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Investigating the interactive efficacy of the enantiomers of Limonene

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ABSTRACT

Limonene is a widely distributed compound present in many essential oils. This cyclic monoterpene has been well studied, but what has been often overlooked is the possible influence of stereochemistry on biological activity, particularly in combination with other essential oil compounds. This study aimed to investigate the *in vitro* antimicrobial inhibition (minimum inhibitory concentration – MIC and anti-quorum sensing – QS), and toxicological screening (brine shrimp lethality assay – BLSA) of the enantiomers of Limonene, alone and in 1:1 combinations with 14 essential oil compounds. When investigated independently, the enantiomers were broadly comparable (MIC 0.25 – 4.00 mg/mL; MQSIC 0.38 – 0.50 mg/mL; ≤5.8% mortality at 48 h). However, variations in the enantiomeric pair were evident when investigated in combination. *S*-(–)-Limonene displayed the most synergy (ΣFIC ≤0.50), in combination with β-caryophyllene against *Cryptococcus neoformans* (MIC = 0.13 mg/mL; ΣFIC = 0.50). *R*-(+)-Limonene reduced the concentration required to block violacein production, lowering the MQSIC to 0.06 mg/mL when paired with Estragole or Geraniol (ΣFQSIC = 0.19 – 0.31). *R*-(+)-Limonene formed non-toxic combinations with γ-Terpinene and α-Terpineol (ΣFPM ≤0.23). The chirality of Limonene affected both efficacy and toxicity in one-third of the combinations investigated, demonstrating that stereoselectivity is critical, yet frequently overlooked, in natural product formulation.

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KEYWORDS
Limonene; enantiomer; antimicrobial; interaction; toxicity; essential oil compounds

1. Introduction

The importance of studying enantiomeric configurations can best be highlighted from the historical catastrophe of thalidomide. Thalidomide was a drug produced in the 1950s that was prescribed as an antiemetic. Thalidomide consists of two rings, a glutarimide and phthalimide ring, with a chiral carbon. The unstable chiral carbon allows for the interconversion of thalidomide between the two enantiomers: *S*-(–)-thalidomide, which is teratogenic, and *R*-(+)-thalidomide, which is non-teratogenic (1). Unfortunately, the drug was produced as a racemic mixture (mixture of equal quantities of enantiomers) and taken by thousands of unsuspecting pregnant women, which resulted in babies born with phocomelia. Enantiomeric configurations have also been shown to impact on antimicrobial activity, where the spectrum of activity may be affected. Some antimicrobials may be available as single enantiomers, such as Linezolid and Erythromycin, or they may be available as a racemate, such as Ofloxacin. The (+)-enantiomer of Linezolid is the active enantiomer (2). Levofloxacin is the (–)-enantiomer of the racemate Ofloxacin, and it binds more effectively to the deoxyribonucleic acid (DNA) gyrase enzyme and topoisomerase IV than its (+)-enantiomeric counterpart and therefore has a broader spectrum of antimicrobial activity (3).

Essential oils are a complex blend of volatile compounds that have shown to be responsible for biological activity (4,5). Limonene, an unsaturated cyclic monoterpene, is one such compound that is frequently observed in several essential oils. This essential oil compound is predominately found in essential oils of citrus peel (6), and several *Pinus* species (7). Limonene has previously demonstrated noteworthy and pathogen-selective antimicrobial activity (8) and the toxicological profile of this monoterpene is well established (9). Limonene has also proven to have anti-quorum sensing (QS) properties (10,11). This process of bacterial communication aids in pathogenesis and virulence, through mechanisms such as biofilm formation and resistant gene mutations (12). However, what is often overlooked in the investigation of Limonene, is the possible impact of the stereochemical configuration of these optically active enantiomers.

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The enantiomers of Limonene can be found in nature as either *R*-(+)-Limonene, *S*-(–)-Limonene or a racemic mixture of the two (Figure 1).

R-(+)-Limonene is more readily available than *S*-(–)-Limonene (13). *R*-(+)-Limonene has been identified as a major constituent in essential oils obtained from *citrus* species, particularly peel extracts (14), and *S*-(–)-Limonene has been identified as one of the major constituents in certain *Mentha* species (Lamiaceae) (15). A study (16) reported that *R*-(+)-Limonene had a broader spectrum of antimicrobial activity than *S*-(–)-Limonene, against the bacterial pathogens investigated, but found no noteworthy trends when investigated against the fungal species. Unfortunately, no quantitative data was given, and the disc diffusion assay was utilized, which has been reported as being unreliable for essential oils and their compounds (17,18). In another study (19), *R*-(+)-Limonene was shown to possess stronger inhibitory action than *S*-(–)-Limonene. In a study against *Escherichia coli*, *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus* and *Staphylococcus epidermidis*, the *S*-(–)-Limonene was more active, with MIC values ranging between 4.00 and 8.00 mg/mL. However, *R*-(+)-Limonene was less active with MIC values ranging between 8.00 and >16.00 mg/mL (20). In terms of antifungal activity, the two enantiomers of Limonene had potent inhibitory activity against clinical isolates of six *Candida* species, and it was reported that against *Candida krusei*, *S*-(–)-Limonene was found to be 10-fold more active (21). Another study found the *S*-(–)-Limonene to be the more active enantiomer against *S. aureus* where the minimum inhibitory concentration (MIC) activity (MIC = 4.00 mg/mL) was three times that of *R*-(+)-Limonene (MIC = 13.00 mg/mL) (22).

In terms of the anti-quorum sensing (anti-QS) activity, 29 essential oil compounds were investigated, including the enantiomers of Limonene, using the biosensor strain *Chromobacterium violaceum*. It was reported that *S*-(–)-Limonene inhibited violacein production, whereas the *R*-(+)-Limonene promoted violacein production (23). The authors of the study emphasized that this observation suggests that more attention must be paid to the chemical composition of essential oils containing such structural analogues. In terms of toxicity, *R*-(+)-Limonene showed toxicity at a concentration almost four times that of *S*-(–)-Limonene. The LC_{50} values were 1.72 mM for *R*-(+)-Limonene and 0.45 mM for *S*-(–)-Limonene (24).

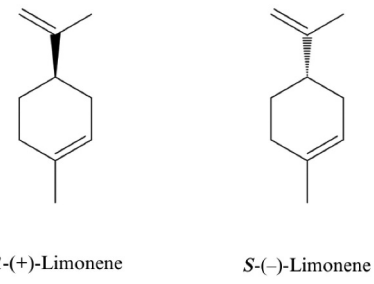

Figure 1. The Structure of the enantiomers of Limonene.

While these studies are important, few studies have investigated the influence of the stereochemistry on the combined interactive activity of Limonene with other essential oils. This is especially relevant as the biological activity of essential oils is often attributed to the combined activity of its constituent molecules (4,5). There are limited studies that have investigated the pharmacological activity and comparison of enantiomers when combined with other compounds. Our earlier study (22) investigated whether micro-organisms respond differently when exposed to both enantiomers of Limonene and the racemate. When combined with 1,8-Cineole, (–)-Limonene displayed antagonism for all ratios investigated, whereas (+)-Limonene displayed synergy that was concentration dependent. This synergy was especially evident with *S. aureus*. While that study demonstrates the impact of variance between enantiomers of Limonene, little is known about the effects with other compounds, the effects against anti-QS activity and toxicity. The aim of this study was thus to conduct *in vitro* antimicrobial (MIC and QS) studies, and toxicological screening of the enantiomers of Limonene, both independently and in combination with a selection of essential oil compounds, to identify variations in activity of the enantiomers when assayed in combination.

