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The role of stereochemistry on the biological activity of essential oil compounds

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THE ROLE OF STEREOCHEMISTRY ON THE BIOLOGICAL ACTIVITY OF ESSENTIAL OIL COMPOUNDS

UNIVERSITY OF THE
WITWATERSRAND,
JOHANNESBURG

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A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Masters of Pharmacy,

2022

DECLARATION

I, Nazihah Hoosen, hereby declare that this dissertation is my own work, with all other sources of information acknowledged by means of a complete reference list. This dissertation is being submitted in fulfilment of the degree of Master of Pharmacy, at the University of the Witwatersrand, Johannesburg. This work has not been submitted before for any degree or examination to this or any other university.

_____

Nazihah Hoosen

Date: 29/07/2022

ABSTRACT

Essential oils and their compounds are often investigated for their biological properties, including their antimicrobial activity, however, what is often overlooked is the influence of the specific stereochemical configuration of the chiral essential oil constituents. The aim of this study was to investigate whether a selection of optical enantiomers related to essential oil chemistry differed in terms of the antimicrobial activity observed, both independently and in combination with a selection of 14 essential oil compounds.

To observe the effects of the compounds against planktonic micro-organisms, the broth micro-dilution assay was undertaken against Gram-positive (*Staphylococcus aureus* and *Enterococcus faecium*) and Gram-negative (*Pseudomonas aeruginosa* and *Klebsiella pneumoniae*) strains, as well as yeasts (*Candida albicans* and *Cryptococcus neoformans*). Combination studies were investigated at equal ratios of 1:1, and results interpreted using the fractional inhibitory concentration (FIC) index. The results of the 1:1 combination study revealed that the most prevalent interaction observed was additivity (56.46%), followed by non-interactive (37.93%) interactions. A total of 5.61% of the combinations were found to be synergistic, most of which was seen against the two yeast pathogens. No antagonism was observed in any of the combinations tested. Although little variation was observed between the enantiomeric pairs independently, a total of 17.18% of the combinations (enantiomer with essential oil compound) exhibited varied interactive efficacy. Overall, (+)-β-Pinene, (–)-Borneol, (–)-α-Pinene and (–)-Limonene often displayed better interactive activity over their enantiomeric counterparts.

Anti-quorum sensing (QS) testing was undertaken with *Chromobacterium violaceum* as the monitor strain, using the broth macro-dilution method in order to obtain a minimum quorum sensing inhibitory concentration (MQSIC). The results revealed that, singularly, all enantiomers and selected essential oil compounds had strong anti-QS activity, with MQSIC values ranging between 0.13 - 0.50 mg/mL. The only exceptions were (–)-α-Pinene and (–)-β-Pinene, which had MQSIC > 0.50 mg/mL. The percentage of violacein inhibition at the MQSIC values ranged between 3.84 - 90.68%. The combination studies were carried out through evaluation of the fractional quorum sensing inhibitory concentration index (ΣFQSIC) and the

fractional percentage violacein reduction index (ΣFPVR). The ΣFQSIC studies revealed that the majority of the combinations were non-interactive (44.90%), followed by additive (20.41%), synergistic (8.16%), and antagonistic (0.51%). In terms of the ΣFQSIC, (+)-Limonene and (+)-Citronellal often (dependent on the compounds in combination) displayed better interactive activity than their enantiomeric counterparts, where variations were observed. The ΣFPVR studies revealed that the majority of the combinations were non-interactive (40.82%), followed by additive (29.08%), and synergistic (4.08%) interactions. In terms of the ΣFPVR, (–)-Camphor, (+)-Borneol and (+)-Menthone often displayed better interactive activity than their enantiomeric counterparts, where variations were observed.

The toxicity of enantiomers and selected compounds, both singularly and in combination, was screened using the brine shrimp lethality assay (BSLA) after an exposure period of 24 hrs and 48 hrs. The only variation observed when investigated independently was between the enantiomers of β-Pinene after 48 hrs, where (+)-β-Pinene had a percentage mortality (PM) of 30.75% and (–)-β-Pinene had a PM of 93.82%. The results of the combination ΣFPM studies revealed that at 24 and 48 hrs, the majority of the combinations were antagonistic (34.69 - 40.82%), followed by non-interactive (17.35 - 30.61%), synergistic (18.37 - 23.47) and additive (9.69 - 15.51%). Variations in terms of the toxicity of the enantiomers were observed in 19.39% and 26.02% of the combinations at 24 and 48 hrs, respectively. Where variations in terms of ΣFPM were observed, (+)-Menthone, (+)-Limonene and (–)-β-Pinene (at 24 and 48 hrs), (–)-Camphor (at 48 hrs) often showed reduced toxicity in combination, when compared to their enantiomeric counterparts.

This study provides further in-depth knowledge of how enantiomers interact when combined with other essential oil compounds. It has been demonstrated that an often-neglected evaluation of the enantiomeric configuration of the essential oil compounds is in fact an important consideration, with the potential to identify therapeutically active combinations with safe toxicological profiles.

DEDICATIONS

"The best form of worship is the pursuit of knowledge."

- The Prophet Muhammed (peace be upon him)

Alhamdulillah, all praise belongs to The Most Gracious.

To my mother, for your endless sacrifice, support and encouragement.

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Draft publication abstract - Appendix B.

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LIST OF ABBREVIATIONS

≈	Approximately equal to
γ	Gamma
°C	Degrees Celsius
%	Percentage
<	Less than
=	Equal to
>	Greater than
≤	Less than or equal to
≥	Greater than or equal to
AHL	Acyl homoserine lactone
AI	Auto-inducer
ATCC	American type culture collection
BSLA	Brine-shrimp lethality assay
CC	Culture control
CFU	Colony forming units
CSLI	Clinical and Laboratory Standards Institute
dm^3	Cubic decimetre
DMSO	Dimethyl sulfoxide
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FIC	Fractional inhibitory concentration
FPM	Fractional percentage mortality
FPVR	Fractional percentage violacein reduction
FQSIC	Fractional quorum sensing inhibitory concentration
g	gram
GC	Gas chromatography
GC-MS	Gas chromatography mass spectrometry
hrs	Hours
IC_{50}	Half-maximal inhibitory concentration
INT	*p*-iodonitrotetrazolium violet
LBA	Luria Bertani agar
LBB	Luria Bertani broth

LC_{50}	Lethal Dose to kill 50.00%
LC_{90}	Lethal Dose to kill 90.00%
LD_{50}	Lethal Concentration to kill 50.00%
mg	Milligram
mg/L	Milligram per litre
mg/mL	Milligram per millilitre
MIC	Minimum inhibitory concentration
min	Minutes
mL	Millilitre
mM	Millimolar
MQSIC	Minimum quorum sensing inhibitory activity
MRSA	Methicillin-Resistant *Staphylococcus aureus*
PM	Percentage mortality
QS	Quorum sensing
QSAR	Quantitative structure activity relationship
rpm	Revolutions per minute
SD	Standard deviation
SDA	Sabourauds Dextrose agar
SDB	Sabourauds Dextrose broth
sec	Seconds
TSA	Tryptone Soya agar
TSB	Tryptone Soya broth
v/v	volume per volume
WHO	World Health Organisation
α	Alpha
β	Beta
µg	Microgram
µg/mL	Microgram per millilitre
µL	Millilitre
ΣFIC	Sum of the fractional inhibitory concentration
ΣFPM	Sum of the fractional percentage mortality
ΣFPVR	Sum of the fractional percentage violacein reduction
ΣFQSIC	Sum of the fractional quorum sensing inhibitory concentration

Chapter 1 - Introduction

1.1 Essential oils and essential oil compounds

Essential oils are aromatic liquids, obtained from plant materials (Chouhan *et al*., 2017). They are highly complex mixtures of volatile secondary metabolites, which comprise of several compounds . These are present within essential oils in variable ratios that correspond with their biological activities (Dhifi *et al*., 2016; Sharifi-Rad *et al*., 2017). The compounds within essential oils can vary in terms of the type and amount of compound present. This depends on a number of factors, including: species, chemotype, geographical location, plant nutrition, soil type, climate and seasonal variations, and the part of the plant from which it is distilled (Pragadheesh *et al*., 2013a; Raut and Karuppayil, 2014). An essential oil may be characterised by two or more major compounds that are present at high concentrations, in comparison to the other compounds that are present in lower or trace amounts (Chouhan *et al*., 2017). The biological activity of essential oils have very often been attributed to the major compounds that are present (Kar *et al*., 2018). However, Miladinović *et al*. (2021) suggested that the minor compounds, or the combination thereof, could be responsible for the antimicrobial activity of an essential oil. The number of individual compounds found in an essential oil may vary from 20 to > 100, and these compounds belong to a variety of different chemical classes (Djilani and Dicko, 2012).

1.2 Classification of essential oil compounds

1.2.1 Terpenes and terpenoids

Essential oil compounds are broadly classified as either unsaturated hydrocarbons (terpenes) or oxygenated hydrocarbons (terpenoids). Terpenes are simple hydrocarbons and are characterised by the presence of one or more double bonds between carbon atoms (Buckle, 2003). They are generally subcategorised into monoterpenes, sesquiterpenes, and diterpenes (Mewalal *et al*., 2017). Terpenoids are more diverse in their chemical structure than terpenes. They contain an oxygen moiety and additional structural arrangements (Gershenzon and Dudareva, 2007). Terpenoids can be further classified based on these structural arrangements,

into: aldehydes (R-CHO), ketones (R-CO-R), alcohols (R-OH, aliphatic) and phenols (R-OH, aromatic) (Clarke, 2008).

1.3 Stereochemistry

Stereochemistry describes the manner in which atoms of a molecule are arranged in a three-dimensional space (Carey and Sundberg, 2007). Chemical compounds that have the same molecular formulae but differ in the arrangement of atoms in the molecule are called isomers (Sekhon, 2013). This means that either the bonding of the atoms is different (structural isomers) or their spatial arrangement is different (stereoisomers). Stereoisomers are further divided into geometrical isomers and optical isomers, more commonly known as enantiomers (Figure 1.1).

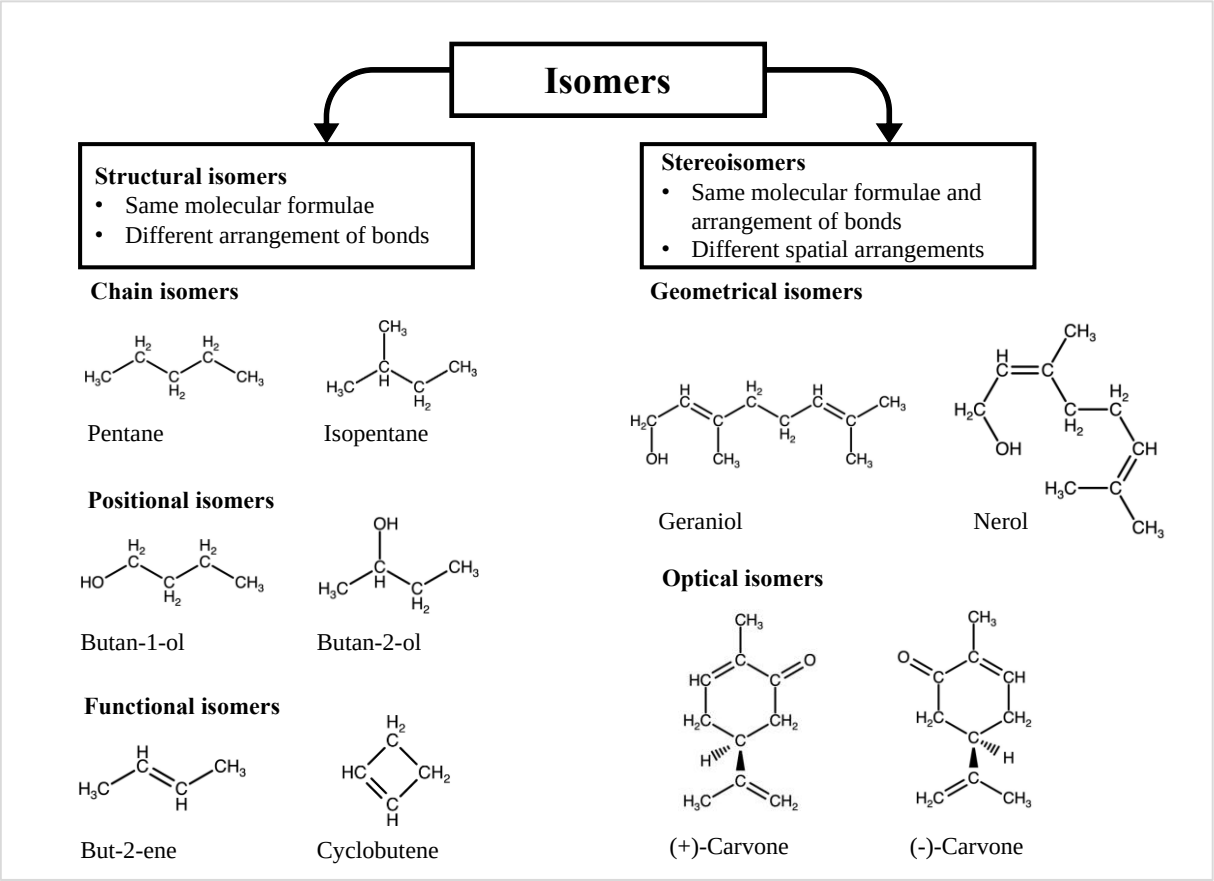


Figure 1.1: The categories and subcategories of isomers, with examples.

1.3.1 Enantiomers

Enantiomers are observed with chiral molecules. A molecule is referred to as chiral when a central carbon atom is attached to four different groups, and the subsequent tetrahedral

molecule is non-superimposable on its mirror image (Figure 1.2). As a result, there are two different ways to place four different substituents in a tetrahedral arrangement around a central carbon atom resulting in two enantiomers.

