Sperandio 2009). Several studies have reported the antimicrobial activities of pure essential oil compounds (Inouye *et al.* 2001; Silva and Fernandes 2010); however, limited studies have been devoted to describing the anti-QS activity of essential oil compounds (Kerekes *et al.* 2013; Burt *et al.* 2014). Due to instability and toxicity concerns, most of these QS-inhibiting compounds have not qualified for clinical trials (Borges *et al.* 2014). The primary objective of this study was to determine the antimicrobial and anti-QS activities of mono- and sesquiterpenes commonly found in commercially available essential oils using a bioreporter strain of *C. violaceum* and *P. aeruginosa*. The secondary objective was to explore the potential role of stereochemistry in optically active compounds and how these subtle structural differences may impact on anti-QS activity.

Results and discussion

Essential oils and their volatile constituents have been extensively studied as antimicrobial agents. However, their antivirulence activity has remained a neglected research opportunity. There are limited studies describing the effect of natural products on virulence factors, for example carvacrol demonstrated QS inhibition in bacteria which limited biofilm formation and/or chitinase production (Kerekes *et al.* 2013; Borges *et al.* 2014; Burt *et al.* 2014). In view of this, we designed a study to document the effect of 29 common volatiles occurring in essential oils on the growth and QS-related processes of two important Gram-negative bacteria: the QS indicator *C. violaceum* and the opportunistic human pathogen *P. aeruginosa*.

Antimicrobial susceptibility testing

Antibacterial activity against *C. violaceum* and *P. aeruginosa* was observed for 55·2% (16/29) of the test compounds at concentrations ranging from 0·032 to 0·5 mg ml^{-1}, while MIC values >0·5 mg ml^{-1} were observed for the remaining 13 compounds. The MIC values for the 29 essential compounds for *C. violaceum* ATCC 12472 are listed in Table 1. The most active molecule was the monoterpene aldehyde citral with an MIC value of 0·032 mg ml^{-1} followed by the monoterpene alcohols thymol and carvacrol (MIC = 0·062 mg ml^{-1}). MICs for the remaining tested compounds ranged from moderate (0·125 mg ml^{-1}) to high (>0·5 mg ml^{-1}).

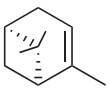
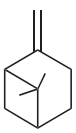
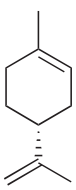
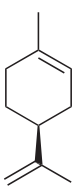
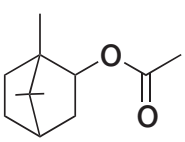
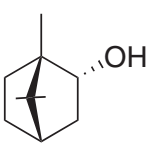
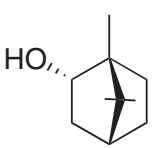
Qualitative antiquorum sensing by agar diffusion assay

The clear zones and the colourless, opaque zones with loss of purple pigmentation around treated discs clearly indicated growth inhibition and QS inhibition, respectively, by these compounds. Of the 29 tested compounds, 22 inhibited violacein production, while seven (+)-α-pinene, (-)-α-pinene, *β*-pinene, (+)-carvone, (+)-limonene, (+)-borneol and bornyl acetate increased violacein production. The 22 active compounds showed varying levels of activity with colourless, opaque zones of violacein inhibition ranging from 9 to 20 mm (Table 1, Fig. S1).

Quantitative evaluation of antiquorum sensing activity in *Chromobacterium violaceum*

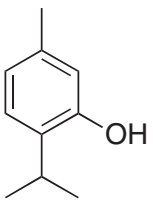
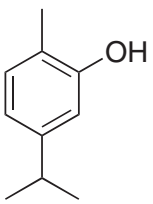
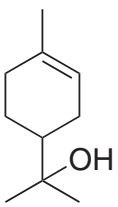
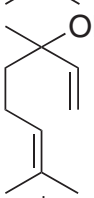
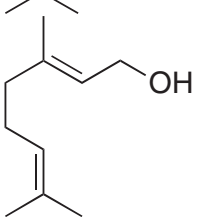
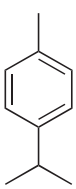
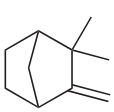
To confirm the effect of these QS-inhibitory compounds, their effect on AHL-mediated violacein pigment production was quantified spectrophotometrically. The minimum QS inhibitory concentrations (MQSIC) of all the compounds have been listed in Table 1. As observed in the agar diffusion assay, violacein production decreased in treated *vs* control cells (Fig. 1a), with >50% inhibition being observed with MQSIC exposure. The most