2. Materials and methods

2.1. Sample selection and preparation

The *R*-(+)-Limonene (LOT 301TI–101) and *S*-(–)-Limonene (LOT BCCD6146) and the selected compounds (Supplementary Table S1) were obtained from Sigma-Aldrich (Johannesburg, South Africa). The compound selection for combination studies was based on previous reports of good antimicrobial activity against most of the pathogens selected for this investigation, as well as availability (25–28). All test compounds had a purity range between 90.00% and 99.00%. For the broth microdilution assay, the samples were dissolved in acetone to yield a starting concentration of 32.00 mg/mL. For the anti-QS analysis, samples were also made up to 32.00 mg/mL but with dimethyl sulfoxide (DMSO, Riedel-de-Haën; concentration 1.60–0.20%) . All

samples were kept in amber bottles at 4 ℃ until further analysis.

2.2. Culture preparation

The micro-organisms investigated for the antimicrobial analysis were selected due to their prevalence in infection and their ability to develop resistance (29–31). Two of each Gram-positive and Gram-negative ESKAPE pathogens were selected, namely: *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecium* (ATCC 27270), *Klebsiella pneumoniae* (ATCC 13887) and *Pseudomonas aeruginosa* (ATCC 27853). In addition, two fungal species were included, namely *Candida albicans* (ATCC 10231) and *Cryptococcus neoformans* (ATCC 14116). Cultures were grown in Tryptone Soya broth (TSB) (Oxoid) at 37 °C for 24 h for bacterial strains and 37°C for 48 h for the yeast strains, except for *C. neoformans*, which was grown in Sabouraud Dextrose broth (SDB) at 37 °C for 72 h. The micro-organisms were kept viable by sub-culturing every two weeks. Bacterial cultures were streaked onto Tryptone Soya agar (TSA) plates and the yeast, *C. neoformans*, was streaked onto Sabourauds Dextrose agar (SDA) and incubated under the optimal incubation conditions to confirm culture purity.

For the QS study, *Chromobacterium violaceum* (ATCC 12472) was used as the biosensor strain. To prepare the culture, *C. violaceum* was incubated, under aerobic conditions, in Luria Bertani broth (LBB) at 30°C for 24 h in an orbital shaker incubator (Labcon) at 140 revolutions per minute (rpm). The culture was then streaked out onto Luria Bertani agar (LBA) and incubated at 30°C for 24 h to check for purity. A single colony from this streak plate was then inoculated into 10.00 mL of LBB and incubated at 30°C for 24 h under constant agitation (140 rpm). After incubation, this culture contained approximately 5×10^6 CFU/mL. This suspension was then diluted by a factor of 10 using LBB as a diluent, to achieve a culture suspension of 5×10^5 CFU/mL, which was then used for the anti-QS assay.

2.3. The minimum inhibitory concentration assay

The MIC assay was performed on the enantiomers and selected compounds (32). A volume of 100.00 µL of sterile TSB/SDB was placed into each well of a 98-well micro-titre plate. The compounds were placed into the top row of the micro-titre plate in volumes of 100.00 µL in acetone, after which serial doubling dilutions were performed to subsequently achieve compound concentrations of 4.00, 2.00, 1.00, 0.50, 0.25, 0.13 and 0.06 mg/mL. A 0.50 McFarland's turbidity standard was prepared by diluting overnight growth of micro-organism into TSB/SBD at 1:100 dilutions and was thereafter added to all the wells of the micro-titre plates. These were sealed and incubated at the appropriate incubation conditions. After incubation, 40.00 µL of a 0.40% *p*-Iodonitrotetrazolium (INT) violet indicator solution was added to the inoculated wells. A change in colour of the wells from clear to pink or red was used as an indication of the presence of microbial growth. Plates were analysed, and the MIC values interpreted as the lowest concentration at which growth was inhibited (visually noted as the lowest concentration having no colour change (33). Noteworthy inhibitory activity is considered as an MIC value of ≤1.00 mg/mL (34). The study was performed in duplicate, and where variations between the enantiomers occurred, a third replicate was performed on a consecutive day. Repetitions and replicates were reported as mean values. To ensure that it was not the solvent itself that was responsible for the antimicrobial effects noted, acetone (32.00 mg/mL) was used as the negative control. To ascertain the susceptibility of the cultures, ciprofloxacin and amphotericin B (Sigma-Aldrich) were used as the positive controls for bacteria and yeasts, respectively. Ciprofloxacin was prepared using sterile water as a vehicle to make up to stock concentrations of 0.01 mg/mL, whilst amphotericin B was first dissolved in dimethyl sulfoxide (DMSO) and then made up to 0.1 mg/mL with sterile water.

2.4. Anti-quorum sensing analysis

The enantiomers and selected compounds were assayed for anti-QS activity at concentrations of 0.50, 0.25, 0.13 and 0.06 mg/mL (23,35). After the compounds and controls were prepared at the test concentrations, they were inoculated with 100.00 µL of *C. violaceum* from the stock suspension. This was then incubated at 30 °C for 24 h at 140 rpm. Following incubation, the results were observed macroscopically and the anti-QS activity of the compounds was indicated by the absence of violacein by determining the minimum quorum sensing inhibitory concentration (MQSIC). To confirm that the inhibition of violacein was attributed to the reduction in QS activity and not to the growth inhibitory activity of the compounds, the samples were thereafter sub-cultured onto LBA. This was confirmed by the growth of *C. violaceum* on the sub-cultured plate after another 24 h. The anti-QS study was performed in duplicate, and where variations between the enantiomers occurred, a third replicate was performed on a consecutive day to confirm the results.

To quantify the extent to which QS was inhibited the optical density (O.D.) of the sample at each

concentration was determined. In order to do this, 1.00 mL aliquots of the compounds at each concentration were centrifuged at 15,000 rpm (LLG-uniCFUGE 5 centrifuge) for 10 min. The pellets were then suspended in 1.00 mL of 100.00% DMSO solution and each sample was vortexed for 20 sec, to solubilize the violacein. Following this step, the samples were then further centrifuged at 15,000 rpm for 7 min to separate the bacterial cells from the solution, and 200.00 µL of the supernatant of each sample was transferred into a well of a 96-well micro-titre plate. The absorbance of the supernatant in each well was then read at O.D of 595 nm using FilterMax F5 multi-mode microplate reader (Molecular Devices). The study was performed in duplicate, and where variations occurred, a third replicate was performed on a consecutive day to confirm the results, and a mean percentage violacein inhibition was calculated using Eq. (1).

$$\text{Percentage violacein production}(\%) = \frac{\text{Culture control @ O.D 595} - \text{Test compound @ O.D 595}}{\text{Culture control @ O.D 595}} \quad (1)$$

The MQSIC was thereafter determined as the effective concentration of the compound at which 50.00 % or greater of the QS activity was inhibited (23).

2.5. Toxicity screening

The Brine Shrimp Lethality Assay (BSLA) was used to investigate the toxicity (36). Artificial seawater was prepared by dissolving 16.00 g of Tropic Marine® sea salt in 500.00 mL of distilled water. Dried brine-shrimp (*Artemia franciscana*) eggs (Ocean Nutrition™) (0.50 g) were then added to the saltwater. The eggs were left for 48 h to hatch under a constant light source. A rotatory pump (Kiho) was included for aeration of the saltwater. Thereafter, 400.00 µL of saltwater (containing approximately 40 – 60 live brine-shrimp) was added to each well of a 48-well microtiter plate, along with 400.00 µL of the sample being tested. The compounds were added at a concentration of 0.50 mg/mL in triplicate per plate. The study was performed in duplicate, and where variations between the enantiomers occurred, a third replicate was performed on a -consecutive day to confirm the results where mean values were reported. The brine-shrimp were examined under a light microscope (Olympus, 40X magnification) before adding the compounds to determine their viability. Any dead brine-shrimp at the initial count was excluded from the percentage mortality (PM) calculation. Dead brine-shrimp were counted at 24- and 48 h intervals by viewing plates under the light microscope. Finally, a lethal dose of 100.00% acetic acid (50.00 µL) was added to each well in order to take a final count of the brine-shrimp and thus calculate the PM at each time interval (Eq. (2)). A PM of 50.00 % or greater is considered to be biologically toxic (36).