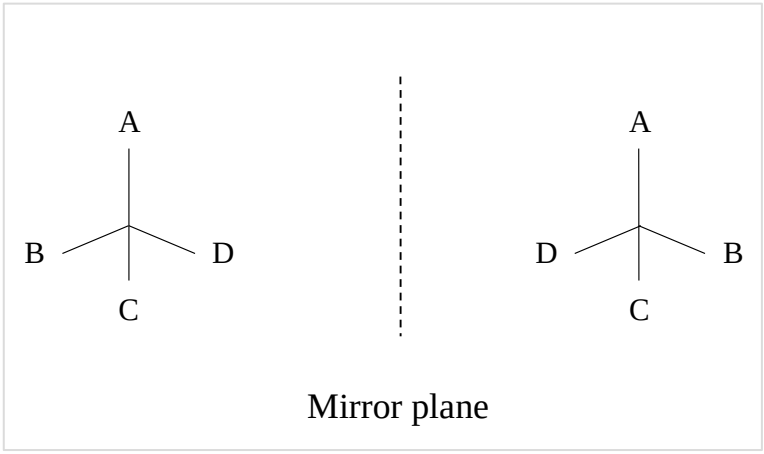


Figure 1.2: Enantiomers of a simple tetrahedral molecule, which are non-superimposable mirror images of each other.

A pair of enantiomers have the same physical properties, such as boiling points, but differ in how they rotate in plane-polarized light (Clarke, 2008). The property of a molecule to rotate plane-polarised light is why they are called optical isomers. If the rotation of plane-polarised light is clockwise it is termed *dextrorotatory*, and the isomer is referred to as the *d*-form of that molecule. Alternatively, if the rotation of plane-polarised light is anti-clockwise it is termed *levorotatory*, and the isomer is referred to as the *l*-form (Cordato *et al*., 2003). The angle of optical rotation that is measured is also expressed as either a positive (+)- or negative (–)-value, depending on the direction of rotation. If the direction of rotation is clockwise (*dextrorotatory*), it is expressed as a (+)-value, and if the direction of rotation is anti-clockwise (*levorotatory*), is expressed as a (–)-value (Hutt and Tan, 1996). A mixture of equal amounts of the (+)- and (–)-forms of a compound are called racemic mixtures, or a racemate. A racemic mixture of the two is indicated by (±) before the name of the compound. The R/S system is also often utilised to distinguish enantiomers, where the atomic number of the atoms attached to the chiral centre determine the order of priority of those atoms. The higher the atomic number the higher its priority, and if the order of priority of the atoms is clockwise, the chirality is denoted as the R-form (*Rectus*), and if it is anti-clockwise it is denoted as the S-form (*Sinister*) (Singh *et al*., 2014).

1.3.2 Enantiomerism in pharmacology

Enantiomers of chiral drugs may have equal affinities for target sites and exert the same pharmacological effect. However, biological receptor systems and enzymes in the body often exhibit stereochemical preference between enantiomers (Aggarwal *et al*., 2002). Hence, they may display varying pharmacological effects, some of which may be due to one enantiomer having a higher binding affinity for target sites, resulting in a more potent effect, or both enantiomers having equal binding affinities, but exert opposed pharmacological activities. This can be easily understood with the example of a drug-receptor model, as depicted in Figure 1.3, where one enantiomer may bind to the desired receptor, while the other may partially bind resulting in a less potent therapeutic effect. In other cases, enantiomers may act on different biological target sites altogether, and produce independent pharmacological activities (Lin *et al*., 2011).

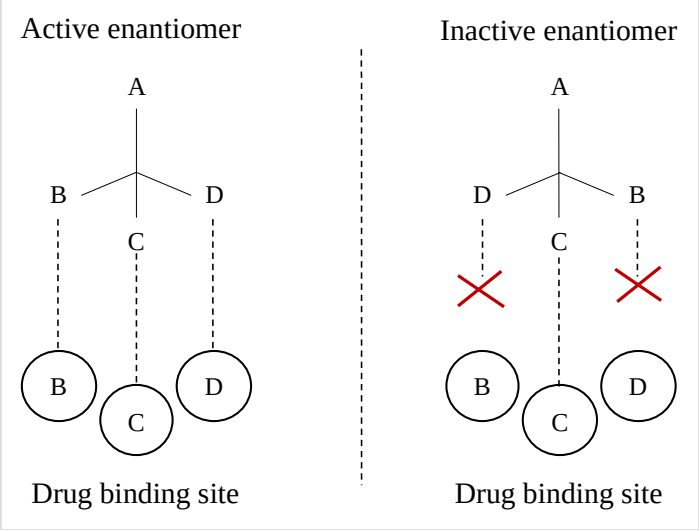


Figure 1.3: Depiction of an enantiomeric pair with different binding affinities for the same drug binding site.

Enantiomers of chiral drugs can differ in their pharmacokinetic and pharmacodynamic properties. An example of a pharmacokinetic difference due to stereoisomerism is the absorption of Methotrexate, where *l*-Methotrexate is more readily absorbed than *d*-Methotrexate (Chhabra *et al*., 2013). Another example is seen with Omeprazole, which is the racemate of the R- and S- enantiomers, and Esomeprazole is the S-enantiomer. Both Omeprazole and Esomeprazole have similar action in inhibiting stomach acid production, however, Esomeprazole is more bio-available than Omeprazole (Andersson *et al*., 2001). *l*-

Propranolol has beta-adrenoceptor-blocking action while *d*-Propranolol is inactive (Chhabra *et al*., 2013). S-Warfarin is more extensively bound to blood albumin than R-Warfarin, and hence less bioavailable due to a lower volume of distribution. In addition, R- and S- Warfarin undergo different metabolic processes, resulting in different half-lives. The half-life of S-Warfarin is 32 hrs, whereas the half-life for R-Warfarin is 54 hrs (Qayyum *et al*., 2019). The bio-availability is R-Verapamil is two-fold more than that of S-Verapamil, due to reduced hepatic first-pass metabolism (Hanada *et al*., 2008).

Pharmacodynamic differences due to stereoisomerism can affect factors such as potency, and in the case of antimicrobial activity, the spectrum of activity may be affected. 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 (Wright *et al*., 2014). 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 (Tunitskaya *et al*., 2011). Elder *et al*. (2020) highlighted that what has not been investigated is if the non-potent enantiomer plays any role in the development of antibiotic resistance.

Enantiomerism may also influence the toxicity displayed by chiral drugs, as a result of the unique structure-activity relationships in biological systems. Therefore, the toxicity between enantiomers may vary as a result of stereoisomers having selective protein-binding, transport enzyme interactions, metabolism and DNA binding (Smith, 2009). An example of this is seen with Dopa, or dihydroxy-3,4-phenylalanine. This drug is a precursor of dopamine that is used in the effective treatment of Parkinson's disease. The toxicity of the *d*-enantiomer of Dopa results in agranulocytosis, therefore, only the *levorotatory* form called L-dopa is currently used as a therapeutic treatment for Parkinsons disease (Nguyen *et al*., 2006). Another well-known example is Thalidomide. The racemate was used in the 1960s for the treatment of morning sickness in pregnant women, but was withdrawn when it was discovered to be teratogenic, causing phocomelia (shortening of the limbs) in infants. Subsequent investigations revealed that both the R- and S-enantiomers of Thalidomide have equivalent therapeutic efficacy, however, the S-enantiomer was found to be teratogenic whereas the R-enantiomer was not (Agranat *et al*., 2002). The use of Thalidomide in its single R-enantiomeric form could have prevented this tragedy.

Due to the difficulty presented with chiral separation, many drugs are still formulated and used therapeutically in their racemic form despite the toxicity that may be present due to the presence of an enantiomer. This often results in the unwanted side effects associated with drugs. This is seen with Ketamine, an anaesthetic drug. The (+)-enantiomer is more potent and less toxic than the (–)-counterpart, yet it is still produced and used therapeutically as a racemic drug (Nguyen *et al*., 2006). A few important examples of chiral drugs have been outlined in the current discussion, however, Hancu and Modroiu (2022) provides a more comprehensive review of the stereoselectivity of chiral drugs. Enantiomerism is an important consideration in the pharmacological, therapeutic or toxic effects of chiral drugs. The advantages of enantiopure drugs include; fewer adverse effects, reduced dosing and simpler pharmacokinetic and pharmacodynamic profiles.

1.3.3 Enantiomers of essential oil compounds

Many terpenes and terpenoids have chiral structures, and they can occur in essential oils as either the (+)- or (–)- enantiomer of that compound, or as a racemate. Various techniques are utilised for chemical profiling, and these include: nuclear magnetic resonance spectrometry, high-performance liquid chromatography, liquid chromatography coupled with mass spectrometry and gas chromatography coupled with mass spectrometry (GC-MS) (Maree *et al*., 2014). Analysis through GC-MS is the preferred method of determining the chemical profile of the compounds in an essential oil (Rubiolo *et al*., 2010). It produces representative and reproducible chromatograms of volatile metabolites, together with the associated mass spectra for each separated component peak, which aids in metabolite identification (Lebanov *et al*., 2021).

Chiral GC-MS is a step further, which allows for the determination of the enantiomeric ratio or enantiomeric distribution, of the chiral compounds present within an essential oil. This is often utilised in the authenticity control of essential oils (Do *et al*., 2015). For example, (–)-Linalool has a greater enantiomeric distribution in essential oils such as *Aniba rosaeodora* Ducke, *Cinnamomum camphora* Nees, and *Lavandula angustifolia* Mill. However, (+)-Linalool has a greater enantiomeric distribution in essential oils such as *Coriandrum sativum* L. and *Citrus aurantium* L. (Aprotosoaie *et al*., 2014). Woolley *et al*. (2012) investigated the variations in the chemical profiles of *Boswellia sacra* Flück. and *Boswellia carteri* Birdw. essential oils, which are frankincense species. Through optical rotation evaluation and chiral

separation, it was determined that α-Pinene was present in *B. carterii* at 48.00%, and had a (–)-optical rotation (−13°), whereas α-Pinene was present in *B. sacra* at 79.00% and had a (+)-optical rotation (+30°). The authors added that the key to distinguishing between frankincense species is through chirality. Satyal and Setzer (2020) analysed samples of *C. sativum* essential oil through chiral GC-MS and found that the enantiomeric distribution of Linalool was 87.00% (+):13.00% (−), α-Pinene was 93.00% (+):7.00% (−), and Camphor was 13.00% (+):87.00% (–).

It is therefore evident that knowledge of the enantiomeric distribution is utilised as a means of authenticating and potentially standardising the use of essential oils. This is particularly important when considering the fact that essential oils are often investigated for the promising biological activities, such as antimicrobial efficacy.

1.4 Antimicrobial activity

The antimicrobial activity of essential oils has been previously investigated and observed against a range of bacterial and fungal pathogens, and the enhanced combined activity has also been reported (Swamy *et al.*, 2016; Chouhan *et al.*, 2017; Orchard and van Vuuren, 2017; Wińska *et al.*, 2019; Reyes-Jurado *et al.*, 2020). In addition, the antimicrobial activity of essential oil compounds, both independently and in combination, have been investigated for their antimicrobial activity (van Zyl *et al.*, 2010; Tariq *et al.*, 2019; Yuan *et al.*, 2019; Ahmad *et al.*, 2021).

1.4.1 Antimicrobial activity of enantiomers of essential oil compounds

Seven chiral essential oils were selected to be the focus of the current investigation, and are outlined in Table 1.1. These chiral compounds were selected in order to have a range of chiral compounds with varied structural groups and based on availability, and were investigated in both their enantiopure (+)- and (–)-forms.

Table 1.1: The enantiomers selected for the investigation

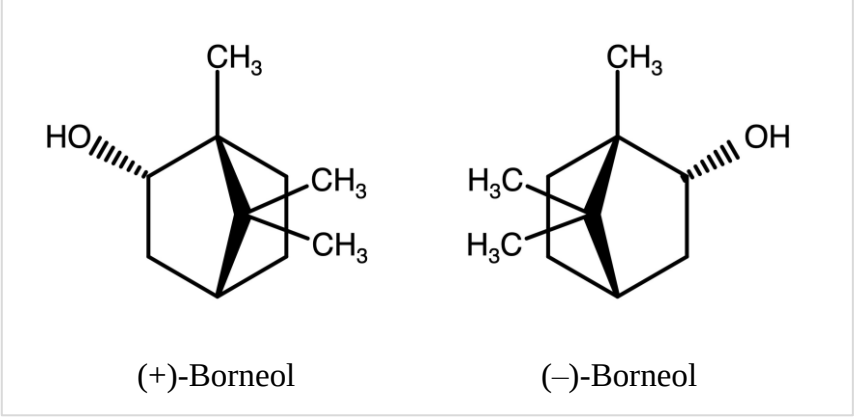
Chiral compound	Chemical formula	Structural classification
Borneol	$C_{10}H_{18}O$	Bicyclic monoterpenoid alcohol
Camphor	$C_{10}H_{16}O$	Bicyclic monoterpene ketone

Chiral compound	Chemical formula	Structural classification
Citronellal	$C_{10}H_{18}O$	Acyclic monoterpenoid aldehyde
Limonene	$C_{10}H_{16}$	Cyclic monoterpene
Menthone	$C_{10}H_{18}O$	Cyclic monoterpenoid ketone
α-Pinene	$C_{10}H_{16}$	Bicyclic monoterpene
β-Pinene	$C_{10}H_{16}$	Bicyclic monoterpene

Upon review of the literature on the influence of enantiomerism on the antimicrobial activity of enantiomers of essential oil compounds, it was determined that the research is substantially lacking. In addition, data regarding the antimicrobial activity of enantiomers of chiral essential oil compounds is limited and inconsistent in terms of the antimicrobial assays used, making it difficult to compare results. A further in-depth analysis of the literature pertaining to the antimicrobial efficacy of each set of enantiomers are given as follows;

1.4.1.1 Borneol

Borneol is a bicyclic monoterpenoid alcohol, and is a structural analogue of Camphor (Granger *et al*., 2005; Almeida *et al.*, 2013) (Figure 1.4). Both enantiomers of Borneol occur in nature, and the racemate occurs less frequently (Tabanca *et al*., 2001). (+)-Borneol has been identified as having a slightly pungent Camphor-like scent with an earthy-peppery note and often occurs in *Rosmarinus* and *Lavandula* species (Lamiaceae), whereas (–)-Borneol has been described as having a less pungent scent and is often found in *Pinus* and *Abies* species (Pinaceae) (Tabanca *et al*., 2001; Panten and Surburg, 2015).