Table 1 Minimum inhibitory concentrations (MIC), minimum quorum sensing inhibitory concentrations (MQSIC) and colourless zones of turbidity (ZOT) at MQSIC of standard essential oil compounds

Compound	Structural	Chemical class	MIC mg ml^{-1}	MQSIC* mg ml^{-1}	ZOT (mm)
(+) α-pinene		Monoterpene hydrocarbon	>0·5	Positive†	0
(-) α-pinene		Monoterpene hydrocarbon	>0·5	Positive	0
β-pinene		Monoterpene hydrocarbon	>0·5	Positive	0
(+) Limonene		Monoterpene hydrocarbon	0·25	Positive	0
(–) Limonene		Monoterpene hydrocarbon	>0·5	0·5	9
Bornyl acetate		Other	>0·5	Positive	0
(+) Borneol		Monoterpene alcohol	0·5	Positive	0
(-) Borneol		Monoterpene alcohol	>0·5	0·5	12

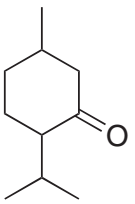
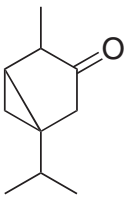
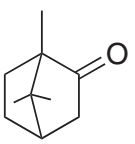
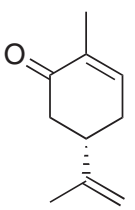
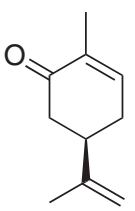
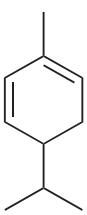
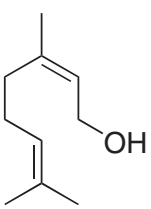
(continued)

Table 1 *(continued)*

Compound	Structural	Chemical class	MIC mg ml^{-1}	MQSIC* mg ml^{-1}	ZOT (mm)
Thymol		Monoterpene alcohol	0·062	0·032	12
Carvacrol		Monoterpene alcohol (cyclic)	0·062	0·032	13
α-Terpineol		Monoterpene alcohol	0·062	0·125	20
Linalool		Monoterpene alcohol (acyclic)	0·5	0·25	11
Geraniol		Monoterpene alcohol (acyclic)	0·125	0·062	10
p-Cymene		Monoterpene hydrocarbon	>0·5	0·25	15
Camphene		Monoterpene hydrocarbon	>0·5	0·5	10

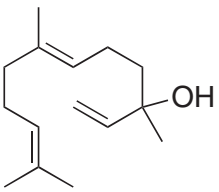
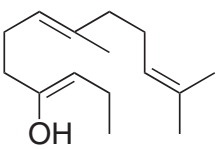
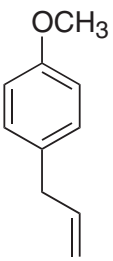
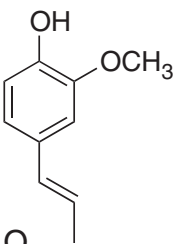
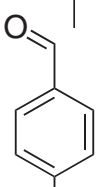
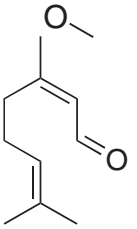
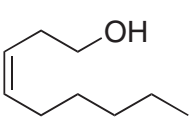
(continued)

Table 1 *(continued)*

Compound	Structural	Chemical class	MIC mg ml^{-1}	MQSIC* mg ml^{-1}	ZOT (mm)
Menthone		Monoterpene ketone	0·25	0·125	14
Thujone		Monoterpene ketone	0·5	0·25	14
Camphor		Monoterpene ketone	>0·5	0·25	14
(+) Carvone		Monoterpene ketone	0·25	Positive	0
(−) Carvone		Monoterpene ketone	0·5	0·25	11
α-Phellandrene		Monoterpene	>0·5	0·5	13
Nerol		Sesquiterpene alcohol (acyclic)	0·25	0·125	16

(continued)