$$\text{PM}(\%) = \frac{\text{Total number of dead brine} - \text{shrimp at 24/48 hrs}}{\text{Total number of dead brine} - \text{shrimp after the addition of 100\% acetic acid}} \quad (2)$$

2.6. Combination analyses

The combined interactive efficacy of the enantiomers of Limonene with the selected compounds was carried out as described previously; however, the enantiomers and selected compounds were combined at a 1:1 ratio in each study and a mean value was obtained, respectively (37). For the antimicrobial analysis, the interactions between the compounds were analysed using the fractional inhibitory concentration index (ΣFIC) using the MIC data and ΣFQSIC using the MQSIC data, using Eqs. **(3)** and (4).

$$\Sigma\text{FIC}^{(i)} = \frac{\text{MIC}\,(a^*)\ \text{in combination with}(b)}{\text{MIC}\,(a)\ \text{independently}}$$
$$\Sigma\text{FIC}^{(ii)} = \frac{\text{MIC}\,(b^*)\ \text{in combination with}(a)}{\text{MIC}\,(b)\ \text{independently}} \quad (3)$$

*Where (a) is the MIC of either enantiomer in the combination and (b) is the MIC of the selected compound used in the combination.

The sum of the FIC, known as the FIC index, is thus calculated as:

$$\Sigma\text{FIC} = \text{FIC}^{(i)} + \text{FIC}^{(ii)} \quad (4)$$

The ΣFIC values for each combination can be interpreted as synergistic (ΣFIC ≤0.5), additive (ΣFIC values >0.5 – 1.0), non-interactive (ΣFIC values >1.0 ≤ 4.0) or antagonistic (ΣFIC values >4.0).

For the anti-QS analysis, Eqs. (3) and (4) were used to calculate the ΣFQSIC, where (a) is the MQSIC of either enantiomer in the combination and (b) is the MQSIC of the selected compound used in the combination. As with the ΣFIC interpretation, the ΣFQSIC was interpreted as either synergistic, additive, non-interactive or antagonistic.

For the anti-quorum sensing analysis, the first step was to determine the effect of the interactions of the enantiomers with the selected compounds at the effective concentration required to disrupt bacterial communication (ΣFQSIC). The second step was to determine the effect of the combinations on the extent to which bacterial communication was affected. This was done by determining the fractional percentage violacein

reduction (ΣFPVR) using Eq. (5). Both steps are important to carry out as the enantiomeric pair may differ in the extent to which they inhibit violacein production at an equivalent effective concentration, and thus, the extent of bacterial communication.

$$\text{Percentage violacein reduction (PVR)} = 100.00\% - \text{Percentage violacein inhibition} \quad (5)$$

Once this was determined for the compounds individually and in combination, Eqs. (3) and (4) were used to calculate the ΣFPVR, where (a) is the PVR of either enantiomer in the combination and (b) is the PVR of the selected compound used in the combination. As with the ΣFIC interpretation, the ΣFQSIC and ΣFPVR were interpreted as either synergistic, additive, non-interactive or antagonistic.

Finally, for the brine shrimp lethality assay, the interactions between the compounds were then analysed using the fractional percentage mortality (ΣFPM) index with the same principal equations where (a) was the PM of either enantiomer in the combination and (b) was the PM of the selected compound used in the combination. The ΣFPM was thus also interpreted as either synergistic (reduced toxicity), additive, non-interactive or antagonistic (enhanced toxicity).

3. Results and discussion

3.1. Antimicrobial analysis

3.1.1. Minimum inhibitory concentration of the enantiomers and selected compounds independently and in combination

As the focus of this investigation was to examine the variability in terms of the combined interactive efficacy of the enantiomers, the antimicrobial inhibitory activity of the selected compounds was initially investigated independently to determine a baseline (Table 1). The *S*-(−)-Limonene displayed mostly similar inhibitory activity when compared to *R*-(+)-Limonene with differences of no more than one dilution factor. Noteworthy activity was noted for both enantiomers against *C. neoformans* and for the *S*-(−)-Limonene against *C. albicans* and *P. aeruginosa*. This has been observed previously (20–22). Eugenol, Geraniol and Isoeugenol displayed the most noteworthy and broad-spectrum inhibitory activity against all six pathogens investigated, with MIC values ranging between 0.25 and 1.00 mg/mL, which has been previously observed (38–40). Menthol and α-Terpineol displayed noteworthy inhibitory activity against the yeast and Gram-negative strains, with MIC values ranging between 0.50 and 1.00 mg/mL, as has been previously observed (41,42). As was observed with the enantiomers investigated independently, the

Table 1. The mean MIC (mg/mL) for enantiomers of Limonene and the selected compounds, with standard deviation (SD) (in parentheses).

	Micro-organism					
	Yeast strains		Gram-negative bacterial strains		Gram-positive bacterial strains	
	C. neoformans ATCC 14116	*C. albicans* ATCC 10231	*P. aeruginosa* ATCC 27853	*K. pneumoniae* ATCC 13887	*S. aureus* ATCC 25923	*E. faecium* ATCC 27270
Enantiomer						
R-(+)-Limonene	**0.50 (±0.00)**	2.00 (±0.00)	1.50 (±0.71)	2.00 (±0.00)	4.00 (±0.00)	4.00 (±0.00)
S-(−)-Limonene	**0.25 (±0.00)**	**1.00 (±0.00)**	**1.00 (±0.00)**	2.00 (±0.00)	4.00 (±0.00)	4.00 (±0.00)
Selected compound						
Camphene	**0.50 (±0.00)**	2.00 (±0.00)	**1.00 (±0.00)**	4.00 (±0.00)	2.00 (±0.00)	4.00 (±0.00)
β-Caryophyllene	**0.25 (±0.00)**	2.00 (±0.00)	1.50 (±0.71)	2.00 (±0.00)	4.00 (±0.00)	4.00 (±0.00)
p-Cymene	**0.50 (±0.00)**	**1.00 (±0.00)**	**1.00 (±0.00)**	2.00 (±0.00)	4.00 (±0.00)	4.00 (±0.00)
Estragole	**0.50 (±0.00)**	2.00 (±0.00)	**1.00 (±0.00)**	2.00 (±0.00)	4.00 (±0.00)	4.00 (±0.00)
Eucalyptol	**0.50 (±0.00)**	2.00 (±0.00)	2.00 (±0.00)	2.00 (±0.00)	2.00 (±0.00)	4.00 (±0.00)
Eugenol	**0.25 (±0.00)**	**0.50 (±0.00)**	**1.00 (±0.00)**	**1.00 (±0.00)**	**1.00 (±0.00)**	**1.00 (±0.00)**
Geraniol	**0.25 (±0.00)**	**0.50 (±0.00)**	**1.00 (±0.00)**	**1.00 (±0.00)**	**1.00 (±0.00)**	**1.00 (±0.00)**
Isoeugenol	**0.25 (±0.00)**	**0.50 (±0.00)**	**1.00 (±0.00)**	**1.00 (±0.00)**	**0.50 (±0.00)**	**1.00 (±0.00)**
Linalyl acetate	**0.25 (±0.00)**	2.00 (±0.00)	1.50 (±0.71)	2.00 (±0.00)	2.00 (±0.00)	4.00 (±0.00)
Menthol	**0.50 (±0.00)**	**1.00 (±0.00)**	**1.00 (±0.00)**	**1.00 (±0.00)**	2.00 (±0.00)	2.00 (±0.00)
Ocimene	**0.25 (±0.00)**	2.00 (±0.00)	**1.00 (±0.00)**	2.00 (±0.00)	4.00 (±0.00)	4.00 (±0.00)
Sabinene hydrate	**0.25 (±0.00)**	**1.00 (±0.00)**	**1.00 (±0.00)**	2.00 (±0.00)	2.00 (±0.00)	2.00 (±0.00)
γ-Terpinene	**0.25 (±0.00)**	2.00 (±0.00)	1.50 (±0.71)	2.00 (±0.00)	2.00 (±0.00)	4.00 (±0.00)
α-Terpineol	**0.50 (±0.00)**	**0.50 (±0.00)**	**1.00 (±0.00)**	**1.00 (±0.00)**	2.00 (±0.00)	2.00 (±0.00)
Controls						
Ciprofloxacin (positive control) (μg/mL)	–	–	$6.25x10^{-4}$ (±0.00)	$7.8x10^{-5}$ (±0.00)	$6.25x10^{-4}$ (±0.00)	$1.25x10^{-3}$ (±0.00)
Amphotericin B (positive control) (μg/mL)	$6.25x10^{-3}$ (±0.00)	$6.25x10^{-3}$ (±0.00)	–	–	–	–
Acetone (negative control)	1.00 (±0.00)	> 4.00 (±0.00)	> 4.00 (±0.00)	> 4.00 (±0.00)	> 4.00 (±0.00)	> 4.00 (±0.00)