(+)-Borneol (–)-Borneol

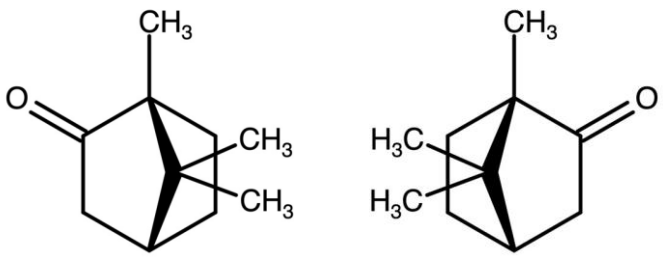
Figure 1.4: The structure of the enantiomers of Borneol.

Tabanca *et al*. (2001) investigated the impact of the chirality of Borneol on antimicrobial activity, and the results varied. In their study, essential oils obtained from parts of *Micromeria cristata* (Hampe) Griseb. collected from three different regions were analysed through GC-MS, and it was found that Borneol was the major compound (27.00 - 39.00%) of these essential oils. In addition, through multidimensional GC-MS analysis, (–)-Borneol was identified as the only enantiomeric form of Borneol in all three samples. The three essential oil samples as well as pure (+)- and (–)- enantiomers of Borneol were then investigated for their antimicrobial activity. This was evaluated against *Proteus vulgaris*, *Enterobacter aerogenes*, *Salmonella typhimurium*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans*. Both enantiomers of Borneol displayed similar inhibitory activity against *P. vulgaris*, *E. aerogenes* and *S. typhimurium* with MIC values of 125.00 µg/mL; and against *E. coli* and *C. albicans* with MIC values of 125.00 µg/mL for (–)-Borneol and 250.00 µg/mL for (+)-Borneol. Similar inhibitory activity was also seen against *S. aureus* and *P. aeruginosa* with MIC values of 125.00 µg/mL for (+)-Borneol and (–)-Borneol had an MIC of 250.00 µg/mL. However, when the MIC values of the (+)-Borneol and (–)-Borneol against *E. coli* were compared to the inhibitory activity of the essential oil samples, one of the three neat essential oil samples still displayed stronger inhibitory activity against this pathogen with an MIC of 62.50 µg/mL. This was also observed against *C. albicans*, where two samples had stronger inhibitory activity than the enantiomers of Borneol, with MIC values of 31.25 µg/mL and 62.50 µg/mL. It was not further investigated whether or not the stronger inhibitory activity of the essential oil samples was due to synergistic interactions of Borneol with the other essential oil compounds present within the oil. Guimarães *et al.* (2019) evaluated the antibacterial of a selection of monoterpenes and terpenoids, amongst which were (+)-Borneol and (–)-Borneol. The antibacterial effects were investigated through minimum inhibitory concentration (MIC) determination against *Bacillus cereus*, *Salmonella typhimurium*, *E. coli* and *S. aureus*. It was reported that, against *B. cereus* and *E. coli*, the enantiomers of Borneol had equivalent antimicrobial activity, with MIC values of 0.12 - 0.25 mg/mL. However, against *S. typhimurium* and *S. aureus*, the inhibitory activity exerted by each of the enantiomers differed, where (–)-Borneol showed stronger inhibitory activity than (+)-Borneol. The MIC values of (+)-Borneol and (–)-Borneol were 8.00 and 0.12 mg/mL, against *S. typhimurium*, respectively, and 0.25 and 0.03 mg/mL against *S. aureus*, respectively. İşcan (2017) also reported that the inhibitory activity of (+)-Borneol was equivalent to (–)-Borneol, when investigated against *S. aureus*, *P. aeruginosa*, *C. albicans*, *E. aerogenes*, *P. vulgaris*, *Serratia marcescens*, *E. coli*, *S. typhimurium*, *Bacillus cereus*, *Bacillus subtilis*, *Listeria monocytogenes* and *Staphylococcus*

epidermidis. A trend was observed in which (+)-Borneol was more active than (–)-Borneol, where variations were observed.

1.4.1.2 Camphor

Camphor is a bicyclic monoterpene ketone (Figure 1.5) and a major compound in essential oils such as *C. camphora* and *Rosmarinus officinalis* L. (Santoyo *et al*., 2005; Pragadheesh *et al*., 2013a). Camphor can occur naturally in its enantiomeric form. In essential oils from the *Ocimum* species (Lamiaceae), (+)-Camphor was found to be the most abundant compound (33.20%); and in essential oils of *Tanacetum* species (Asteraceae), (–)-Camphor was found to be the most abundant enantiomer (14.00%) (Tabanca *et al*., 2007; Pragadheesh *et al*., 2013b).



(+)-Camphor (–)-Camphor

Figure 1.5: The structure of the enantiomers of Camphor.

Tabanca *et al*. (2007) reported antimicrobial activity of essential oils obtained from *Tanacetum argenteum* subsp. *flabellifolium* (Boiss. and Heldr.) against *S. aureus*, *P. aeruginosa*, *E. aerogenes* and *P. vulgaris* with MIC values of 125.00 µg/mL. Through multidimensional GC-MS analysis of the *T. argenteum* essential oil, it was found that, in addition to Camphor being the major constituent of the oil, the enantiomeric distribution of (–)-Camphor was 100.00%. This means that no (+)-Camphor was detected, and Camphor was present in this essential oil in the (–) enantiomeric form only. The study concluded that the antimicrobial activity observed may be directly associated with the enantiomeric configuration of its constituents or the presence of synergy between the major and minor constituents within the oil, but did not further investigate the antimicrobial activity of those compounds or the interactions between them that may have been responsible for the inhibitory activity observed. Viljoen *et al*. (2003) investigated the effects of (+)-Camphor and (–)-Camphor through time-kill studies and found

the effects of both enantiomers to be negligible against *C. albicans* (no total reduction of CFUs seen after 1 hr of incubation). İşcan (2017) also reported that the inhibitory activity of (+)-Camphor was equivalent to (–)-Camphor, when investigated against *S. aureus, P. aeruginosa, C. albicans, E. aerogenes, P. vulgaris, S. marcescens, E. coli, S. typhimurium, B. cereus, B. subtilis, L. monocytogenes* and *S. epidermidis.* It was observed, however, that (+)-Camphor was moderately more active than (–)-Camphor against *C. albicans*.

Pragadheesh *et al*. (2013a) investigated the antimicrobial activity and seasonal variations of essential oils of 12 *C. camphora* leaf collections, particularly the variation in the quantities and proportions of compounds present in the essential oils through GC-MS analysis. The investigation was carried out against a phyto-pathogenic fungus, *Choanephora cucurbitarum*. It was determined that Camphor was the major constituent (68.40 - 81.20%) in the 12 oil samples investigated. Through chiral GC-MS analysis, it was found that (+)-Camphor had an enantiomeric distribution of 99.40% and (–)-Camphor had an enantiomeric distribution of < 1.00%. The antifungal activity of the enantiomers of Camphor were tested against *C. cucurbitarum* and it was found that (+)-Camphor inhibited approximately 70.00% of the growth after 24 hrs, whereas (–)-Camphor only inhibited approximately 50.00% of the growth. In addition, the *C. camphora* essential oil still showed greater inhibitory activity than the (+)-Camphor when examined independently, which may have been attributed to certain combinations of the compounds or enantiomers present. Yet, this was not investigated within the study. In another study conducted by Pragadheesh *et al*. (2013b), essential oils from two *Ocimum* species, *Ocimum canum* Sims, and *Ocimum kilimandscharicum* Gürke, were investigated for their antifungal activity against *Rhizoctonia solani* and *C. cucurbitarum,*. The oils were characterised through chiral analysis, and it was observed that, in addition to Camphor being the major constituent (33.20% and 66.50%, respectively) of the two oils, the enantiomeric distribution of (+)-Camphor was recorded as 99.00%. However, the major constituent is not always responsible for the antimicrobial activity of an essential oil, and hence studies such as the current investigation are important.

1.4.1.3 Citronellal

Citronellal is an acyclic monoterpenoid aldehyde (Figure 1.6) and has been described as having a lemon-like scent (Fatima and Luqman, 2021; Quintans-Júnior *et al*., 2011). This compound has been identified as being a major constituent in the essential oils *Eucalyptus citriodora*

Hook. and *Corymbia citriodora* Hook. (Aguiar *et al*., 2014; De Araújo-Filho *et al*., 2019). Cahyono *et al.* (2014) identified Citronellal as the major compound in the essential oil *Cymbopogon winterianus* Jowitt, and through enantioselective GC analysis, found that the enantiomeric distribution of Citronellal was 88.21% of (R)-(+)-Citronellal. (–)-Citronellal was characterised as the main component of the essential oils of *Citrus hystrix* DC. and *Backhousia citriodora* F.Muell. (Sato *et al*., 1990; Doran *et al.*, 2001).

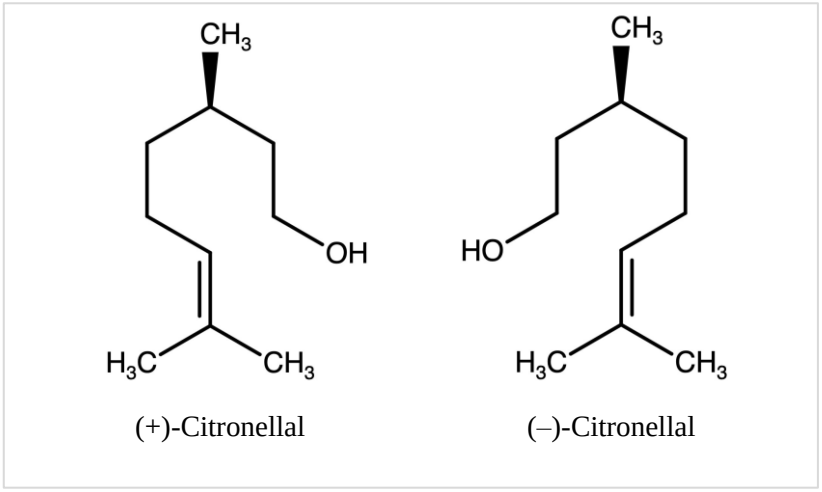


Figure 1.6: The structure of the enantiomers of Citronellal.

Carrillo-Hormaza *et al.* (2015) reported that (–)-S-Citronellal and (+)-R-Citronellal displayed varied activity when investigated against *Enterobacter cloacae* with MIC values of 5.00 and > 5.00 µg/mL respectively. Ngan *et al.* (2012) reported that the enantiomers of Citronellal had equivalent MIC values > 2.50 mg/mL against *S. aureus* and *Klebsiella pneumoniae*. İşcan (2017) also reported that the inhibitory activity of (+)-Citronellal was equivalent to (–)-Citronellal, when investigated against *S. aureus, P. aeruginosa, C. albicans, E. aerogenes, P. vulgaris, S. marcescens, E. coli, S. typhimurium, B. cereus, S. aureus, L. monocytogenes* and *S. epidermidis.* However, İşcan (2017) reported that when tested against *B. subtilis,* the MIC values differed. (+)-Citronellal was more active with an MIC value of 0.25 mg/mL, whereas (–)-Citronellal had an MIC value of 1.00 mg/mL.

1.4.1.4 Limonene

Limonene is an unsaturated cyclic monoterpene (Figure 1.7), with the (+)-enantiomer being more readily available than the (–)-enantiomer (Bonaccorsi *et al*., 2011). (–)-Limonene has been described as having a turpentine-like scent, whereas (+)-Limonene has a more citrus-like

scent (Laska, 1999). (+)-Limonene has been identified as a major constituent in essential oils obtained from citrus plants, particularly peel extracts (John *et al.*, 2017), and (–)-Limonene has been identified as one of the major constituents in certain *Mentha* species (Lamiaceae) (Shutava *et al*., 2014).

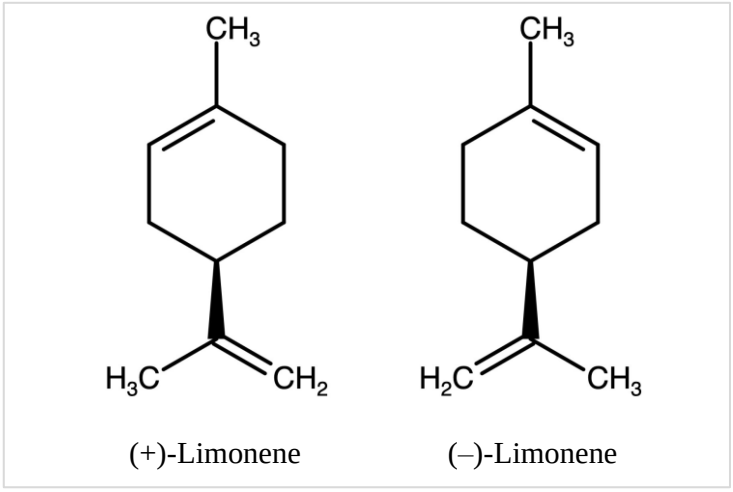


Figure 1.7: The structure of the enantiomers of Limonene.