Table 1 *(continued)*

Compound	Structural	Chemical class	MIC mg ml^{-1}	MQSIC* mg ml^{-1}	ZOT (mm)
Nerolidol		Sesquiterpene alcohol (acyclic)	>0·5	0·5	12
Farnesol		Acyclic sesquiterpene alcohol (acyclic)	>0·5	0·5	11
Estragole		Phenylpropene	0·125	0·062	13
Isoeugenol		Phenylpropanoid	0·125	0·062	13
p-Anisaldehyde		Aldehyde	>0·5	0·25	12
Citral		Monoterpene aldehyde (acyclic)	0·032	0·016	15
cis-3-Nonen-1-ol		Other	0·25	0·125	16

*Minimum quorum sensing inhibitory concentration (MQSIC) was determined as the lowest concentration at which violacein production was inhibited by ≥50%.

†Positive refers to increased violacein production rather than inhibition.

prominent violacein inhibition (>90%) was observed with 0·125 mg ml^{-1} of α-terpineol and *cis*-3-nonen-1-ol.

To gain a better understanding of structure–activity relationships, the effect of stereochemistry of chiral compounds was investigated. The (+)-enantiomers tested showed an increase in violacein production, while the (-)-enantiomers (except (-)-α-pinene) inhibited violacein production (Fig. 1b).

It was also observed that the growth of the bacteria was not affected as the entire cell counts from the treated groups showed no significant difference when compared to the control. For all experiments, the solvent controls showed similar cell growth and violacein production as observed in control cells.

Quantitative evaluation of antiquorum sensing activity in *Pseudomonas aeruginosa*

In an effort to further explore and confirm the anti-QS potential of these volatiles, the inhibition of *P. aeruginosa* pyocyanin production was investigated. Pyocyanin, a QS factor in *P. aeruginosa* characterized by a blue pigment, was inhibited by 18 of the 29 assayed compounds (Fig. 2a). The pyocyanin inhibitory profile was very different from that observed for violacein inhibition. Thujone was responsible for ~75% inhibition of *P. aeruginosa* ATCC 27853 pyocyanin production. Even though the results of pyocyanin inhibition of all the compounds, except estragole, farnesol, *p*-anisaldehyde and (−)-α-pinene, are congruent with the results observed for violacein inhibition, the level of pyocyanin inhibition by these test compounds was lower than violacein inhibition in *C. violaceum*.

The pyocyanin inhibition results for the different enantiomers, however, followed the same pattern as observed for violacein inhibition (Fig. 2b). The most prominent pyocyanin inhibitory activity was observed for (−)-borneol.

Microbial pathogenesis depends on numerous virulence factors, which collectively contribute to initiate and maintain infections. During pathogenesis, compromise of a single virulence factor may be compensated for by other virulence factors. However, the loss of essential virulence factors attenuates pathogenicity of the micro-organism (Schaber *et al.* 2004). QS networks regulate expression of essential pathogenic virulence factors which in turn regulate microbial pathogenesis (Rutherford and Bassler 2012). Therefore, the interruption of QS can be an effective strategy to control disease-causing pathogens.

Of 29 tested compounds, 44·8% (13/29) demonstrated higher MIC values (>0·5 mg ml^{-1}) while these compounds exhibited ≥50% AHL-mediated QS inhibition at lower concentrations (0·125–0·5 mg ml^{-1}). To assess whether the inhibition of violacein and/or pyocyanin production in *C. violaceum* and *P. aeruginosa*, respectively, could be attributed to growth reduction or AHL inhibition, cultures grown at MQSIC were subcultured onto agar plates revealing that none of these concentrations showed an effect on the bacterial growth. The major advantage of this approach of inhibiting virulence factor production is that it is preferential to develop antivirulence agents rather than antimicrobial agents as the latter results in selection pressure on microbial communities to acquire resistance (Rasmussen and Givskov 2006).

The results observed in Fig. 1a,b demonstrate that the majority of tested compounds, irrespective of their MIC values, inhibited violacein production. Previous studies have reported similar results obtained for extracts and essential oils from medicinal plants (e.g. Zahin *et al.* 2010). Kerekes *et al.* (2013) observed that the monoterpene limonene had no inhibitory effect on the production of violacein, α-pinene and linalool demonstrated a quantity-dependent inhibitory effect, while the terpene alcohol, terpinene-4-ol was most effective in violacein inhibition. Borges *et al.* (2014) also reported that some phenolic compounds such as gallic acid and ferulic acid displayed antimicrobial activities but did not interfere with the QS.