n = 2 replicates, with third consecutive replicate to confirm variations between enantiomers; bold = noteworthy activity (MIC ≤ 1.00 mg/mL). Noteworthy activity only considered for < 1.00 mg/mL for C. neoformans due to the value for negative control noted

yeasts were most susceptible, particularly *C. neoformans*, followed by the Gram-negative bacterial strains.

For the MIC combination studies (Table 2), the enantiomers of Limonene interacted either synergistically, additively or were non-interactive with the selected compounds. No antagonism was observed. Synergy was observed for selected combinations with Limonene against *C. neoformans* and *S. aureus*. Compared to the MIC assessment of the enantiomers independently where minimal variation between the enantiomers was observed, the combination studies revealed a greater degree of variation. Of the 168 combinations investigated, 56 combinations demonstrated differences in the MIC values between the enantiomers. Furthermore, in those 56 combinations where variations were observed, *S*-(–)-Limonene often interacted synergistically or additively in combination, where *R*-(+)-Limonene was non-interactive. This was most often observed against *C. neoformans*. It was noted that *S*-(–)-Limonene was the more antimicrobially active enantiomer when combined with *p*-Cymene, Eucalyptol, Eugenol, Isoeugenol, Linalyl acetate and Ocimene. Our previous study was the only other one that could be found that investigated and compared the interactive efficacy of the enantiomers of Limonene with 1,8-Cineole (Eucalyptol), the results of which, against *C. neoformans* and *P. aeruginosa*, correlate with those reported in the current study (22). Overall, the combination of the enantiomers of Limonene with β-Caryophyllene against *C. neoformans*, and in combination with γ-Terpinene against *S. aureus*, had the greatest variation observed in terms of the interactive efficacy. Against *C. neoformans*, *R*-(+)-Limonene in combination with β-Caryophyllene was non-interactive (ΣFIC = 3.00), and *S*-(–)-Limonene in combination with β-Caryophyllene interacted synergistically (ΣFIC = 0.50). Against *S. aureus*, *R*-(+)-Limonene interacted synergistically with γ-Terpinene (ΣFIC = 0.38) and *S*-(–)-Limonene was non-interactive (ΣFIC = 1.50).

3.1.2. Anti-quorum sensing analysis

The results of the anti-QS analysis of the enantiomers and selected compounds are reported in Table 3. When investigated independently, the enantiomers of Limonene did not vary in terms of their anti-QS activity. Both *R*-(+)-Limonene and *S*-(–)-Limonene had similar average MQSIC values of 0.50 and 0.38 mg/mL, respectively. There is a scarcity of studies in which the enantiomeric configuration of essential oil compounds is considered when evaluating the anti-QS activity. One such study (23) could be found that investigated the anti-QS activity of the Limonene enantiomers, where (+)-Limonene promoted the production of violacein by approximately 20.00%, whereas (–)-Limonene inhibited the production of violacein by 20.00% at the highest concentration investigated (0.50 mg/mL). The current study found that both enantiomeric forms had concentration-dependent inhibition of violacein, with 53.71% of violacein inhibition observed at the MQSIC of 0.38 – 0.50 mg/mL for both enantiomeric forms. These discrepancies may be due to differences in quantification methods, compound purity, or experimental conditions. Notably, while *R*-(+)-limonene showed lower MQSIC values in several combinations, indicating enhanced potency, *S*-(–)-limonene often achieved greater violacein reduction at these concentrations. This difference in combination activity may be attributed to differences in molecular interactions with bacterial membranes or quorum sensing regulatory proteins. *R*-(+)-limonene may more effectively interfere with early QS signal generation or receptor binding (e.g. AHL-LuxR type systems), therefore lowering the concentration needed to initiate inhibition. In contrast, *S*-(–)-limonene may exert stronger effects downstream, potentially by disrupting transcriptional responses or inhibiting violacein biosynthesis enzymes more robustly. These enantiomer-specific effects were also evident in combination studies, where synergistic or antagonistic outcomes varied depending on the pairing, further supporting the outcome that stereochemistry modulates both the stage and strength of QS pathway interference. These results highlight the necessity of evaluating both inhibitory thresholds and phenotypic outcomes when assessing anti-QS efficacy. Even if the compound doesn't block the initial QS signal very strongly, it may still strongly suppress violacein production. Future mechanistic studies targeting specific QS regulators and violacein biosynthesis pathways are warranted to determine the enantiomer-specific modes of action. The MQSIC values and percentage of violacein inhibition were also determined for the selected compounds. The compounds *p*-Cymene, Eugenol, Isoeugenol, Menthol, Ocimene, γ-Terpinene and α-Terpineol showed the most noteworthy anti-QS activity (inhibited the production of violacein by ≥ 50.00%). This noteworthy anti-QS activity has been previously reported (43–45). In fact, a study evaluated the correlation between the anti-QS activity of 40 commercial essential oils and the metabolomic profiles of the oils and identified Eugenol, Geraniol and Menthol as putative biomarkers responsible for the anti-QS activity observed, which correlates to the findings of the current investigation (46).

The anti-QS activity of the combinations of the enantiomers with the selected compounds was carried out, and the percentage violacein inhibition of the

Table 2. The mean MIC (mg/ml) with standard deviation for the 1:1 combinations in the antimicrobial analysis, with ΣFIC value and interaction classification (in parentheses).