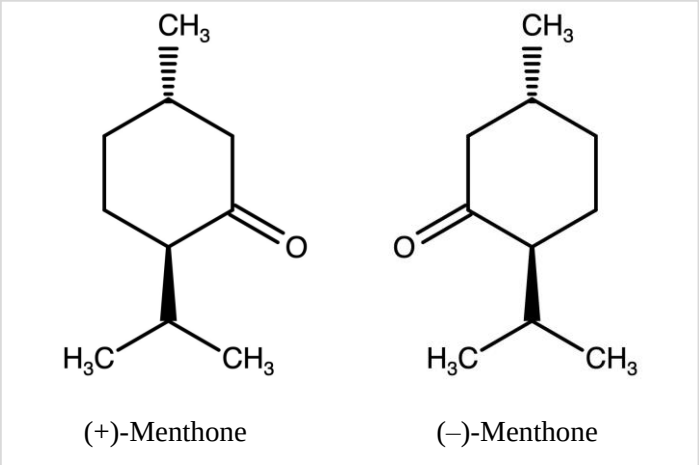
Lis-Balchin *et al*. (1996) reported a substantial difference in the spectrum of antimicrobial activity of the (+)-Limonene and (–)-Limonene against 25 different Gram-positive and Gram-negative bacteria, including 20 strains of *L. monocytogenes* and eight fungal species. No quantitative data was given, however, it was reported through the disc diffusion assay, that (+)-Limonene inhibited 22 bacterial species, while (–)-Limonene inhibited 19 bacterial species. Similarly, against the *L. monocytogenes* strains tested, (+)-Limonene inhibited 13 of the 20 strains, while (–)-Limonene only inhibited seven. Therefore, (+)-Limonene demonstrated a broader spectrum of activity. However, no trend was observed against the fungal species investigated, which included *Alternaria alternata*, *Chaetomium* spp., *Fusarium culmorum*, *Penicillium citratum* and four *Aspergillus* species. The investigation is limited in the sense that the disc diffusion method was utilised, and it was not specified which bacterial strains were evaluated. The emphasis was on the number of strains against which there was efficacy, and no further detail was given. In addition, the unreliability of the disc diffusion method has been reported (Janssen *et al*., 1987; Kalemba and Kunicka, 2003; Ríos and Recio, 2005; Cos *et al*., 2006). İşcan (2017) reported that against *E. coli*, *B. cereus*, *B. subtilis*, *S. aureus*, and *S. epidermidis*, (–)-Limonene was more active, with MIC values ranging between 4.00 - 8.00 mg/mL. However, (+)-Limonene was less active with MIC values ranging between 8.00 - > 16.00 mg/mL. In terms of antifungal activity, Iraji *et al*. (2020) reported that the two

enantiomers of Limonene had potent inhibitory activity against clinical isolates of six *Candida* species, namely: *Candida glabrata*, *Candida tropicalis*, *Candida dubliniensis*, *Candida parapsilosis*, *Candida krusei* and *C. albicans*. The MIC values of (+)-Limonene ranged between 0.69 - 8.69 µg/mL, and 0.50 - 5.29 µg/mL for (–)-Limonene. Therefore, the variation in the antifungal activity between the enantiomers of Limonene were reported to be mostly negligible. However, against *C. krusei*, (–)-Limonene was found to be ten-fold more active.

In terms of antibacterial activity, a study conducted by van Vuuren and Viljoen (2007) found the (–)-Limonene to be the more active enantiomer against *S. aureus* where the inhibitory activity (MIC = 4.00 mg/mL) was three times that of (+)-Limonene (MIC = 13.00 mg/mL). In another study, conducted by Aggarwal *et al*. (2002), (+)-Limonene was shown to possess stronger inhibitory action than (–)-Limonene. Essential oils of *Mentha spicata* L. and *Anethum sowa* Roxb. (Indian dill) were investigated and through gas chromatography (GC) analysis, it was determined that amongst the major compounds of *M. spicata* was (–)-Limonene (27.30%), and in *A. sowa*, was (+)-Limonene (21.40%). The antimicrobial activity of the two oils as well as the two enantiomeric forms of Limonene were investigated against 12 human-pathogenic bacterial species and seven fungal species, using the broth dilution assay. The essential oil of *M. spicata*, which possessed the (–)-Limonene, displayed stronger antibacterial activity than *A. sowa*, which contained the (+)-Limonene. The study also reported that (–)-Limonene investigated independently had little activity and in comparison, (+)-Limonene was highly active. Therefore, the essential oil containing (–)-Limonene displayed stronger inhibitory activity than the essential oil containing (+)-Limonene, whereas, independently, (+)-Limonene displayed stronger antimicrobial activity than (–)-Limonene. While there is potential for the enantiomers of Limonene to vary in their antimicrobial inhibitory activity, there is a need for a more comprehensive study into the influence of enantiomerism on the combined activity of Limonene with other compounds.

1.4.1.5 Menthone

Menthone is a cyclic monoterpenoid, and the ketone analogue of Menthol (Schmitz *et al*., 2015) (Figure 1.8). (–)-Menthone is often the most abundant enantiomer of Menthone present in the essential oils of *Mentha piperita* L. (peppermint). (+)-Menthone is a constituent in the essential of *Nepeta japonica* Willd. (Ravid *et al.*, 1994). Menthone has a minty odour, similar to that of Menthol, and also occurs naturally in peppermint oil (Schmitz *et al*., 2015).



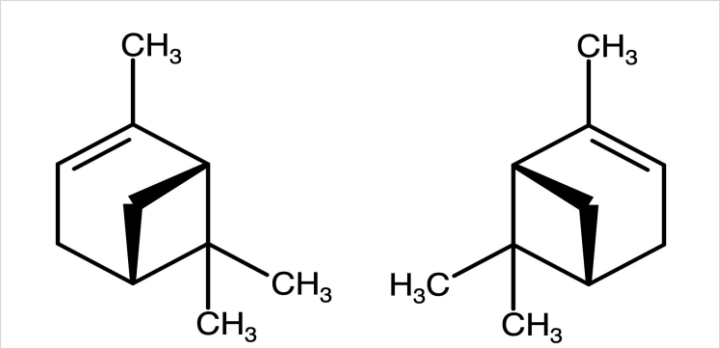
(+)-Menthone (–)-Menthone

Figure 1.8: The structure of the enantiomers of Menthone.

The antimicrobial properties of Menthone has been previously highlighted (Kamatou *et al*., 2013). Studies that evaluated the comparative inhibitory activity of the enantiomers of Menthone are considerably limited. Iraji *et al*. (2020) reported that, against clinical isolates of *C. albicans*, the enantiomers of Menthone had similar MIC values of 14.21 mM and 26.33 mM for (+)-Menthone and (–)-Menthone, respectively. The study evaluated and compared the antifungal activity of the enantiomers of Menthone against isolates of *Candida* species and found (+)-Menthone to be two to ten-fold more active against all the isolates investigated. To the best of my knowledge, this is the only investigation in which both of the enantiomers of Menthone were evaluated and compared in the same study. Furthermore, the combination of the different enantiomers and their effects when combined with other compounds has not been adequately studied.

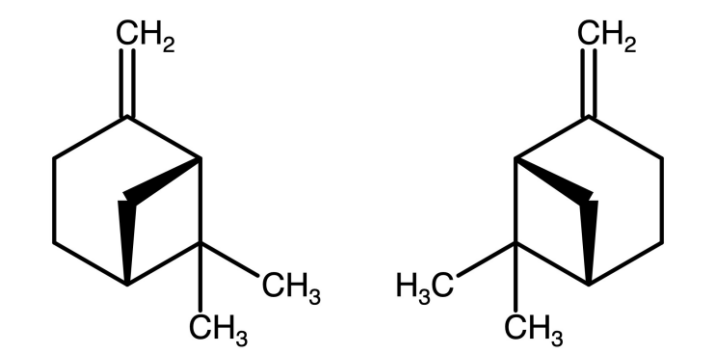
1.4.1.6 α-Pinene and β-Pinene

Pinenes are bicyclic monoterpenes with two structural isomers: α-Pinene and β-Pinene, that each occur as (+) and (–) enantiomeric forms in nature (Figures 1.9 and 1.10). α-Pinene is the main secondary metabolite in many conifer-derived essential oils (Allenspach *et al*., 2020). α-Pinene with its volatile and hydrophobic properties, has a fresh pine scent and woody flavour (Vespermann *et al*., 2017). β-Pinene has been described as having a woody-green pine-like odour (Lasekan and Abbas, 2012). The (+)- and (–)-forms of β-Pinene have been found in the essential oils from several species belonging to the families of Artemisae and Cupressaceae, and the (–)-form is a constituent of several essential *Citrus* species (Vespermann *et al*., 2017).



(+)-α-Pinene (–)-α-Pinene

Figure 1.9: The structure of the enantiomers of α-Pinene.



(+)-β-Pinene (–)-β-Pinene

Figure 1.10: The structure of the enantiomers of β-Pinene.

Of the enantiomers selected for the current investigation, there was more research into the influence of enantiomerism on the antimicrobial activity of α-Pinene, and the overall finding is that (+)-α-Pinene is the more active enantiomer. Lis-Balchin *et al*. (1999) investigated the antimicrobial activity of enantiomerically pure (+)-α-Pinene and (–)-α-Pinene against 25 pathogenic bacterial strains, 20 strains of *L. monocytogenes* and three filamentous fungi, using the disc diffusion method. The 25 bacterial species were a combination of Gram-positive and Gram-negative species, some of which included: *B. subtilis*, *Streptococcus faecalis*, *E. coli*, *E. aerogenes*, *K. pneumoniae*, *P. vulgaris*, *P. aeruginosa* and *S. aureus*. The three filamentous fungi investigated were *Aspergillus niger*, *Aspergillus ochraceus* and *Fusarium culmorum*. The antibacterial activity of the enantiomers were reported in terms of whether or not growth was inhibited, and no quantitative data was given. The study reported that (–)-α-Pinene displayed a broader spectrum of activity of the two enantiomers, inhibiting 18 of the 25 bacteria tested, of which 11 out of 16 were Gram-positive and six out of nine were Gram-negative. This was also

observed with the *L. monocytogenes* strains, where (–)-α-Pinene showed broader inhibitory activity compared to the positive enantiomer, but (+)-α-Pinene was the more active of the two against Gram-positive *L. monocytogenes* strains. When tested against the fungal species, (+)-α-Pinene showed greater inhibitory activity against *A. niger* and *A. ochraceus*, but little difference in inhibitory activity was observed against *F. culmorum*. This study does highlight that different enantiomers of a chiral compound can have different antimicrobial activity, however, the study reports on the spectrum of activity, rather than the variations between enantiomers on inhibitory activity. Filipowicz *et al.* (2003) found that similar inhibitory activity was observed between the enantiomers of α-Pinene, against *S. aureus*, Methicillin-Resistant *Staphylococcus aureus* (MRSA), *Acinetobacter baumannii* and *C. albicans*. Nikitina *et al.* (2009) investigated the antifungal effects of the enantiomers of α-Pinene through the disc diffusion method (eight-day incubation) against *A. niger*, *A. fumigatus*, *Penicillium tardum*, *Penicillium chrysogenum* and *C. albicans*, as well as yeast and dermatomycete strains isolated from the skin of patients. The study reported that in comparison to (–)-α-Pinene, (+)-α-Pinene had moderate antifungal activity, however, no quantitative data was given.

Iraji *et al*. (2020) reported that the antifungal potency of (+)-α-Pinene was greater than (–)-α-Pinene, against clinical isolates of six *Candida* species. The study reported that the antifungal activity of (+)-α-Pinene ranged between 0.57 - 2.33 μg/mL, whereas the antifungal activity of (–)-α-Pinene ranged between 3.46 - 84.73 μg/mL. Therefore, (+)-α-Pinene displayed considerably stronger antifungal activity than (–)-α-Pinene. Dhar *et al*. (2014) assessed the activity of (+)-α-Pinene and (–)-α-Pinene against *Micrococcus luteus*, *S. aureus*, *E. coli* and *C. albicans*. It was reported that (+)-α-Pinene exhibited modest action against the selected micro-organisms, while (–)-α-Pinene did not exhibit antimicrobial activity at the tested concentration. It can therefore be seen that (+)-α-Pinene has often been reported to be the more active enantiomer. The antimicrobial activity of the enantiomers of α-Pinene and β-Pinene were investigated by da Silva *et al*. (2012) by determining the inhibitory activity against MRSA, *C. albicans*, *Cryptococcus neoformans* and *Rhizopus oryzae*, a parasitic fungi that is the common cause of zygomycosis in immunocompromised patients (Ibrahim *et al*., 2005). It was reported that the (+)-enantiomers of α-Pinene and β-Pinene exhibited inhibitory activity against the pathogens investigated, with MIC values ranging from 117.00 - 6250.00 μg/mL. The (–)-enantiomers exhibited no antimicrobial activity up to 20.00 mg/mL. In terms of the evaluation of the comparative antimicrobial activity of the enantiomers of β-Pinene, very limited research has been previously undertaken.

1.4.2 The combined antimicrobial efficacy of enantiomers of essential oil compounds

Studies have previously characterised the enantiomeric composition of essential oil compounds and attributed their antimicrobial activity to the most predominantly occurring enantiomer without conducting any studies to verify this (Sakhanokho *et al*., 2013; Lawal *et al*., 2014; Dudai *et al*., 2017; Damasceno *et al*., 2019). It is also important to note that the antimicrobial activity of an essential oil is not necessarily due to the activity of its major compound, and that the minor compounds may play a role. In addition, the activity may be as a result of the combination of certain compounds. Of the chiral compounds selected for the current investigation, data on the comparative antimicrobial activity between enantiomeric pairs in combination could only be found for Camphor, Limonene, α-Pinene and β-Pinene, and this was undertaken on limited test micro-organism strains.

In a study conducted by Viljoen *et al*. (2003), the enantiomer-specific antimicrobial activity of Camphor was investigated. The antimicrobial activity of the essential oil of *Osmitopsis asteriscoides* L. against *C. albicans*, *S. aureus*, and *P. aeruginosa* was investigated through three different antimicrobial assays (disc diffusion, MIC by micro-titre plate and time-kill studies). The time-kill assays revealed that the essential oil had strong fungicidal activity against *C. albicans* and bacteriostatic activity against *S. aureus* in a concentration-dependent manner. However, poor antibacterial activity was observed against *P. aeruginosa*. Through GC-MS analysis, it was determined that the sample of *O. asteriscoides* essential oil comprised of 40 compounds, with 1,8-Cineole and (–)-Camphor being the major constituents at 60.00% and 12.00% respectively. This means that Camphor only accumulates in the (–)-form in this plant as no (+)-Camphor was detected. The antimicrobial activities of 1,8-Cineole and (–)-Camphor were investigated both independently and in combination against *C. albicans*, with 1,8-Cineole showing antifungal efficacy independently, and a synergistic antimicrobial effect when tested in combination with (–)-Camphor. The study deduced that the positive antimicrobial activity seen in the *O. asteriscoides* essential oil may be due to the synergistic antimicrobial effect of these two compounds in combination, but also noted that 1.00% of the essential oil still had greater microbicidal activity than the combination of compounds studied and suggested that the influence of other minor compounds may have been responsible for this.