The diversity in inhibitory potential observed for the different essential oils (Fig. 1) could be related to diversity in their chemical structures. Jaramillo-Colorado *et al.* (2012) observed that citral was more effective in inhibiting QS mediated by long chain AHLs. The mechanism of violacein inhibition for the essential oils is most likely related to AHL mimicry and signal reception inhibition. Many monoterpene essential oils although not structurally similar to AHLs share similarity to a C6 (short chain) linear carbon skeleton (Jaramillo-Colorado *et al.* 2012) and could therefore compete with short chain AHL-based violacein production. Thus, the structural diversity of essential oil compounds would allow a wider spectrum of activity against micro-organisms producing diverse short- and long chain AHLs.

The increased violacein production observed for seven essential oils is in agreement with previous findings where some compounds from pea seedlings appeared to inhibit QS in one bacterium while promoting QS in others (Teplitski *et al.* 2000). However, further research is required to determine the QS-enhancing mechanisms of (+)-enantiomers.

Although the QS systems of *C. violaceum* and *P. aeruginosa* both consist of the LuxI/LuxR homologues, *P. aeruginosa* has three QS systems, namely *las, rhl* and *pqs*. Pyocyanin production is regulated by the *pqs* system, whose signal molecule is called *Pseudomonas* quinolone signal (PQS) (Alymanesh *et al.* 2013; Zhou *et al.* 2013). The differences observed in violacein and pyocyanin inhibition by essential oil constituents could be attributed to their effect on different QS systems in the two organisms tested. Thus, thujone and citral were more effective