	Enantiomers											
	C. neoformans		*C. albicans*		*P. aeruginosa*		*K. pneumoniae*		*S. aureus*		*E. faecium*	
Selected compound	*R*-(+)-Limonene	*S*-(–)-Limonene	*R*-(+)-Limonene	*S*-(–)-Limonene	*R*-(+)-Limonene	*S*-(–)-Limonene	*R*-(+)-Limonene	*S*-(–)-Limonene	*R*-(+)-Limonene	*S*-(–)-Limonene	*R*-(+)-Limonene	*S*-(–)-Limonene
Camphene	**0.25** ±0.00 (0.50; Syn)	**0.25** ±0.00 (0.75; Add)	2.00 ± 0.00 (1.00; Add)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.83; Add)	**1.00** ± 0.00 (1.00; Add)	2.00 ± 0.00 (0.75; Add)	4.00 ± 0.00 (1.50; Ind)	4.00 ± 0.00 (1.50; Ind)	4.00 ± 0.00 (1.50; Ind)	4.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 (1.00; Add)
β-Caryophyllene	***1.00*** ±0.00 (3.00; Ind)	**0.13** ±0.00 (0.50; Syn)	2.00 ± 0.00 (1.00; Add)	**1.00** ± 0.00 (0.75; Add)	1.50 ± 0.71 (1.00; Add)	2.00 ± 0.00 (1.67; Ind)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 (1.00; Add)
p-Cymene	**0.50** ±0.00 (1.00; Add)	**0.13** ±0.00 (0.38; Syn)	2.00 ± 0.00 (1.50; Ind)	**1.00** ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.67; Ind)	**1.00** ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 (1.00; Add)
Estragole	**0.19** ±0.09 (0.38; Syn)	**0.25** ±0.00 (0.75; Add)	2.00 ± 0.00 (1.00; Add)	**1.00** ± 0.00 (0.75; Add)	2.00 ± 0.00 (1.67; Ind)	**1.00** ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (0.50; Syn)	4.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 (1.00; Add)
Eucalyptol	**0.75** ±0.35 (1.50; Ind)	**0.25** ±0.00 (0.75; Add)	2.00 ± 0.00 (1.00; Add)	**1.00** ± 0.00 (0.75; Add)	2.00 ± 0.00 (1.17; Ind)	**1.00** ± 0.00 (0.75; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 (1.50; Ind)	2.00 ± 0.00 (0.75; Add)	4.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 (1.00; Add)
Eugenol	**0.50** ±0.00 (1.50; Ind)	**0.25** ±0.00 (1.00; Add)	**1.00** ± 0.00 (1.25; Ind)	**1.00** ± 0.00 (1.50; Ind)	**1.00** ± 0.00 (0.83; Add)	**1.00** ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.50; Ind)	**1.00** ± 0.00 (0.75; Add)	2.00 ± 0.00 (1.25; Ind)	2.00 ± 0.00 (1.25; Ind)	2.00 ± 0.00 (1.25; Ind)	2.00 ± 0.00 (1.25; Ind)
Geraniol	**0.25** ±0.00 (0.75; Add)	**0.25** ±0.00 (1.00; Add)	**0.50** ± 0.00 (0.63; Add)	**0.50** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.83; Add)	2.00 ± 0.00 (2.00; Ind)	2.00 ± 0.00 (1.50; Ind)	2.00 ± 0.00 (1.50; Ind)	2.00 ± 0.00 (1.25; Ind)	2.00 ± 0.00 (1.25; Ind)	2.00 ± 0.00 (1.25; Ind)	2.00 ± 0.00 (1.25; Ind)
Isoeugenol	**0.25** ±0.00 (0.75; Add)	**0.25** ±0.00 (1.00; Add)	**0.50** ± 0.00 (0.63; Add)	**0.50** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.83; Add)	**1.00** ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.50; Ind)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (1.13; Ind)	**1.00** ± 0.00 (1.13; Ind)	2.00 ± 0.00 (1.25; Ind)	2.00 ± 0.00 (1.25; Ind)
Linalyl acetate	***1.00*** ±0.00 (3.00; Ind)	**0.25** ±0.00 (1.00; Add)	2.00 ± 0.00 (1.50; Ind)	**1.00** ± 0.00 (0.75; Add)	2.00 ± 0.00 (1.33; Ind)	1.50 ± 0.71 (1.25; Ind)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 (1.50; Ind)	4.00 ± 0.00 (1.50; Ind)	4.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 (1.00; Add)
Menthol	**0.50** ±0.00 (1.00; Add)	**0.50** ±0.00 (1.50; Ind)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (1.00; Add)	**1.00** ± 0.00 (0.83; Add)	**1.00** ± 0.00 (1.00; Add)	**1.00** ± 0.00 (0.75; Add)	2.00 ± 0.00 (1.50; Ind)	2.00 ± 0.00 (0.75; Add)	2.00 ± 0.00 (0.75; Add)	2.00 ± 0.00 (0.75; Add)	2.00 ± 0.00 (0.75; Add)
Ocimene	**0.25** ±0.00 (0.75; Add)	**0.13** ±0.00 (0.50; Syn)	2.00 ± 0.00 (1.00; Add)	**1.00** ± 0.00 (0.75; Add)	2.00 ± 0.00 (1.67; Ind)	**1.00** ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 (1.00; Add)
Sabinene hydrate	**0.19** ±0.09 (0.56; Add)	**0.25** ±0.00 (1.00; Add)	2.00 ± 0.00 (1.50; Ind)	**1.00** ± 0.00 (1.00; Add)	**1.00** ± 0.00 (0.83; Add)	2.00 ± 0.00 (2.00; Ind)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (0.75; Add)	2.00 ± 0.00 (0.75; Add)	4.00 ± 0.00 (1.50; Ind)	4.00 ± 0.00 (1.50; Ind)
γ-Terpinene	**0.50** ±0.00 (1.50; Ind)	**0.25** ±0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.67; Add)	2.00 ± 0.00 (1.67; Ind)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	***1.00*** ± 0.00 (0.38; Syn)	*4.00* ± 0.00 (1.50; Ind)	4.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 (1.00; Add)
α-Terpineol	**0.38** ±0.18 (0.75; Add)	**0.25** ±0.00 (0.75; Add)	**0.50** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (1.50; Ind)	2.00 ± 0.00 (1.67; Ind)	2.00 ± 0.00 (2.00; Ind)	2.00 ± 0.00 (1.50; Ind)	2.00 ± 0.00 (1.50; Ind)	2.00 ± 0.00 (0.75; Add)	2.00 ± 0.00 (0.75; Add)	4.00 ± 0.00 (1.50; Ind)	2.00 ± 0.00 (0.75; Add)
Controls												
Ciprofloxacin (positive control) (μg/mL)	–		–		$6.25x10^{-4}$ (±0.00)		$7.8x10^{-5}$ (±0.00)		$6.25x10^{-4}$ (±0.00)		$1.25x10^{-3}$ (±0.00)	
Amphotericin B (positive control) (μg/mL)	$6.25x10^{-3}$ (±0.00)		$6.25x10^{-3}$ (±0.00)		–		–		–		–	
Acetone (negative control)	1.00 (±0.00)		>4.00 (±0.00)		>4.00 (±0.00)		>4.00 (±0.00)		>4.00 (±0.00)		>4.00 (±0.00)	

n = 2 replicates, with third consecutive replicate to confirm variations between enantiomers; bold = noteworthy activity (MIC ≤ 1.00 mg/mL), noteworthy activity only considered for <1.00 mg/mL for *C. neoformans* due to the value for negative control noted; *italics* = variations in MIC values between enantiomers in combination that is more than one well-dilution difference;

Table 3. The percentage violacein inhibition and mean MQSIC of the enantiomers and the selected compounds, with standard deviation (SD) (in parentheses).

Compound	Conc. mg/mL	Percentage Violacein inhibition (%)	MQSIC (mg/mL) ± (SD)
Enantiomer			
R-(+)-Limonene	0.50	**53.71**	0.50 ± (0.00)
	0.25	43.36	
	0.13	21.09	
	0.06	15.63	
S- (–)-Limonene	0.50	**53.71**	0.38 ± (0.18)
	0.25	44.73	
	0.13	31.25	
	0.06	21.29	
Selected compound			
Camphene	0.50	36.86	>0.50± (0.00)
	0.25	**54.33**	
	0.13	32.27	
	0.06	26.75	
β-Caryophyllene	0.50	**57.05**	0.50 ± (0.00)
	0.25	39.9	
	0.13	34.25	
	0.06	27.84	
p-Cymene	0.50	76.12	0.06 ± (0.00)
	0.25	75.64	
	0.13	74.61	
	0.06	**72.08**	
Estragole	0.50	76.6	0.13 ± (0.00)
	0.25	76.28	
	0.13	**76.28**	
	0.06	44.4	
Eucalyptol	0.50	**61.86**	0.50 ± (0.00)
	0.25	48.24	
	0.13	35.11	
	0.06	35.77	
Eugenol	0.50	75.64	0.06 ± (0.00)
	0.25	75.64	
	0.13	69.23	
	0.06	**66.87**	
Geraniol	0.50	75.96	0.25 ± (0.00)
	0.25	**76.44**	
	0.13	42.95	
	0.06	46.66	
Isoeugenol	0.50	76.12	0.06 ± (0.00)
	0.25	75.8	
	0.13	73.26	
	0.06	**74.65**	
Linalyl acetate	0.50	**63.46**	0.50 ± (0.00)
	0.25	43.59	
	0.13	45.33	
	0.06	40.86	
Menthol	0.50	76.76	0.06 ± (0.00)
	0.25	76.76	
	0.13	76.6	
	0.06	**56.42**	
Ocimene	0.50	76.92	0.06 ± (0.00)
	0.25	76.6	
	0.13	73.76	
	0.06	**59.18**	
Sabinene hydrate	0.50	73.24	0.13 ± (0.00)
	0.25	71.96	
	0.13	**70.72**	
	0.06	28.23	
γ-Terpinene	0.50	74.68	0.06 ± (0.00)
	0.25	72.76	
	0.13	66.02	
	0.06	**54.59**	
α-Terpineol	0.50	76.76	0.06 ± (0.00)
	0.25	76.76	
	0.13	76.6	
	0.06	**56.42**	