A study conducted by van Vuuren and Viljoen (2007) investigated whether micro-organisms respond differently when exposed to both enantiomers of Limonene and/or its racemate, when

combined with 1,8-Cineole with which is frequently co-occurs in nature. The MIC assay was conducted against *S. aureus*, *E. faecalis*, *B. cereus*, *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *Moraxella catarrhalis* and *C. neoformans*. In combination 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*. This activity is interesting to note because even though (–)-Limonene displayed stronger inhibitory activity when the enantiomers were investigated individually, it was (+)-Limonene that displayed synergy in combination with 1,8-Cineole whereas (–)-Limonene combinations resulted in antagonism; which was mostly observed for combinations of racemic mixtures of Limonene enantiomers with 1,8-Cineole. The authors emphasised that the chirality of the molecules influences the antimicrobial activity, and that this activity is highly pathogen-specific. The antimicrobial activity of the (+)-enantiomers of α-Pinene and β-Pinene were investigated by da Silva *et al*. (2012) by determining the inhibitory activity (MIC assay) against MRSA, *C. albicans*, *C. neoformans* and *R. oryzae*. The antimicrobial activity of combinations of the commercial antibiotics Amphotericin B and Ciprofloxacin, with the (+)-enantiomers of α-Pinene and β-Pinene were evaluated. It was found that Ciprofloxacin in combination with (+)-α-Pinene or (+)-β-Pinene exhibited synergistic activity against MRSA. Combinations of Amphotericin B and the (+)-enantiomers of α-Pinene or β-Pinene displayed a non-interactive effect on all the fungal species investigated, and no antagonism was reported for any of the combinations investigated. The (–)-enantiomers were not evaluated and compared.

To the best of my knowledge, the investigations discussed were the only studies in which the enantiomeric forms of the essential oil compounds were identified and comparatively evaluated to one another. The lack of research in this regard is evident and thus warrants further in-depth analysis.

1.4.3 Antimicrobial analysis

The 'ESKAPE' acronym is used to describe a group of highly virulent Gram-positive and Gram-negative bacterium, which are: *Enterococcus faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter* spp. In February 2017, the World Health Organisation (WHO) prioritised the ESKAPE pathogens as either 'critical', 'high' or 'medium' priority pathogens requiring research and development of new antibiotics. The pathogens, *A. baumannii*, *P. aeruginosa*, *K. pneumoniae* and various *Enterobacter* spp. were categorised as

'critical' priority; and *E. faecium* and *S. aureus* were categorised as 'high' priority pathogens (WHO, 2017). These bacteria are commonly implicated in hospital acquired (nosocomial) infections amongst immunocompromised and critically ill patients, and are identified as pathogens prone to potential mechanisms of drug resistance (Santajit and Indrawattana, 2016). The acronym 'ESKAPE' also expresses the ability of these bacteria to "escape" the lethal effects of antibiotics, which is responsible for extensive morbidity and mortality seen in patients and the burden on healthcare resources (Ma *et al*., 2020).

Ramsamy *et al.* (2018) retrospectively reported the prevalence of the ESKAPE pathogens in nine public hospitals in KwaZulu-Natal, South Africa, over a period of five years between 2011 and 2015. The study reported that *S. aureus* was most frequently isolated (n = 24495, 38.00%), followed by *K. pneumoniae* (n = 14282, 22.00%). In a study conducted by Perovic *et al*. (2018), one of the aims was to determine the number of cases for each of the ESKAPE pathogens isolated from blood cultures in 2016, across 16 public hospitals in South Africa. The study found that of the 5265 Enterobacteriaceae identified, 53.00% were identified as *K. pneumoniae*, 35.00% were identified as *E. coli* and 12.00% were identified as *Enterobacter cloacae*. Of the 2318 Gram-negative bacteria identified, 71.00% were identified as *A. baumannii* and 29.00% were identified as *P. aeruginosa*. In another study, conducted by Ismail *et al.* (2019), 28920 cases of infection caused by the ESKAPE pathogens and *E. coli* were reported in 2016, and 32293 in 2017, across the two health sectors in South Africa (public and private).

In addition to bacterial diseases, South Africa's fungal disease burden is substantial and broad in scope. Schwartz *et al.* (2019) reviewed the incidence and prevalence of fungal infections reported in literature, in South Africa and globally, considering the immunocompromised state of the patients. It was estimated that 8357 cases of HIV-associated *Cryptococcal* meningitis occur each year in South Africa, based on surveillance data from the National Institute for Communicable Diseases (GERMS, 2019). A review published by Singh and Urhekar (2013) reported that since 1995 (to 2013), *Candida* species have become the fourth most common cause of nosocomial Candidemia (third most in intensive care units) and are associated with a crude mortality rate of 39.00%. In South Africa, symptomatic vulvovaginal candidiasis is one of the most common causes of women to seek advice in primary healthcare facilities (Apalata *et al*., 2014). Mnge *et al.* (2017) found that in a tertiary hospital in the Eastern Cape, 95 out of 209 (45.5%) *Candida* isolates obtained from clinical specimens were identified as *C. albicans*, making it the most prevalent species identified. *C. albicans* usually has an inherently low

virulence, except in immunocompromised patients with impaired physiological and cellular barriers (Mnge *et al*., 2017). Apalata *et al.* (2014) found that there was a positive correlation seen between women who developed symptomatic vulvovaginal candidiasis and HIV positive women on uncontrolled anti-retroviral therapy and a high viral load.

The relevance of investigating these pathogens is clearly highlighted by their prevalence in infection requiring hospitalisation in developing countries, including South Africa, and their ability to develop resistance. It is for these reasons that the WHO has promoted the initiative to stimulate research and development into therapeutic alternatives against these pathogens. Based on these factors, the pathogens outlined in Table 1.2 have been selected for the antimicrobial analysis for the current investigation. The selection of two of each Gram-positive and Gram-negative bacteria, and two yeast pathogens was to firstly, focus the investigation on common disease-causing pathogens with a high prevalence not only in South Africa, but globally. Thereafter, to ensure that the different groups of micro-organisms are covered.

Table 1.2: The pathogens selected for the antimicrobial analysis in this study

Group	**Micro-organism**	**Description**	**Reference**
Gram-positive bacteria	*Staphylococcus aureus* ATCC 25923	Forms part of the normal intestinal tract flora and skin commensals. Involved in acute and chronic wound infections. Common cause of skin infections.	Pendleton *et al.* (2013); Navidinia (2016).
	Enterococcus faecium ATCC 27270	Forms part of the normal intestinal tract flora. Persistently found in water sources, ground, meat and dairy products. Frequently implicated in healthcare-associated infections, particularly in immunocompromised patients. Opportunistic bacteria with the potential to cause severe infectious diseases, such as bacteraemia, endocarditis, wound infections and meningitis.	Pendleton *et al.* (2013); Navidinia (2016).

Group	Micro-organism	Description	Reference
Gram-negative bacteria	*Klebsiella pneumoniae* ATCC 13887	Frequently implicated in lower respiratory tract infection and catheter-associated urinary tract infection. Virulence is invasive and intrinsic in nature, due to fimbrial adhesions and a thick capsule.	Podschun and Ullmann (1998); Pendleton *et al.* (2013); Navidinia (2016).
	Pseudomonas aeruginosa ATCC 27853	Intrinsic ability to develop resistance due to the few porins in its outer membrane, resulting in the inability or difficulty of antibiotics to penetrate the cell. Able to survive extreme conditions. Common cause of infection in cancer patients and burn victims.	Pendleton *et al.* (2013); Navidinia (2016); Ma *et al.* (2020).
Fungi	*Candida albicans* ATCC 10231	Forms part of the normal flora of the mucous membranes of upper respiratory tract and female genitalia. Can cause candidiasis in immunocompromised individuals.	Singh and Urhekar (2013).
	Cryptococcus neoformans ATCC 14116	Mainly causes pulmonary and central nervous system infection (*Cryptococcal* meningitis). Primary route of infection is inhalation. Ranks fourth amongst most common causes of infection in sub-Saharan Africa.	(Park *et al*., 2009; La Hoz and Pappas, 2013).

In vitro antimicrobial susceptibility tests are often performed on disease-causing micro-organisms that tend to exhibit multi-drug resistance (EUCAST, 2003). A variety of testing methods are used to determine the antimicrobial activity of pure compounds, including the broth micro-dilution method. Dilution methods are the most suitable method of susceptibility testing, as it allows for the quantification of antimicrobial activity (Balouiri *et al*., 2016). The MIC value is the lowest concentration of the sample investigated that is required to inhibit the

growth of a specific micro-organism (EUCAST, 2003). The advantages of this test method include its reproducibility, the small amount of sample required, and the low cost allowing large numbers of replicates (Benkova *et al*., 2020). The standardization of the widely used broth micro-dilution method by the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST), particularly in terms of the concentration of inoculum used, makes it a reliable method that yields results that can be accurately reproduced and compared. As such, the broth micro-dilution method was determined to be the most suitable, reliable, and accurate means of assessing antimicrobial activity.

1.5 Anti-quorum sensing activity

Compounds that have antimicrobial activity may still eventually develop resistance. It is therefore preferable to target the factors that regulate pathogenesis. Amongst the pathogenic traits used by micro-organisms to increase virulence is quorum sensing (QS). Quorum sensing is a bacterial cell-to-cell communication process through which bacterium are able to communicate adaptive resistance responses to one another and initiate different physiological processes collectively. These QS-regulated physiological processes often aid virulence, and include motility, pathogenicity, formation of biofilms, genetic competence development, sporulation, generation and secretion of proteolytic enzymes (Ghosh *et al*., 2022).

The process of QS relies on the biosynthesis, secretion, and assimilation of auto-inducers (AIs), which in turn leads to the production of signalling molecules (Asfour, 2018). The production of signalling molecules is dependent on the mechanism of auto-inducing and can differ in Gram-negative and Gram-positive bacteria (Xavier and Bassler, 2003). These signalling molecules are divided into three types: 1) Auto-inducer 2 (AI-2) in Gram-positive and Gram-negative bacteria, allowing for interspecies communication, 2) Auto-inducing peptides or oligopeptides in Gram-positive bacteria, allowing for intercellular communication, and 3) N-acyl homoserine lactones (AHLs) in Gram-negative bacteria, to monitor density population in QS-regulated gene expression control (Ghosh *et al*., 2022).

Chromobacterium violaceum is a Gram-negative bacterium that is commonly found in soil and water in tropical and subtropical regions (Kumar, 2012). This bacterium produces a characteristic purple pigment called ‘violacein’, the production of which is regulated by AHL-

mediated QS (Hossain *et al*., 2017). Violacein is an easily observable and quantifiable QS-regulated trait (Kothari *et al*., 2017). While violacein production is itself not a factor that aids virulence, its regulation by AHL signal molecules allows for the screening of compounds that inhibit this, and other virulence factors that are AHL-mediated, some of which include gene regulation, antibiotic resistance, and aspects of biofilm formation (Chu *et al*., 2011). As such, *C. violaceum* is a suitable biosensor strain for compounds that inhibit AHL-mediated QS inhibition (Adonizio *et al*., 2006; Szabó *et al*., 2010).

Early anti-QS agents were first identified from algal and fungal origins. *Delisea pulchra* (red marine alga) and was found to contain halogenated Furanones, which are compounds that competitively block AHLs due to their structural similarity (Manefield *et al*., 2001). Natural Furanones and their synthetic analogues have shown considerable anti-QS activity in some species (Wu *et al*., 2004). Their use, however, is limited in that the Furanones are too reactive and may be too toxic for treatment of bacterial infections in humans. As such, there has been an increasing interest in alternative antipathogenic agents that that are non-toxic anti-QS inhibitors (Hentzer and Givskov, 2003).

1.5.1 Anti-quorum sensing activity of enantiomers of essential oil compounds

Studies in which the anti-QS activity of essential oil compounds have been previously evaluated, and several review articles have been published (Silva *et al*., 2016; Khan *et al*., 2018; Reichling, 2020;). What is evident, however, is the considerable lack of anti-QS research on comparative enantiomers. One study successfully demonstrated that there is in fact variations in the anti-QS activity between enantiomeric pairs of chiral essential oil compounds. Ahmad *et al*. (2015) investigated the anti-QS activity of 29 essential oil compounds, including the enantiomers of α-Pinene, Limonene and Borneol, against violacein production in *C. violaceum*. The minimum quorum sensing inhibitory concentration (MQSIC) of the compounds were determined and it was reported that the (–)-enantiomers of Limonene and Borneol inhibited violacein production, whereas the (+)-enantiomers promoted violacein production. The authors of the study emphasised that this observation means that more attention must be paid to the chemical composition of essential oils containing such structural analogues.

A study investigated the anti-QS activity of the enantiomers of Limonene against a biosensor strain other than *C. violaceum*. Zheng *et al.* (2020) investigated the QS inhibitory activity of

(+)-Limonene and (–)-Limonene against the Gram-negative biosensor strain *Vibrio campbelli*, at concentrations of 0.001% (1 x10^5 mg/mL) and 0.0001% (1 x10^6 mg/mL) and reported that both enantiomeric forms of Limonene similarly inhibited bioluminescence in *V. campbelli*. To the best of my knowledge, these are the only investigations in which the focus was to evaluate, and more specifically, compare the anti-QS activity of essential oil compounds in their enantiomeric forms. No data could be found in which the enantiomers of Camphor, Citronellal and Menthone were evaluated for their anti-QS activity. Moreover, no investigations could be found in which the combined anti-QS activity of essential oil compounds were evaluated. The need for the current study is therefore highlighted.