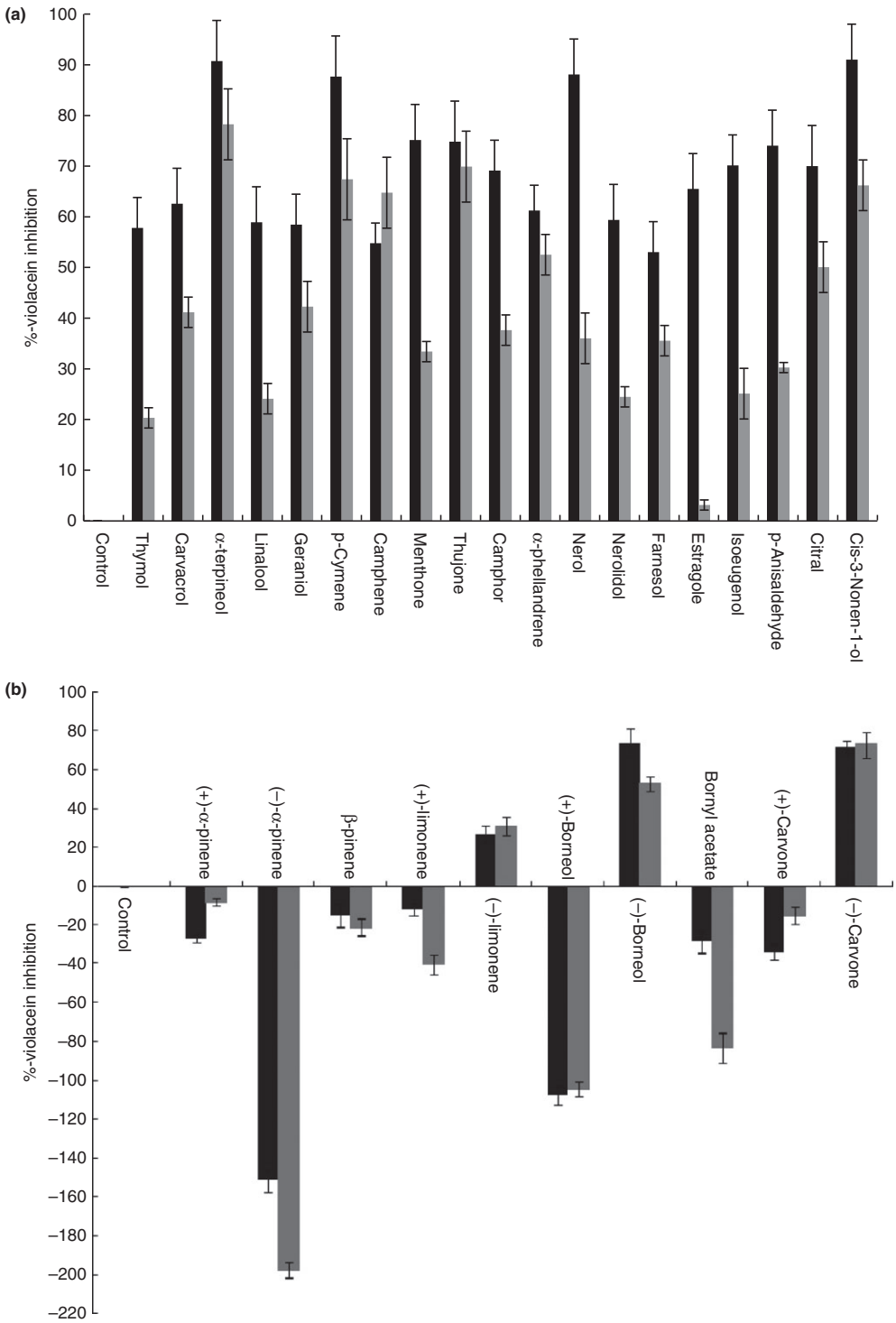


Figure 1 (a) Quantitative analysis of *Chromobacterium violaceum* ATCC 12472 violacein inhibition by selected essential oil compounds following minimum quorum sensing (QS) inhibitory concentration (black bars) and 0·5× minimum QS inhibitory concentration (MQSIC) (grey bars) exposures. Bars represent the mean of three independent experiments ± SD. (b) Quantitative analysis of *C. violaceum* ATCC 12472 violacein inhibition by different essential oil enantiomers following MQSIC (black bars) and 0·5× MQSIC (grey bars) exposures. Bars represent the mean of three independent experiments ± SD.

against the *Pseudomonas pqs* system for pyocyanin inhibition, while α-terpineol and *cis*-3-nonen-1-ol were more effective violacein inhibitors.

The findings of this study highlight the importance of evaluating the unexplored anti-QS properties of natural products. The observed inhibition of QS in *C. violaceum* and *P. aeruginosa* suggests that plant volatiles may interfere with QS in other clinically important pathogens using similar AHL signalling molecules and systems. From the results obtained, albeit preliminary, it is plausible to suggest that QS-inhibitory compounds of natural origin may inspire the formulation of a new generation of antimicrobial agents to control the infectious pathogens. Apart from the applications in the field of medicine, this strategy may become relevant in other fields, for example agriculture, food technology and aquaculture. Therefore, further in depth studies are encouraged to explore the contribution of structural analogues, enantiomers and the role of specific moieties to assess the mechanism of anti-QS action of natural products.

Materials and methods

Bacterial strains, media and culture conditions

Bioreporter bacterial strains *C. violaceum* ATCC 12472 and *P. aeruginosa* ATCC 27853 were routinely cultured in Luria–Bertani (LB) broth medium (1% peptone, 0·5% yeast extract, 0·5% NaCl, per 100 ml distilled water) at 30°C for 24 h with shaking at 150 ***g***. For all the experiments, a single colony was inoculated in 10 ml of LB media and incubated overnight at 30°C with agitation.

Antimicrobial activity

The antimicrobial activity of 29 selected essential oil compounds (Table 1; Sigma-Aldrich, Steinheim, Germany) was determined by the broth microdilution method (CLSI 2003). Essential oil compounds were prepared in 100% DMSO and then further diluted in distilled water to the desired concentrations. DMSO was always ≤1% of the total volume. All the compounds were tested at concentrations ranging from 0·004 to 0·5 mg ml^{-1} in Mueller–Hinton broth. MIC is defined as the minimum concentration of a compound at which there was no visible growth of the test strain. Results were calculated as a mean of experiments performed in triplicate.