n = 2 replicates, with third consecutive replicate to confirm variations between enantiomers; **bold** = mean percentage violacein inhibition that is ≥ 50.00%; MQSIC of positive control (Vanillin) = 0.23 (± 0.05) mg/mL (79.73% violacein inhibition); MQSIC of negative control = > 0.50 mg/mL (1.51% violacein inhibition at 0.50 mg/mL).

combinations at the varying concentrations was determined. The interactive efficacy (ΣFQSIC) in terms of the effect of the combinations on the effective concentration to inhibit QS was then determined. This was then followed by determining the interactive efficacy in terms of the extent to which QS is inhibited (ΣFPVR) (Table 4).

The MQSIC values ranged between 0.06 and 0.50 mg/mL. However, a few combinations had MQSIC values >0.50 mg/mL. This was evident with both enantiomers of Limonene in combination with either β-Caryophyllene or Linalyl acetate, and *S*-(−)-Limonene in combination with Camphene. The most noteworthy combinations, which inhibited violacein by ≥50.00% at the lowest concentrations tested, were seen by both enantiomers in combination with Estragole, Eugenol, Geraniol, Isoeugenol, Menthol and α-Terpineol. It was noted that variations ≥20% were evident with selected compounds *p*-Cymene, Estragole, Isoeugenol, Ocimene and α-Terpineol.

The greatest variation in violacein inhibition was observed with *p*-Cymene with over a 40-fold difference when combined with the two enantiomers of Limonene. The combined *R*-(+)-Limonene with *p*-Cymene inhibited violacein by an average of 40.72% at a concentration of 0.06 mg/mL, whereas *S*-(−)-Limonene when combined with *p*-Cymene inhibited violacein only by an average of 0.98% at the same concentration.

In terms of the comparative MQSIC values between the enantiomeric pairs in combination, the results were mostly equivalent, with two exceptions. In combination with *p*-Cymene with *R*-(+)-Limonene had a MQSIC of 0.19 mg/mL, whereas *p*-Cymene with *S*-(−)-Limonene had a combined MQSIC of 0.50 mg/mL. In addition, when combined with α-Terpineol, *R*-(+)-Limonene had a MQSIC of 0.06 mg/mL, whereas α-Terpineol with *S*-(−)-Limonene had a MQSIC of 0.25 mg/mL.

While no other anti-QS study could be found comparing combinations with Limonene enantiomer, some studies evaluated the anti-QS activity of essential oils where Limonene and the selected compounds, as studied herein, were constituents (47,48). While similarities could be drawn, it is important to consider the influence of all major and minor compounds within the neat essential oils, as these interactions may further complicate the outcome.

In terms of the ΣFQSIC, 10.71% of the combinations were synergistic, 32.14% were additive, 35.71% were non-interactive and 21.43% were non-interactive. Antagonism was observed with one combination (*S*-(−)-Limonene with *p*-Cymene). Synergy was observed with both enantiomers of Limonene in combination with Geraniol, with ΣFQSIC values of 0.19 and 0.42 for *R*-(+)-Limonene and *S*-(−)-Limonene, respectively. Synergy was also observed with *R*-(+)-Limonene in combination with Estragole (ΣFQSIC = 0.31). The most noteworthy variation observed in terms of ΣFQSIC was between the enantiomers of Limonene in combination with *p*-Cymene, where *R*-(+)-Limonene was non-interactive (ΣFQSIC of 1.69), whereas *S*-(−)-Limonene interacted antagonistically (ΣFQSIC = 4.67) with *p*-Cymene.

The ΣFPVR studies revealed that 50.00% of the combinations were additive, 28.57% were non-interactive and 21.43% were undefined (no endpoint value hence could not be definitively used in calculation). No synergy or antagonism was observed. In terms of variations between the enantiomers' interactive ability to inhibit violacein. Ocimene, *R*-(+)-Limonene interacted additively (ΣFPVR = 0.96), whereas *S*-(−)-Limonene was non-interactive (ΣFPVR = 1.16). *R*-(−)-Limonene interacted additively in combination with either *p*-Cymene, Eucalyptol, Geraniol, Isoeugenol or Menthol, with ΣFPVR values ranging between 0.61 and 0.99, whereas *R*-(+)-Limonene was non-interactive, with ΣFPVR values ranging between 1.02 and 1.28. This means that in terms of the ΣFPVR, which is the extent to which violacein is inhibited, it was the *S*-(−)-Limonene that was more active. This is interesting given that *R*-(+)-Limonene interacted more favourably in terms of ΣFQSIC. *R*-(+)-Limonene interacted with selected compounds in a way that reduced the combined effective concentration to inhibit bacterial communication by more than 50.00%, when compared to *S*-(−)-Limonene. However, *S*-(−)-Limonene interacted with selected compounds in way that increased the extent to which bacterial communication was inhibited, and therefore had increased anti-QS activity, when compared to *R*-(+)-Limonene at the same concentration.

3.2. Toxicity screening

When comparatively investigating the Limonene enantiomers for toxicity in the BSLA (Table 5), both configurations were found to be non-toxic after 24 and 48 h. The low toxicity of the Limonene enantiomers has been previously reported (49–51). Furthermore, a comprehensive review was undertaken on the toxicological data, safety and risk evaluation of *R*-(+)-Limonene which categorized this compound as having low toxicity (52). The focus of this investigation was to examine the variability of toxicity when the enantiomers were in combination with a selection of compounds. Camphene, β-Caryophyllene, Eucalyptol, Linalyl

Table 4. The Mean Percentage Violacein Inhibition (%), MQSIC, ΣFQSICand ΣFPVR at MQSIC of the 1:1 Combinations of the enantiomers of limonene and selected compounds at the varying concentrations.