1.5.2 The broth anti-quorum sensing macro-dilution assay

While authors Ahmad *et al*. (2015) evaluated the anti-QS activity of essential oil compounds using the broth dilution method, a more comprehensive approach was undertaken by Kharsany (2019). The author evaluated the anti-QS activity of bioactive compounds using both the broth micro-dilution and macro-dilution methods comparatively and reported inconsistencies where the percentage violacein inhibition results from the lower concentrations tested in the micro-dilution method were much higher than those obtained from the macro-dilution method. As the MQSIC is interpreted quantitatively as the lowest concentration that yields a percentage inhibition of over 50.00%, reporting on MQSIC values using the micro-dilution method would give different values and thus could not be used. This variation was attributed to the growth pattern of *C. violaceum*, which forms a purple-pigmented ring on the walls of the container in which it grown. The macro-dilution allowed for the test tubes to be vortexed thus incorporating this culture residue into the broth. This, however, is not possible to do in microtitre plate conditions. As such, the broth macro-dilution was determined to be more suitable, reliable and accurate.

1.6 Toxicity of enantiomers of essential oil compounds

Natural products, such as essential oils, are often perceived to be safe and non-toxic. However, their safety profiles are often understudied, creating the potential for adverse reactions either alone, or concomitantly with other natural products or therapeutic agents (Gaston *et al*., 2020). Essential oil compounds at high concentrations are toxic. This is often attributed to their highly lipophilic nature and the damage caused to cell membranes (Dhifi *et al*., 2016). This is one of

the limiting factors in its therapeutic use. While common essential oils and essential oil compounds have been investigated for their toxicity previously (Tisserand and Young, 2014; Falleh *et al*., 2020), the influence of stereochemistry on the toxicity profiles of the essential oil compounds is often poorly understood and there is an obvious lack of data in this regard. A further in-depth analysis of the comparison with literature (where available) are given as follows;

1.6.1.1 Borneol

Nunes *et al*. (2018) investigated the toxicological profiles of (–)-Borneol and its derivatives using the BSLA and found (–)-Borneol to be non-toxic up to a concentration of 1.00 mg/mL. However, (+)-Borneol was not evaluated.

1.6.1.2 Camphor

Tak *et al.* (2006), investigated the acaricidal activities of (1S)-(–)-Camphor against the copra mite, *Tyrophagus putrescentiae* (mould mite), using direct contact and vapour phase toxicity bioassays. It was reported that (1S)-(–)-Camphor was toxic. The investigation is limited in that Camphor was not evaluated in its (+)-enantiomeric form.

1.6.1.3 Citronellal

Fouad *et al*. (2021) investigated the toxicity of the enantiomers of Citronellal against *Sitophilus oryzae* (rice weevil) through contact, fumigant and repellency assays and the overall finding was that across the three assays, (–)-Citronellal displayed greater toxicity than (+)-Citronellal in terms of LC_{50} and LC_{90} values.

1.6.1.4 Limonene

Giatropoulos *et al.* (2012) reported R-(+)-Limonene and S-(–)-Limonene were toxic against *Aedes albopictus* (tiger mosquito), with equivalent LC_{50} values of 35.99 mg/mL and 34.89 mg/L. Fouad *et al*. (2021) investigated the toxicity of the enantiomers of Limonene against *S. oryzae* and the overall finding was that both enantiomers of Limonene displayed equivalent toxicity in terms of LC_{50} and LC_{90} values in terms of the contact and fumigant assays. Fouad

and da Camara (2017) evaluated the insecticidal activity of R-(+)-Limonene and S-(–)-Limonene against *Sitophylus zeamais* (maize weevil) at concentrations ranging between 40.00 - 60.00 μL/mL, to evaluate their toxicity through contact, fumigant, and ingestion assays. The difference reported between the two enantiomers in the fumigant and contact test were not appreciable, however, the ingestion test revealed that of R-(+)-Limonene was more toxic than S-(–)-Limonene. Batista *et al.* (2019) investigated the antileishmanial activity of the enantiomers of Limonene and reported that (+)-Limonene showed toxicity at a concentration almost four times that of (–)-Limonene, therefore showing reduced toxicity in comparison. The LC_{50} values were 1.72 mM for (+)-Limonene and 0.45 mM for (–)-Limonene.

1.6.1.5 Menthone

Enantiomeric comparison studies on Menthone are limited. Giatropoulos *et al.* (2018) reported similar LC_{50} values of 53.90 mg/L and 59.00 mg/L for (+)-Menthone and (–)-Menthone, respectively, against *A. albopictus* (tiger mosquito). Fouad *et al*. (2021) investigated the toxicity of the enantiomers of Menthone against *S. oryzae*, and the overall finding was that (+)-Menthone was slightly less toxic, but not to an appreciable extent, in terms of LC_{50} and LC_{90} values, when evaluated through the contact and fumigant assays.

1.6.1.6 α-Pinene and β-Pinene

Michaelakis *et al.* (2009), evaluated the insecticidal activity of the (+)- and (–)- enantiomers of α-Pinene and β-Pinene on *Culex pipiens* (common house mosquito) and reported LC_{50} values of 0.06 mg/mL for both enantiomers of α-Pinene and 0.07 mg/mL and 0.04 mg/mL for (+)-β-Pinene and (–)-β-Pinene respectively. Traboulsi *et al.* (2002), also reported LC_{50} values of 0.06 mg/mL for the two enantiomers of α-Pinene against *C. pipiens*. Giatropoulos *et al.* (2012) investigated the larvicidal activity of the enantiomers of α-Pinene and β-Pinene against the mosquito *A. albopictus* and reported LC_{50} values of 68.68 mg/L and 72.30 mg/L for (+)- and (–)-α-Pinene, respectively, and 47.33 mg/L and 42.39 mg/L for (+)- and (–)-β-Pinene respectively (after 24 hrs). Thus, the enantiomers of each compound were found to display similar toxicity profiles. However, Vourlioti-Arapi *et al.* (2012) reported equivalent LC_{50} values for the enantiomeric pairs of α-Pinene and β-Pinene against *C. pipiens*. The LC_{50} values reported were 0.08 mg/mL and 0.07 mg/mL for (+)-β-Pinene and (–)-β-Pinene, respectively, and 0.08 mg/mL and 0.09 mg/mL for (+)-α-Pinene and (–)-α-Pinene, respectively. The study

reported that amongst the essential oils investigated for their larvicidal activity, when the contained amount of (–)-α-Pinene was more than 50.00%, the LC_{50} values of the essential oils ranged from 65.69 - 96.69 mg/L, while for amounts between 19.00 - 50.00% the respective LC_{50} values ranged from 55.84 - 65.55 mg/L. Therefore, the higher the concentration of (–)-α-Pinene present, the lower the observed toxicity of the essential oil. Fouad *et al*. (2021) investigated the toxicity of the enantiomers of α-Pinene against *S. oryzae*, and the overall finding was that (+)-α-Pinene was less toxic that (–)-α-Pinene in terms of LC_{50} and LC_{90} values, when evaluated through the contact, fumigant, and repellency assays.

1.6.2 Combined toxicity of enantiomers of essential oil compounds

Investigations in which the enantiomeric forms of the essential oils were evaluated in combination (i.e. when each enantiomeric form was combined with another sample) were limited, where, to the best of my knowledge only one study could be found. Pavela (2014) investigated the acute toxicity of 30 aromatic compounds against larvae from *S. littoralis* (leaf worm) at a dose of 0.30 mg/larva after 24 hrs of exposure. The study evaluated the individual and combined toxicity profiles and reported the interactions to be either synergistic, additive, antagonistic or non-interactive. The following enantiomers were included in the investigation: R-(+)-Limonene, (–)-β-Pinene, (+)-Camphor and (–)-Borneol. It was reported that (–)-Borneol and Camphor had a low PM value when investigated alone, however, enhanced toxicity was observed in combination with other compounds. However, the enantiomeric counterparts of these compounds were not evaluated and compared. Citronellal (racemate), Menthone and α-Pinene were also investigated, however, their enantiomeric distribution was not specified.

1.6.3 The brine-shrimp lethality assay (BSLA)

The brine-shrimp lethality assay is a useful tool in the preliminary assessment of toxicity, and has been used previously to evaluate the toxicity of essential oils (Orchard *et al*., 2019). Hatched *Artemia salina* (brine-shrimp) nauplii are exposed to the test compound for a specified period of time and the toxicity of the compound is correlated to the mortality of the brine-shrimp. Although this method does not provide information on the mechanism of toxicity, the data obtained from this assay can be backed up by more specific bioassays, once the toxic potential of the compounds has been evaluated (Pisutthanan *et al.*, 2004; Gadir, 2012; Naidu *et al*., 2014). The advantages of this toxicological screening method are its rapidity, simplicity,

inexpensiveness relative to specific bioassays, and high degree of repeatability (Hamidi *et al*., 2014). The aim of the current investigation includes identifying potential variations in the toxicity of enantiomers of essential oil compounds, thus the BSLA is a suitable method. It has therefore been selected for this study as a screening tool for toxicity.

1.7 Rationale and motivation for the study

The substantial lack of data in terms of the stereoselective antimicrobial, anti-QS, and toxicity of essential oil compounds is evident. Although the data available in this regard is considerably limited, there is potential for enantiomers of essential oil compounds to differ in their antimicrobial inhibitory activity, anti-QS activity, and toxicity. In addition, there is in particular a lack of research in terms of the influence of stereochemistry on the combined antimicrobial, anti-QS and toxic effects of essential oil compounds. What needs to be taken cognisance of, is that the essential oil compounds of enantiomers of those compounds do not necessarily act independently within the essential oil. They are combined with other major and minor compounds, and the interactions with those compounds often result in increased or decreased bio-activity of the essential oil. The need for a comprehensive study that investigates the influence of the stereochemical configuration of essential oil compounds is needed. As such, the current investigation evaluated a selection of enantiomeric pairs (Table 1.1) for variations in their inhibitory antimicrobial efficacy, anti-QS activity and toxicity profiles. Furthermore, the influence of stereochemistry on their combined interactive efficacy is the focus of this study.

In order to investigate the combined activity of the enantiomers, a selection of essential oil compounds were combined with the enantiomers. The compounds were selected based on having been identified as having good antimicrobial activity against the majority of the pathogens selected for this investigation, as well as availability. As such, these compounds have been referred to in the current investigation as the selected compounds to avoid confusion with the enantiomeric compounds, referred to as the enantiomers. The selected compounds have been outlined in Table 1.3.

Table 1.3: The compounds selected to be combined with the enantiomers

Selected compound	**Pathogen against which the compound is antimicrobially active**	**Reference**
Camphene	*C. albicans*	Maree *et al.* (2014)
β-Caryophyllene	*P. aeruginosa*	de Rapper *et al*. (2021)
p-Cymene	*P. aeruginosa* *C. neoformans*	Owen *et al.*, (2019); de Rapper *et al*. (2021)
Estragole	*S. aureus*	Maree *et al.* (2014)
Eucalyptol	*S. aureus*	van Vuuren and Viljoen (2007)
Eugenol	*S. aureus* *K. pneumoniae* *C. albicans*	Maree *et al.* (2014); Orchard *et al.* (2017); de Rapper *et al*. (2021)
Geraniol	*S. aureus* *C. neoformans*	Maree *et al.* (2014); Orchard *et al.* (2017)
Isoeugenol	*C. albicans*	Maree *et al.* (2014)
Linalyl acetate	*C. albicans* *C. neoformans*	Maree *et al.* (2014); Orchard *et al.* (2017)
Menthol	*C. albicans*	Orchard *et al.* (2017)
Ocimene	*S. aureus* *K. pneumoniae* *C. albicans*	Orchard *et al.* (2017); de Rapper *et al*. (2021)
Sabinene hydrate	*C. neoformans*	Maree *et al.* (2014)
γ-Terpinene	*P. aeruginosa* *C. neoformans*	Maree *et al.* (2014); Owen *et al.*, (2019)
α-Terpineol	*C. neoformans*	Maree *et al.* (2014)

1.8 Aims and objectives

The aim of this study was to conduct an *in vitro* antimicrobial analysis into the inhibitory and anti-QS activity of enantiomeric pairs of chiral essential oil compounds, both independently and in combination with a selection of compounds. Furthermore, the toxicological screening was studied.

The objectives that followed were;

- To determine the antimicrobial activity of a selection of enantiomers and compare variability of efficacy using the MIC assay.
- To determine the interactive antimicrobial activity of the enantiomers in combination with the selected compounds by calculating the fractional inhibitory concentration (ΣFIC) and compare the differences between enantiomers.
- To determine the anti-QS activity of the enantiomers and compare the difference, using the MQSIC assay and quantification of the percentage violacein inhibition.
- To determine the interactive antimicrobial activity of the enantiomers in combination with the selected compounds by calculating the fractional quorum sensing inhibitory concentration (ΣFQSIC) and the fractional percentage violacein reduction (ΣFPVR) and compare the differences between enantiomers.
- To screen the toxicity of the enantiomers and compare the difference, between enantiomers using the brine-shrimp lethality assay.
- To determine the interactive toxicity of the enantiomers in combination with the selected compounds by calculating the fractional percentage mortality (ΣFPM) and differences between enantiomers.

Chapter 2 - Antimicrobial inhibitory studies on enantiomers and combinations with the selected compounds

2.1 Introduction

As highlighted in Chapter 1, there is a lack of research investigating and comparing the stereoselective antimicrobial inhibitory activity of chiral essential oil compounds. What was also highlighted in Chapter 1 was the substantial lack of research into the varied efficacy each of the enantiomeric forms of a chiral compound when combined with other essential oil compounds. Thus, the current chapter aimed to examine this variability in terms of antimicrobial activity, through minimum inhibitory concentration (MIC) determination of the enantiomers, and their combined interactive efficacy. To achieve this goal, the enantiomeric pairs were combined with a selection of compounds. The selected compounds were initially investigated independently in order to determine a baseline when comparing the combined antimicrobial inhibitory activity with the enantiomers.