Qualitative biosensor bioassay for detecting anti-QS activity of volatile constituents

For qualitative screening of anti-QS activity of essential oil constituents, inhibition of violacein pigment production by biosensor strain *C. violaceum* ATCC 12472 was measured. The biosensor assay was carried out as described previously with slight modifications (Chenia 2013). Briefly, *C. violaceum* cells (10^5 cells ml^{-1}) were mixed in molten LB agar (~40°C) and poured into a Petri dish. Sterile filter discs (9 mm; Oxoid, Basingstoke), impregnated with varied concentrations (MIC, 0·5 × MIC and 0·25 × MIC) of the test compounds (dissolved in 1% DMSO) in 10 μl final volume, were placed on solid agar and incubated at 30°C for 24 h. A disc impregnated with 10 μl of 1% DMSO served as a negative control. The diameter of the clear zones indicating inhibition and the colourless, opaque zones against a purple background were recorded in mm after 24 h. Clear zones around the discs indicated complete growth inhibition, while colourless, opaque halos around the discs indicated inhibition of QS by the test compounds.

Quantitative detection of violacein inhibition in presence of test compounds

To quantify the anti-QS activity of the essential oil compounds, the percentage inhibition of violacein pigment was quantified in treated *vs* untreated *C. violaceum* ATCC 12472 as described previously (Chenia 2013). The *C. violaceum* cells were subcultured at least twice and grown for 24 h at 30°C on LB agar plates. The adjusted inoculum suspension of 5×10^6 CFU ml^{-1} was diluted 1 : 10 in media to yield a final inoculum concentration of 5×10^5 CFU ml^{-1}. To confirm the ability of the test compounds to inhibit violacein production, different concentrations ranging from 0·016 to 0·5 mg ml^{-1} were added to 5 ml of LB broth. From the above cultured stock, 100 μl of *C. violaceum* ATCC 12472 was added to all tubes. In all experiments, DMSO was added similarly as the negative control. Tubes were incubated at 30°C for 24 h with agitation. Following incubation, 1 ml aliquots were centrifuged at 12 000 ***g*** for 10 min to pellet bacteria containing violacein. The supernatant was discarded and the pellet was resuspended in 1 ml of DMSO to solubilize the violacein. To remove bacterial cells, suspensions were centrifuged using the same operational procedure as mentioned above. Violacein was quantified from the supernatant by recording the absorbance at $OD_{585\ nm}$ using a Bio-tek ELx800 UV-Vis Universal microplate reader. The percentage of violacein inhibition was calculated using the following formula as described by Packiavathy *et al.* (2012):

$$\text{Percentage of violacein inhibition} = [(\text{control } OD_{585\,nm} - \text{test } OD_{585\,nm})/\text{control } OD_{585\,nm} \times 100$$

On the basis of the inhibition of violacein production with respect to control, the MQSIC of the test com-