Selected compound	Conc.mg/mL	*R*-(+)-Limonene				*S*-(–)-Limonene			
		% violacein inhibition	Mean MQSIC (mg/mL)	ΣFQSIC	ΣFPVR at MQSIC	% violacein inhibition	Mean MQSIC (mg/mL)	ΣFQSIC	ΣFPVR at MQSIC
Camphene	0.50	49.12	>0.50	nd; nd	nd; nd	**53.52**	0.50 ± 0.00	nd; nd	nd; nd
	0.25	40.82				38.67			
	0.13	25.10				39.45			
	0.06	24.41				31.64			
β-Caryophyllene	0.50	32.23	>0.50	nd; nd	nd; nd	19.14	>0.50	nd; nd	nd; nd
	0.25	13.77				16.6			
	0.13	13.18				4.00			
	0.06	9.67				7.71			
p-Cymene	0.50	67.09	*0.19* ± (0.09)	1.69; Ind	1.03; Ind	**76.37**	*0.50* ± 0.00	4.67; Ant	0.69; Add
	0.25	**64.26**				47.07			
	0.13	49.02*				22.85*			
	0.06	40.72*				0.98*			
Estragole	0.50	86.72	0.06 ± (0.00)	0.31; Syn	0.95; Add	87.11	0.13 ± 0.00	0.67; Add	0.58; Add
	0.25	86.72				86.82			
	0.13	77.64				**81.93**			
	0.06	**70.31***				48.34*			
Eucalyptol	0.50	66.11	0.38 ± (0.18)	0.88; Add	1.10; Ind	**72.85**	0.50 ± 0.00	1.33; Ind	0.61; Add
	0.25	**50.49**				31.74			
	0.13	46.09				37.21			
	0.06	35.16				20.90			
Eugenol	0.50	86.82	0.06 ± (0.00)	0.56; Add	0.56; Add	86.91	0.06 ± 0.00	0.58; Add	0.95; Add
	0.25	86.91				86.62			
	0.13	82.91				81.54			
	0.06	**79.00**				**64.45**			
Geraniol	0.50	87.01	0.06 ± (0.00)	0.19; Syn	1.28; Ind	86.72	0.13 ± 0.00	0.42; Syn	0.79; Add
	0.25	86.13				86.82			
	0.13	78.91				**75.68**			
	0.06	**60.16**				45.90			
Isoeugenol	0.50	86.72	0.06 ± (0.00)	0.56; Add	1.08; Ind	86.82	0.13 ± 0.00	1.17; Ind	0.99; Add
	0.25	86.72				87.01			
	0.13	74.41				**67.97**			
	0.06	**64.55***				41.11*			
Linalyl acetate	0.50	28.32	>0.50	nd; nd	nd; nd	40.23	>0.50	nd; nd	nd; nd
	0.25	22.66				27.34			
	0.13	24.12				24.61			
	0.06	26.17				16.80			
Menthol	0.50	86.72	0.06 ± (0.00)	0.56; Add	1.02; Ind	86.72	0.13 ± 0.00	1.17; Ind	0.63; Add
	0.25	87.11				86.82			
	0.13	74.80				**72.27**			
	0.06	**54.00**				38.87			
Ocimene	0.50	86.82	0.25 ± (0.00)	2.25; Ind	0.96; Add	86.82	0.25 ± 0.00	2.33; Ind	1.16; Ind
	0.25	**58.20**				**50.29**			
	0.13	49.41				32.62			
	0.06	47.46*				25.98*			

(*Continued*)

Table 4. (Continued).

Selected compound	Conc.mg/mL	*R*-(+)-Limonene				*S*-(–)-Limonene			
		% violacein inhibition	Mean MQSIC (mg/mL)	ΣFQSIC	ΣFPVR at MQSIC	% violacein inhibition	Mean MQSIC (mg/mL)	ΣFQSIC	ΣFPVR at MQSIC
Sabinene hydrate	0.50	86.82	0.13 ± (0.00)	0.63; Add	1.35; Ind	86.91	0.13 ± 0.00	0.67; Add	1.37; Ind
	0.25	71.58				74.32			
	0.13	**51.46**				**51.37**			
	0.06	36.33				34.47			
γ-Terpinene	0.50	75.59	0.13 ± (0.00)	1.13; Ind	0.97; Add	68.95	0.25 ± 0.00	2.33; Ind	0.90; Add
	0.25	56.54				**59.47**			
	0.13	**55.37**				37.89			
	0.06	34.28				17.38			
α-Terpineol	0.50	86.72	*0.06* ± (0.00)	0.56; Add	0.93; Add	87.01	*0.25 ± 0.00*	2.33; Ind	0.63; Add
	0.25	86.91				86.91			
	0.13	77.54				**77.34**			
	0.06	**66.02***				45.61*			

n = 2 replicates, with third consecutive replicate to confirm variations between enantiomers; **bold** = mean percentage violacein inhibition at lowest concentration to inhibit ≥ 50.00%; *= difference in percentage violacein inhibition that is ≥ 20.00%; italics = variations in MQSIC values between enantiomers in combination, that is more than one well-dilution difference; underlined = variations in interactive efficacy between enantiomers in combination; Syn = synergy, Add = additive, Ind = non-interactive, Ant = antagonistic; nd = undefined due to MQSIC value of the compounds or the combination thereof being > 0.50 mg/mL; MQSIC of positive control (Vanillin) = 0.23 (±0.05) mg/mL (79.73% violacein inhibition); MQSIC of negative control = > 0.50 mg/mL (1.51% violacein inhibition at 0.50 mg/mL).

acetate, *p*-Cymene, Sabinene hydrate and γ-Terpinene displayed non-toxic PM values, ranging between 0.85% – 6.87%, at 24 h, and 1.11 – 23.79% at 48 h. The non-toxic nature of *p*-Cymene, γ-Terpinene and β-Caryophyllene have been reported previously (53,54).

The results of the BSLA (after 24 and 48 h of exposure) conducted on the 1:1 combinations of the enantiomers with the selected compounds are given in Table 6. It is evident that in terms of toxicity analysis through BSLA screening, variations were observed.

R-(+)-Limonene often interacted more favourably in combination when compared to *S*-(–)-Limonene. This was particularly evident in combination with γ-Terpinene and α-Terpineol.

Variations in terms of the PM (toxic or non-toxic) were observed with the enantiomers of Limonene in combination with *p*-Cymene, γ-Terpinene and α-Terpineol after 24 and 48 h. In combination with these three selected compounds, *R*-(+)-Limonene was non-toxic after 24 and 48 h, with PM values ranging between 0.98% –29.63% at 24 h, and 2.41% – 33.33% at 48 h. However, in combination with the same three selected compounds, *S*-(–)-Limonene was found to be toxic at both 24 and 48 h. In combination with *p*-Cymene and γ-Terpinene, *S*-(–)-Limonene had a PM of 100.00% from 24 h; and in combination with α-Terpineol, *S*-(–)-Limonene had a PM of 51.93% at 24 h and 80.01% at 48 h. The favourable interaction of *R*-(+)-Limonene with γ-Terpinene and α-Terpineol is further identified and highlighted through the ΣFPM studies. After both 24 and 48 h, *R*-(+)-Limonene interacted synergistically with α-Terpineol, with ΣFPM values of 0.20 and 0.23, respectively. The combination of *S*-(–)-Limonene with α-Terpineol was, conversely, antagonistic at both 24 and 48 h (ΣFPM = 55.60 and 22.24, respectively). This trend was also observed with γ-Terpinene, which interacted synergistically and additively with *R*-(+)-Limonene at 24 and 48 h (ΣFPM = 0.22 and 0.61), respectively. In contrast, the interaction with *S*-(–)-Limonene was antagonistic from 24 h (ΣFPM = 115.50).

Table 5. Mean PM (%) of enantiomers of Limonene and the selected compounds, against brine shrimp.

Enantiomer	Average PM at 24 h (%)	Average PM at 48 h (%)
R-(+)-Limonene	**4.82**	**5.77**
S-(–)-Limonene	**0.48**	**1.85**
Selected compound		
Camphene	**1.84**	**3.77**
β-Caryophyllene	**4.42**	**10.10**
Estragole	81.00	82.45
Eucalyptol	**0.85**	**1.11**
Eugenol	100.00	100.00
Geraniol	100.00	100.00
Isoeugenol	100.00	100.00
Linalyl acetate	**3.18**	**23.79**
Menthol	100.00	100.00
Ocimene	**42.16**	55.04
p-Cymene	**6.87**	**10.60**
Sabinene hydrate	**1.47**	**10.43**
γ-Terpinene	**4.17**	**6.94**
α-Terpineol	**13.99**	63.17

n = 2 replicates, with third consecutive replicate to confirm variations between enantiomers; **bold** –PM values < 50.00%; PM of positive control (Potassium dichromate) = 100.00%; PM of negative controls: 2.00% DMSO = 0.49%, 50.00% acetone = 1.08%, salt water = 0.83%.