2.2 Materials and methods

2.2.1 Preparation of compounds and controls

A selection of enantiomeric pairs (Table 1.1), and a selection of compounds with which to combine with the enantiomers**,** were included in this investigation (Table 1.3). The enantiomers and selected compounds were obtained from Sigma-Aldrich and stored in a refrigerator (4 °C) and away from light. All compounds had a purity range between 90.00 - 99.00%. The compounds were dissolved in acetone (Merck) to make up to a starting concentration of 32.00 mg/mL and kept in amber bottles at 4 °C until further analysis (Equation 2.1).

$$\text{Volume of acetone to add } (\mu L) = \frac{(\text{Weight of compound (mg) x } 1000)}{(32 \text{ mg/mL})}$$

Equation 2.1

2.2.2 Preparation of cultures

Six American Type Culture Collection (ATCC) strains were prepared for use in this study. Two Gram-positive, two Gram-negative and two yeast strains were selected. The two Gram-positive strains selected were *Staphylococcus aureus* (ATCC 25923) and *Enterococcus faecium* (ATCC 27270). The Gram-negative strains were *Pseudomonas aeruginosa* (ATCC 27853) and *Klebsiella pneumoniae* (ATCC 13887), and the yeast strains were *Candida albicans* (ATCC 10231) and *Cryptococcus neoformans* (ATCC 14116). A waiver for the use of these micro-organisms was obtained from the University of the Witwatersrand Human Research Ethics Committee (reference number W-CP-201028-2, Appendix C)

Cultures were grown in Tryptone Soya broth (TSB) (Oxoid) at 37 °C for 24 hrs for bacterial strains and 37 °C for 48 hrs for the yeast strains, except for *C. neoformans*, which was grown in Sabourauds Dextrose broth (SDB) at 37 °C for 72 hrs. The micro-organisms were kept viable by sub-culturing every two weeks. Bacterial cultures were streaked onto Tryptone Soya agar (TSA) plates, and incubated under the optimal incubation conditions in order to confirm purity of cultures. The yeast, *C. neoformans*, was streaked onto Sabourauds Dextrose agar (SDA).

2.2.3 The minimum inhibitory concentration (MIC) assay

The MIC assay was performed on the enantiomers and selected compounds, as described by Orchard *et al*., (2019). The broth micro-dilution method is a practical approach to obtaining a MIC of a test sample. The MIC is defined as the lowest concentration that inhibits visible growth of a micro-organism after an optimal incubation period. 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 (van Vuuren *et. al*., 2010). Noteworthy inhibitory activity is considered to be an MIC value ≤ 1.00 mg/mL (Orchard and van Vuuren, 2017). The study was performed in duplicate, and where variations between the enantiomers occurred, a third replicate was performed on the consecutive day to confirm the results and a mean value was obtained. Figure 2.1 demonstrates a final plate layout from which the results of the micro-dilution assay were observed, after sufficient time (4 - 6 hrs, depending on pathogen studied) for the INT to develop its colour.

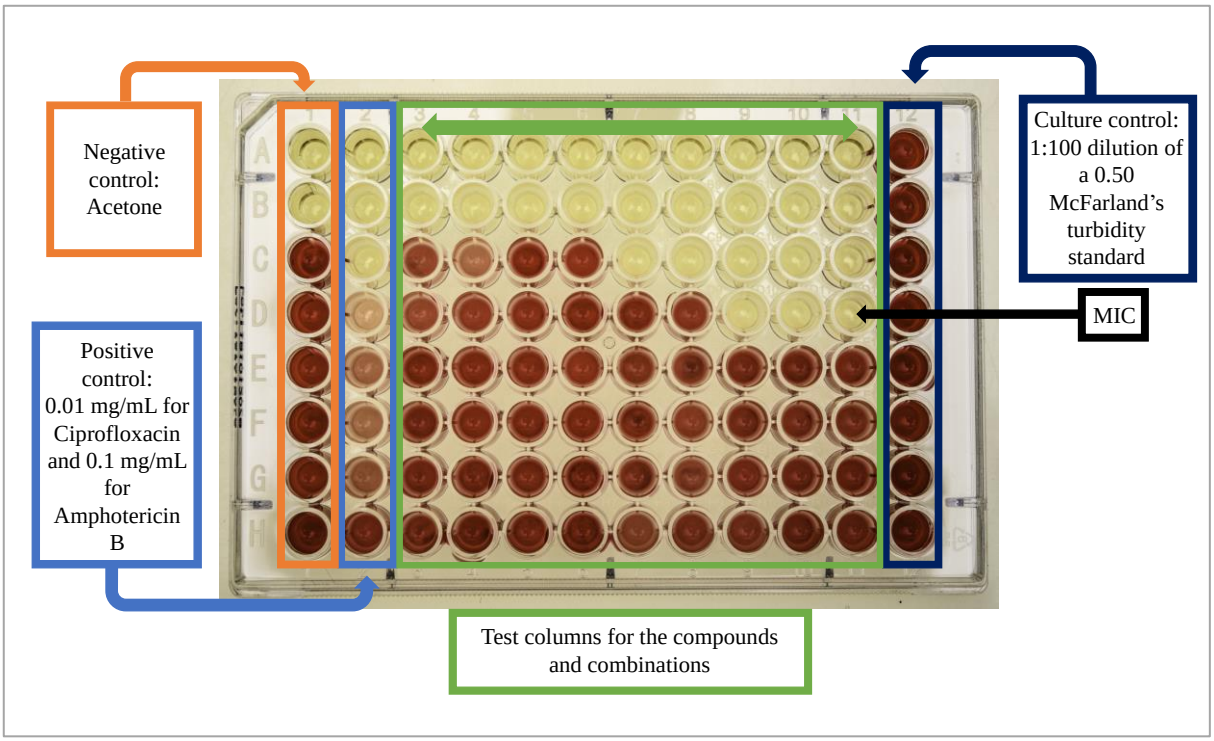


Figure 2.1: Experimental layout and interpretation of a microtiter plate used in the MIC assay.

In order to ensure that it was not the solvent itself that was responsible for the antimicrobial effects noted, acetone was prepared at 32.00 mg/mL and used as the negative control in this study. To ascertain the susceptibility of the cultures, Ciprofloxacin and Amphotericin B (both

obtained from 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) (Riedel-de-Haën) and then made up to 0.1 mg/mL with sterile water.

2.2.4 The interactive efficacy studies

Combination MIC studies were carried out on the enantiomers with the selected compounds as described in Section 2.2.3. However, instead of 100.00 µL of sample, 50.00 µL of the enantiomer and 50.00 µL of the selected compound were combined to investigate a 1:1 ratio. The mean MIC values were obtained, and the fractional inhibitory concentration index (ΣFIC) for the combinations was calculated (Equation 2.2).

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

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

Equation 2.2

The sum of the FIC, known as the FIC index, was calculated using Equation 2.3:

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

Equation 2.3

The ΣFIC was interpreted as either synergistic, additive, non-interactive or antagonistic (van Vuuren and Viljoen, 2011; Ramulondi, 2017). This is described in detail in Table 2.1.

Table 2.1: Interaction classification based on the ΣFIC value

ΣFIC	Interaction classification	Description
≤ 0.50	Synergistic	The effective concentration of the two compounds combined required to inhibit microbial growth was

ΣFIC	Interaction classification	Description
		markedly lower than each of the two compounds when investigated independently.
> 0.50 - 1.00	Additive	There was a decrease in the effective concentration required to inhibit microbial growth, of the two compounds when they interact, as compared to the two compounds when tested independently, however, not to the extent seen with the synergistic combinations.
> 1.0 ≤ 4.0	Non-interactive	The two compounds had no effect in combination with one another. This means that there were no changes to the inhibition, as compared to the two compounds independently.
> 4.00	Antagonistic	The effective concentration required to inhibit bacterial growth of the two compounds combined was markedly higher than each of the two compounds when tested independently, as a result of the unfavorable compound interaction.

Once the interactive efficacy of the enantiomers in combination with the selected compounds was determined, the variations between the enantiomeric pairs in combination with the same selected compound were classified (Table 2.2).

Table 2.2: Classification of comparative interaction observed in the 1:1 combination studies between enantiomeric pairs in combination with the selected compounds

Description of type of interaction between enantiomers and selected compounds in combination	**Referred to as:**
One enantiomeric form in combination with a selected compound demonstrated synergy, while the other enantiomeric form was non-interactive when combined with the same compound.	Synergy versus non-interactive

Description of type of interaction between enantiomers and selected compounds in combination	**Referred to as:**
One enantiomeric form in combination with a selected compound demonstrated synergy, while the other enantiomeric form demonstrated additivity in combination with the same compound.	Synergy versus additive
One enantiomeric form in combination with a selected compound demonstrated synergy, while the other enantiomeric form demonstrated antagonism in combination with the same compound.	Synergy versus antagonism
One enantiomeric form in combination with a selected compound demonstrated additivity, while the other enantiomeric form was non-interactive when combined with the same compound.	Additive versus non-interactive
One enantiomeric form in combination with a selected compound demonstrated additivity, while the other enantiomeric form displayed antagonism when combined with the same compound.	Additive versus antagonism
One enantiomeric form in combination with a selected compound was non-interactive, while the other enantiomeric form displayed antagonism when combined with the same compound.	Non-interactive versus antagonism

2.3 Results and discussion

2.3.1 The antimicrobial efficacy the of enantiomers

The MIC determination of the enantiomers were carried out against the bacterial and yeast pathogens, and the results are given in Table 2.3. For the investigation against the yeasts, the MIC values ranged between 0.50 - 2.00 mg/mL (*C. albicans*) and 0.13 - 1.00 mg/mL (*C. neoformans*). When investigated against the Gram-negative bacteria (*P. aeruginosa* and *K. pneumoniae*) and the Gram-positive bacteria (*S. aureus* and *E. faecium*), the MIC values ranged

between 1.00 - 4.00 mg/mL. Concerning the comparative activity of the enantiomers against Gram-positive versus Gram-negative strains, similar activity was displayed against both classes. The most noteworthy inhibitory activity of the enantiomers was observed against *C. neoformans*. The variation in the MIC values that were observed between the two enantiomers of each chiral compound was not more than one well-dilution difference, and therefore not appreciable. However, a pattern was observed in terms of the enantiomeric form of each chiral compound that was responsible for moderately stronger inhibitory activity, as compared to the other enantiomeric form. (+)-α-Pinene showed moderately stronger inhibitory activity than (–)-α-Pinene against all the pathogens investigated. (–)-Limonene displayed moderately stronger inhibitory activity against the yeast pathogens and *P. aeruginosa*, when compared to (+)-Limonene. This was also observed with (+)-Camphor, when compared to (–)-Camphor. (–)-β-Pinene displayed moderately stronger inhibitory activity against *C. albicans* and *P. aeruginosa*, when compared to (+)-β-Pinene. And finally, (+)-Borneol displayed moderately stronger inhibitory activity against the yeast pathogens, when compared to (–)-Borneol. Citronellal and Menthone displayed equivalent inhibitory activity between their enantiomers, against the pathogens. Upon review of the literature, it was evident that there is a scarcity of investigations in which the enantiomeric forms of essential oil compounds are considered when evaluating their inhibitory activity. A further in-depth analysis of each set of enantiomers are given as follows;

2.3.1.1 Borneol

Guimarães *et al.* (2019) reported a considerable variation in the inhibitory activity between (+)-Borneol and (–)-Borneol against *S. aureus*, with MIC values of 0.25 and 0.03 mg/mL, respectively, whereas the current study found that both enantiomers of Borneol had equivalent MIC values (2.00 mg/mL) against *S. aureus*. The variation observed may be due to the use of different test strains, however, the ATCC strain number of *S. aureus* was not reported in the previous study. This highlights the importance of reporting the strain numbers of the pathogens being investigated, as the susceptibility profiles may differ. The findings of the current investigation are in line with Tabanca *et al*. (2001), which reports that both enantiomers of Borneol displayed similar inhibitory activity against *C. albicans* with MIC values of 0.25 and 0.13 mg/mL for (+)-Borneol and (–)-Borneol, respectively. It was also reported that the enantiomers of Borneol displayed similar inhibitory activity against *S. aureus* and *P. aeruginosa*, which correlated to the findings of the current investigation.

Table 2.3: The mean MIC (mg/mL) for the enantiomers, with standard deviation (in parentheses)

Enantiomer	**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
(+)-Borneol	**0.13 (±0.00)**	**0.50 (±0.00)**	**1.00 (±0.00)**	2.00 (±0.00)	2.00 (±0.00)	2.00 (±0.00)
(–)-Borneol	**0.25 (±0.00)**	**0.75 (±0.35)**	**1.00 (±0.00)**	2.00 (±0.00)	2.00 (±0.00)	2.00 (±0.00)
(+)-Camphor	**0.25 (±0.00)**	**1.00 (±0.00)**	2.00 (±0.00)	2.00 (±0.00)	4.00 (±0.00)	4.00 (±0.00)
(–)-Camphor	**0.38 (±0.18)**	**1.00 (±0.00)**	4.00 (±0.00)	2.00 (±0.00)	2.00 (±0.00)	4.00 (±0.00)
(+)-Citronellal	1.00 (±0.00)	**1.00 (±0.00)**	**1.00 (±0.00)**	2.00 (±0.00)	**1.00 (±0.00)**	2.00 (±0.00)
(–)-Citronellal	1.00 (±0.00)	**1.00 (±0.00)**	**1.00 (±0.00)**	2.00 (±0.00)	**1.00 (±0.00)**	2.00 (±0.00)
(+)-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)
(–)-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)
(+)-Menthone	**0.25 (±0.00)**	2.00 (±0.00)	1.50 (±0.71)	2.00 (±0.00)	4.00 (±0.00)	4.00 (±0.00)
(–)-Menthone	**0.25 (±0.00)**	2.00 (±0.00)	1.50 (±0.71)	2.00 (±0.00)	4.00 (±0.00)	4.00 (±0.00)
(+)-α-Pinene	**0.25 (±0.00)**	**1.00 (±0.00)**	**1.00 (±0.00)**	2.00 (±0.00)	2.00 (±0.00)	2.00 (±0.00)
(–)-α-Pinene	**0.38 (±0.18)**	1.50 (±0.71)	2.00 (±0.00)	4.00 (±0.00)	4.00 (±0.00)	4.00 (±0.00)
(+)-β-Pinene	**0.25 (±0.00)**	2.00 (±0.00)	2.00 (±0.00)	2.00 (±0.00)	2.00 (±0.00)	4.00 (±0.00)
(–)-β-Pinene	**0.25 (±0.00)**	**1.00 (±0.00)**	1.50 (±0.71)	2.00 (±0.00)	2.00 (±0.00)	2.00 (±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; positive control (Ciprofloxacin against bacterial pathogens, Amphotericin B against yeast pathogens) = 6.25×10^{-3} (±0.00) mg/mL (*C. neoformans* and *C. albicans*), 6.25×10^{-4} (±0.00) mg/mL (*P. aeruginosa* and *S. aureus*), 7.8×10^{-5} (±0.00) mg/mL (*K. pneumoniae*), and 1.25×10^{-3} (±0.00) mg/mL (*E. faecium*); negative control (acetone) = > 4.00 (±0.00) mg/mL (*C. albicans*, *P. aeruginosa*, *K. pneumoniae*, *S. aureus* and *E. faecium*), 1.00 (±0.00) mg/mL (*C. neoformans*).