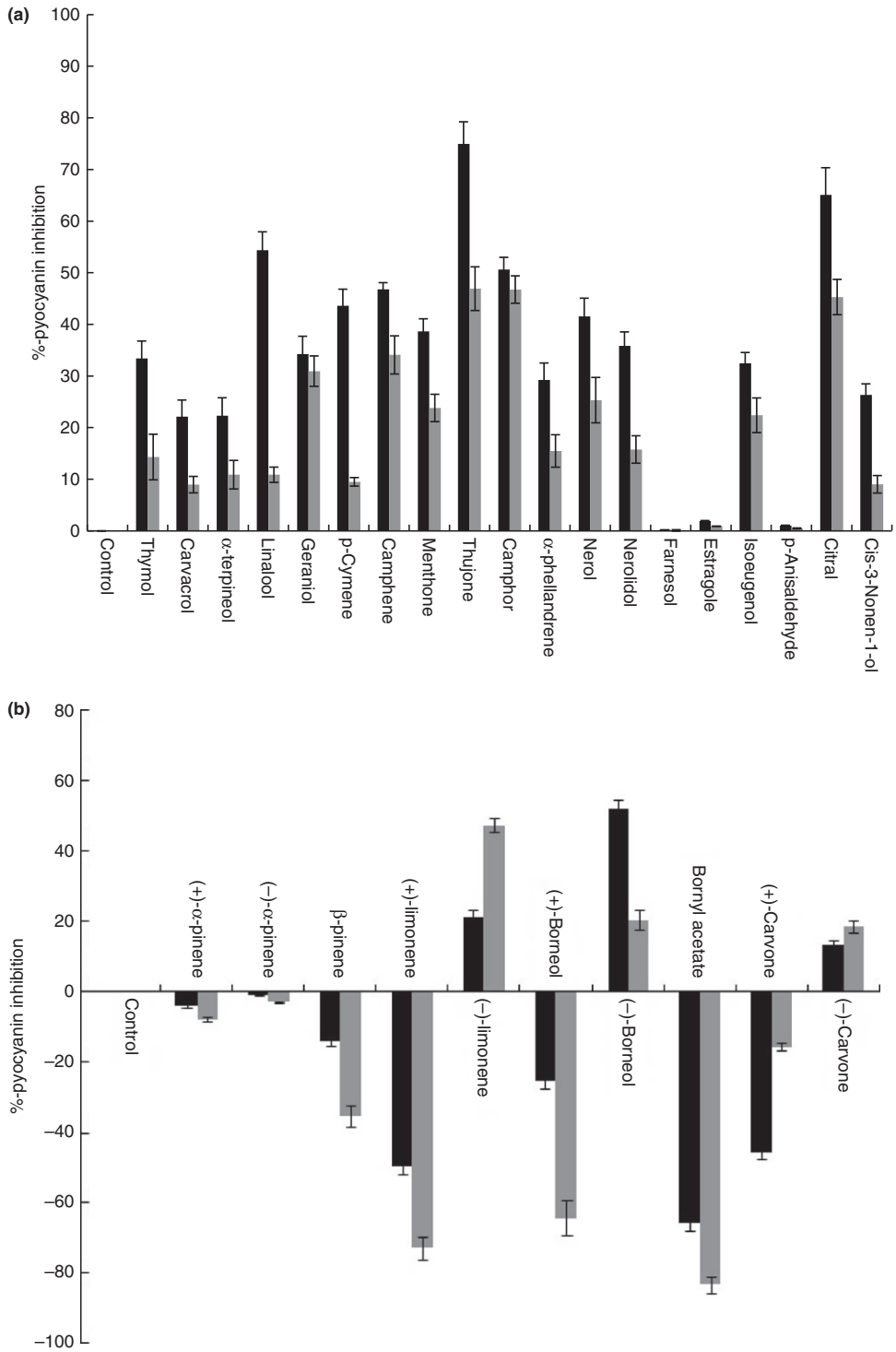


Figure 2 (a) Quantitative analysis of pyocyanin inhibitory activities of selected essential oil compounds following growth of *Pseudomonas aeruginosa* ATCC 27853 at minimum quorum sensing (QS) inhibitory concentrations (black bars) and 0·5× minimum QS inhibitory concentration (MQSIC) (grey bars). Bars represent mean of three independent experiments ± SD. (b) Quantitative analysis of pyocyanin inhibitory activities of selected essential oil enantiomers following growth of *P. aeruginosa* ATCC 27853 at MQSICs (black bars) and 0·5× MQSICs (grey bars). Bars represent mean of three independent experiments ± SD.

pounds was determined as the lowest concentration at which violacein production was inhibited by ≥50%.

Determination of QS inhibition in *Pseudomonas aeruginosa* by pyocyanin assay

To quantify the inhibitory potential of the test compounds on QS-mediated virulence in *P. aeruginosa*, pyocyanin was extracted and estimated from 18 h *P. aeruginosa* ATCC 27853 cells. Test compounds, ranging from 0·062 to 0·5 mg ml^{-1}, were added to 5 ml of freshly prepared *P. aeruginosa* culture, and tubes were incubated at 37°C for 24 h. Following incubation, cultures were centrifuged, 3 ml of chloroform was added to the resulting supernatant and tubes were vortexed vigorously. The chloroform layer was then separated and mixed with 1 ml of 0·2 mol l^{-1} HCl and centrifuged for 10 min at 12 000 ***g***. The HCl layer was separated and OD was measured at 520 nm using a Thermo Electron Heλios α spectrophotometer. The percentage of pyocyanin inhibition was calculated similarly as mentioned above.

Statistical analysis

All the experiments were performed in triplicate. All results were expressed in terms of mean ± standard deviation (SD).

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Conflict of Interest

No conflict of interest declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Representative plates showing antimicrobial and antiquorum sensing activities of α-terpineol (a) and thymol (b) at their respective MIC and MQSIC values against *Chromobacterium violaceum* ATCC 12472.