Very few studies have focused on the combined interactive toxicity of essential oil compounds (55–57). A study investigated the acute toxicity of 30 aromatic compounds against the larvae of *S. littoralis* at a dose of 0.30 mg/larva at 24 h of exposure. *R*-(+)-Limonene was included; however, the enantiomeric counterpart was not evaluated (58). These previous studies that were evaluated reported synergy to be potentiated; in other words, toxicity was or enhanced, as this is relevant to the desired insecticidal and repellent activity. The current study, however, considered synergy to be reduced toxicity in combination, as this is the desired requirement for anti-infective studies. What also needs to be considered is the source of the Limonene.

The observed synergy of the enantiomers of Limonene may be attributed to membrane-disruptive mechanisms (59). Limonene is a lipophilic monoterpene known to integrate into and destabilize microbial membranes, increasing their permeability and fluidity. This membrane disruptive action can facilitate the enhanced intracellular access of the compounds with which it is combined or co-occurs, leading to additive or synergistic antimicrobial activity. Furthermore, it is suggested that Limonene interferes with intracellular signalling pathways, disrupting QS regulated processes, due to its ability to modulate gene expression (60). Notably, *S*-(–)-Limonene has been reported to exhibit greater membrane permeabilization in certain Gram-positive and fungal organisms compared to its *R*-(+)-enantiomeric counterpart, potentially due to enantioselective interactions with membrane lipids or protein channels.

In the case of quorum-sensing inhibition, *R*-(+)-Limonene may act by interfering with the signal transduction pathways or directly disrupting the membrane-associated localization of quorum-sensing receptors, which are often embedded within the lipid bilayer. These effects could potentiate the activity of co-administered anti-QS terpenes such as Geraniol or Estragole by improving their bio-availability or stability in the membrane environment.

Table 6. The mean percentage mortality, ΣFPM and interaction classification (in parentheses) for the 1:1 combinations at 24 and 48 h.

Enantiomer	Selected compound	PM (%)	ΣFPM 24 h	PM (%)	ΣFPM 48 h
R-(+)-Limonene	Camphene	**2.45**	0.92 (Add)	**5.15**	1.13 (Ind)
	β-Caryophyllene	**1.04**	0.23 (Syn)	**16.82**	2.29 (Ind)
	p-Cymene	***29.63***	5.23 (Ant)	***33.33***	4.46 (Ant)
	Estragole	**14.48**	1.59 (Ind)	**35.63**	3.30 (Ind)
	Eucalyptol	**0.00**	nd	**6.01**	3.23 (Ind)
	Eugenol	100.00	10.88 (Ant)	100.00	9.17 (Ant)
	Geraniol	100.00	10.88 (Ant)	100.00	9.17 (Ant)
	Isoeugenol	100.00	10.88 (Ant)	100.00	9.17 (Ant)
	Linalyl acetate	**0.00**	nd	**3.26**	0.35 (Syn)
	Menthol	***41.43***	4.51 (Ant)	89.66	8.22 (Ant)
	Ocimene	**8.55**	0.99 (Add)	***16.82***	1.61 (Ind)
	Sabinene hydrate	**0.00**	nd	**5.57**	0.75 (Add)
	γ-Terpinene	***0.98***	0.22 (Syn)	***3.84***	0.61 (Add)
	α-Terpineol	***1.44***	0.20 (Syn)	***2.41***	0.23 (Syn)
S-(−)-Limonene	Camphene	**0.00**	nd	**7.69**	3.10 (Ind)
	β-Caryophyllene	**0.00**	nd	**2.04**	0.65 (Add)
	p-Cymene	*100.00*	110.78 (Ant)	*100.00*	31.72 (Ant)
	Estragole	**1.55**	1.62 (Ind)	**46.56**	12.85 (Ant)
	Eucalyptol	**0.00**	nd	**2.53**	1.82 (Ind)
	Eugenol	100.00	104.00 (Ant)	100.00	27.50 (Ant)
	Geraniol	100.00	104.00 (Ant)	100.00	27.50 (Ant)
	Isoeugenol	100.00	104.00 (Ant)	100.00	27.50 (Ant)
	Linalyl acetate	**0.51**	0.61 (Add)	**2.01**	0.58 (Add)
	Menthol	*78.46*	81.60 (Ant)	97.78	26.89 (Ant)
	Ocimene	**4.85**	5.07 (Ant)	*95.31*	26.60 (Ant)
	Sabinene hydrate	**2.27**	3.13 (Ind)	**37.14**	11.81 (Ant)
	γ-Terpinene	*100.00*	115.50 (Ant)	*100.00*	34.20 (Ant)
	α-Terpineol	*51.93*	55.60 (Ant)	*80.01*	22.24 (Ant)

n = 2 replicates, with third consecutive replicate to confirm variations between enantiomers; **bold** - PM values < 50.00 %; *italics* = variations in PM between enantiomers in combination; underlined = variations in interactive efficacy between enantiomers in combination; Syn = synergy, Add = additive, Ind = non-interactive, Ant = antagonistic, nd = undefined; PM of positive control (Potassium dichromate) = 100.00%; PM of negative controls: 2.00% DMSO = 0.49%, 50.00% acetone = 1.08%, salt water = 0.83%.

4. Conclusion

When the enantiomers of Limonene were investigated independently across the three assays, they displayed equivalent antimicrobial and anti-QS activity and displayed low toxicity in the BSLA assay. Where variations were observed in the MIC assay, *S*-(−)-Limonene often displayed moderately stronger antimicrobial activity, particularly against *C. neoformans*. The combination studies revealed greater variations between the enantiomers of Limonene in combination with selected essential oil compounds. In the MIC assay, *S*-(−)-Limonene was the more frequently active enantiomer where variations were observed, particularly against *C. neoformans*. However, it was noted that while *S*-(−)-Limonene often had more noteworthy interactions, *R*-(+)-Limonene had considerably stronger antimicrobial activity in certain combinations against certain pathogens, than *S*-(−)-Limonene. The combination anti-QS studies revealed that in terms of the ΣFQSIC, combined effect to reduce the concentration needed to inhibit violacein production, *R*-(+)-Limonene often interacted more favourably, as compared to *S*-(−)-Limonene. However, *S*-(−)-Limonene was more active in terms of the ΣFPVR, which is the extent to which violacein was inhibited. The combination studies in the BSLA assay revealed that where variations were observed, *R*-(+)-Limonene often interacted more favourably in combination as compared to *S*-(−)-Limonene.

Across the three assays, the combination of the enantiomers with *p*-Cymene, γ-Terpinene and α-Terpineol revealed the most variable results. This was particularly evident with γ-Terpinene, where *R*-(+)-Limonene was found to be interact considerably more favourably than *S*-(−)-Limonene. *R*-(+)-Limonene and γ-Terpinene were synergistic against *S. aureus* and displayed additivity against *P. aeruginosa*. This combination was also additive in terms of ΣFPVR (0.97) and displayed considerably low toxicity at 24 and 48 h (0.98% and 3.84%, respectively). In contrast, *S*-(−)-Limonene had a combined PM of 100.00% from 24 h, with γ-Terpinene.

Our findings demonstrate the critical role of stereochemistry in modulating biological activity, highlighting a key, yet often overlooked, dimension in natural

product research. The distinct bio-activities observed among enantiomers assayed alone and, in combination with other molecules, emphasize the importance of considering chirality in pharmacological evaluations, as failing to do so may lead to misinterpretations of efficacy, toxicity and mechanism of action. This study reinforces the imperative need for stereochemical characterization (albeit cumbersome) in drug discovery and development, ensuring a more precise and biologically relevant understanding of natural product interactions.

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