Tabanca *et al*. (2001) reported that the enantiomers of Borneol had equivalent inhibitory activity against *S. aureus* and *P. aeruginosa*, with MIC values of 0.13 mg/mL for (+)-Borneol, and 0.25 mg/mL for (–)-Borneol. Against *C. albicans*, MIC values of 0.25 mg/mL for (+)-Borneol, and 0.13 mg/mL for (–)-Borneol, were reported. While the results were not always congruent, the overall finding was that there was equipotent activity between the enantiomers, which is consistent with the findings of the current investigation. Another investigation, conducted by İşcan (2017), also reported that the inhibitory activity of (+)-Borneol was equivalent to (–)-Borneol, when investigated against *S. aureus*, *P. aeruginosa* and *C. albicans*. This correlates with the findings (Table 2.3) of the current investigation.

2.3.1.2 Camphor

A review conducted by Chen *et al.* (2013) reports on the biological activity of Camphor and Camphor-containing essential oils, and highlights the applications of chiral Camphor molecules. Iraji *et al*. (2020) reported that (–)-Camphor had an MIC value of 7.41 mM (1.13 mg/mL) against *C. albicans*, which is confirmed in the current investigation. The activity of (–)-Camphor was attributed to its ketone structure, allowing for the interaction with the fungal cell wall through its lipophilicity, and penetration into the cell-membrane (Iraji *et al*., 2020).

In terms of the comparative antimicrobial inhibitory activity between the enantiomers of Camphor, Viljoen *et al.* (2003) investigated the effects of (+)-Camphor and (–)-Camphor through time-kill studies and found the effects of the enantiomers to be negligible against *C. albicans* (no total reduction of CFUs seen after 1 hr of incubation). The current study reports, through MIC determination, that the enantiomers of Camphor have noteworthy inhibitory activity against *C. albicans* (MIC value of 1.00 mg/mL) after 48 hrs of incubation. Both the current study and Viljoen *et al.* (2003) are in agreement in terms of the enantiomeric pairs of Camphor displaying equivalent inhibitory activity against *C. albicans*. İşcan (2017) reported that the MIC values of (+)-Camphor was equivalent to (–)-Camphor, when investigated against *S. aureus*, *P. aeruginosa* and *C. albicans*. In addition, the study reported that (+)-Camphor showed moderately stronger inhibitory activity against *C. albicans*, as compared to (–)-Camphor, which correlates with the findings of the current investigation.

2.3.1.3 Citronellal

De Oliveira *et al.* (2017) reported that the MIC value of S-(–)-Citronellal was 0.26 mg/mL against *C. albicans*, whereas the current study reports an MIC value of 1.00 mg/mL. This variation may be due to the use of different ATCC strains. In terms of the comparative antimicrobial inhibitory activity between the enantiomers of Camphor, Ngan *et al.* (2012) reported that both enantiomers of Citronellal had MIC values > 2.50 mg/mL against *S. aureus* and *K. pneumoniae*, while the current study reports values of 1.00 mg/mL (against *S. aureus*) and 2.00 mg/mL (against *K. pneumoniae*). The ATCC strains differed from those used in the current study, which may account for the discrepancy in MIC values. However, the finding that both enantiomeric forms of Citronellal have equivalent inhibitory activity correlates to the findings of the current investigation. İşcan (2017) also reported that against *S. aureus*, *P. aeruginosa* and *C. albicans*, the enantiomers of Citronellal displayed equivalent inhibitory activity to one another. In addition, the MIC values reported in the current study against *S. aureus* and *C. albicans* correspond with those reported by İşcan (2017). Carrillo-Hormaza *et al.* (2015) reported that (–)-S-Citronellal and (+)-R-Citronellal had MIC values of 5.00 and > 5.00 µg/mL, respectively, against *Enterobacter cloacae*. While *E. cloacae* was not investigated in the current study, the overall finding that the variation in the inhibitory activity between the enantiomeric pair is not appreciable, correlates to the current investigation.

2.3.1.4 Limonene

Lis-Balchin *et al.* (1996) reported that (+)-Limonene displayed a broader spectrum of activity than (–)-Limonene when investigated against 25 different Gram-positive and Gram-negative bacteria, 20 strains of *Listeria monocytogenes* and eight fungal species. The current study found that the spectrum of activity of the enantiomers of Limonene, against the six pathogens, were equivalent. However, it is important to note that a broader spectrum of activity does not equate to stronger inhibitory activity. Aggarwal *et al*. (2002) reported that (–)-Limonene had little inhibitory activity against 12 bacterial and seven fungal species, and that in comparison, (+)-Limonene was highly active. However, the pathogens investigated in the current study were not included in the investigation. The findings of the current study are in line with the findings of van Vuuren and Viljoen (2007), who reported that the inhibitory activity of (–)-Limonene (MIC = 4.00 mg/mL) was three times that of (+)-Limonene (MIC = 13.00 mg/mL), against *S. aureus*. The current investigation reports MIC values of 4.00 mg/mL for both enantiomers of

Limonene. However, 4.00 mg/mL was the highest concentration tested in this investigation, which may account for this discrepancy in MIC values. It is important to note that van Vuuren and Viljoen (2007) also investigated the racemate of Limonene, and found that the inhibitory activity differed from that of the pure enantiomers, suggesting a possible interaction between the enantiomeric pairs when combined. This highlights the importance of evaluating chiral essential oil compounds in their enantiopure forms. Iraji *et al*. (2020) investigated the inhibitory activity of the enantiomers of Limonene against clinical isolates of *C. albicans* and reported MIC values of 2.83 mM (0.41 mg/mL) and 5.29 mM (0.72 mg/mL) for (+)-Limonene and (–)-Limonene, respectively. The discrepancy in MIC values may be due to different strains of *C. albicans* investigated. However, the overall finding that the variation in the inhibitory activity between the enantiomers of Limonene is negligible, which corresponds with the current study. The MIC values reported for the enantiomers of Limonene against *C. albicans* are in agreement with İşcan (2017), as well as the overall finding that (–)-Limonene is moderately more active than (+)-Limonene.

2.3.1.5 Menthone

Kapp *et al.* (2020), reported an MIC value of 2.50 mg/mL for (–)-Menthone against *S. aureus*, which is in line with the findings of the current study. In terms of the comparative inhibitory activity of the enantiomers of Menthone, only a few studies could be found. Iraji *et al*. (2020) reported that, against clinical isolates of *C. albicans*, the enantiomers of Menthone had similar MIC values of 14.21 mM (2.19 mg/mL) and 26.33 mM (4.06 mg/mL) for (+)-Menthone and (–)-Menthone, respectively. This correlates to the findings of the current investigation. İşcan (2017) also reported that that the enantiomeric pairs of Menthone displayed equivalent inhibitory activity against *C. albicans, P. aeruginosa* and *S. aureus*, which is in line with the findings of the current study, in terms of the variations between the enantiomers of Menthone not being more than one well-dilution difference, and therefore not appreciable.

2.3.1.6 α-Pinene

A review conducted by Salehi *et al.* (2019) highlights the antimicrobial inhibitory activity of the enantiomers of α-Pinene and β-Pinene. Another review, conducted by Allenspach and Steuer (2021), highlights the enantioselective antimicrobial inhibitory activity of (+)-α-Pinene, as well as the lack of research into the inhibitory activity of (–)-α-Pinene. Nikitina *et al.* (2009)

reported that in comparison to (–)-α-Pinene, (+)-α-Pinene had moderate antifungal activity against *C. albicans* (no quantitative data reported), which is consistent with the findings of the current investigation. Da Silva *et al.* (2012) reported that only (+)-α-Pinene had inhibitory activity against *C. albicans* and *C. neoformans*, with MIC values of 3.13 mg/mL and 0.12 mg/mL, respectively. The MIC values against *C. neoformans* correspond to the findings of the current investigation, whereas an MIC value of 1.00 mg/mL is reported against *C. albicans*. In addition, the previous study reported that (–)-α-Pinene displayed no inhibitory activity up to a concentration of 20.00 mg/mL, whereas the current investigation found that both enantiomers had inhibitory activity against *C. albicans* and *C. neoformans*, with MIC values ranging between 0.25 - 1.50 mg/mL. Eduardo *et al.* (2018), reported that (+)-α-Pinene had an MIC of 2.50 µL/mL (2.50 mg/mL) against *S. aureus*, which correlates to the findings of the current investigation. Van Zyl *et al.* (2006) reported that (+)-α-Pinene did not have inhibitory activity up to concentrations of 234.90 mM (32.00 mg/mL) against the *S. aureus*, and 88.10 mM (12.00 mg/mL) against *C. albicans*. The current investigation reports an MIC value of 2.00 mg/mL for (+)-α-Pinene against *S. aureus*, and 1.00 mg/mL against *C. albicans*. The current study conducted a micro-dilution assay, which is a more accurate measure of activity for essential oil constituents, when compared to the disc diffusion assay, the results of which is often inconsistent (Janssen *et al*., 1987; Kalemba and Kunicka, 2003; Ríos and Recio, 2005; Cos *et al.*, 2006).

In terms of the comparative inhibitory activity of the enantiomers of α-Pinene, Iraji *et al*. (2020) reported a significant ($p < 0.05$) variation against clinical isolates of *C. albicans*, with MIC values of 1.86 mM (0.25 mg/mL) and 49.90 mM (6.80 mg/mL) for (+)-α-Pinene and (–)-α-Pinene, respectively. This has been attributed, by the authors, to the stereoselective inhibition pathway against micro-organisms. However, the current study reports MIC values of 1.00 and 1.50 mg/mL for (+)-α-Pinene and (–)-α-Pinene, respectively. The discrepancy in MIC values may be due to different test strains investigated, however, the overall finding that (+)-α-Pinene is more active than (–)-α-Pinene correlates with the findings of the current investigation. Filipowicz *et al.* (2003) found that similar inhibitory activity was observed against *S. aureus*, between the enantiomers of α-Pinene. Lis-Balchin *et al.* (1999) reported (+)-α-Pinene to be the more active enantiomer, and Dhar *et al.* (2014) reported that the inhibitory activity of (+)-α-Pinene was moderately stronger than that of (–)-α-Pinene, against *S. aureus* and *C. albicans*, which correlates with the findings of the current investigation. Ložienė *et al.* (2018) also evaluated the inhibitory activity of (1R)-(+)-α-Pinene, against *S. aureus* and *C. albicans*. The

study reported that (1R)-(+)-α-Pinene had an MIC value of 0.01% ($1x10^{-4}$ mg/mL) against *S. aureus* and 0.0002 % ($2x10^{-6}$ mg/mL) against *C. albicans*. However, the current investigation reports MIC values of 2.00 and 1.00 mg/mL, against *S. aureus* and *C. albicans*, respectively. The discrepancy in MIC values may be due to different strains investigated. While the inhibitory activity of (–)-α-Pinene was not evaluated and compared, Ložienė *et al.* (2018) did evaluate isolated α-Pinene fractions with different enantiomeric compositions. The fraction in which (–)-α-Pinene was greater than (+)- α-Pinene had MIC values of 0.01% ($1x10^{-4}$ mg/mL) each, against both *S. aureus* and *C. albicans*, whereas the fraction in which (–)-α-Pinene and (+)-α-Pinene were equivalent had MIC values of 0.20% ($2x10^{-3}$ mg/mL) each, against both *S. aureus* and *C. albicans*. This highlights the stereoselective inhibitory activity observed for the enantiomers of α-Pinene and correlates to the overall finding of the current investigation that (+)-α-Pinene is more active than (–)-α-Pinene.

2.3.1.7 β-Pinene

Filipowicz *et al.* (2003) reported MIC values of 1.60 µL/mL (1.60 mg/mL) for (–)-β-Pinene against both *S. aureus* and *C. albicans*, which correlates with the findings of the current investigation. Da Silva *et al*. (2012) reported that (+)-β-Pinene had inhibitory activity against *C. albicans* and *C. neoformans*, with MIC values of 0.19 mg/mL and 0.23 mg/mL, respectively, whereas (–)-β-Pinene was not antimicrobially active. The MIC values against *C. neoformans* correspond to the findings of the current investigation, while an MIC value of 2.00 mg/mL is reported against *C. albicans*. In addition, the study reported that (–)-β-Pinene displayed no inhibitory activity up to a concentration of 20.00 mg/mL, whereas the current investigation found that both enantiomers had inhibitory activity against *C. albicans* and *C. neoformans*, with MIC values ranging between 0.25 - 2.00 mg/mL. Van Zyl *et al.* (2006) also reported that (+)-β-Pinene showed inhibitory activity at 22.00 mM (3.00 mg/mL) against *S. aureus* and 7.30 mM (1.00 mg/mL) against *C. albicans*, which is in line with the findings of the current investigation.

2.3.2 The antimicrobial efficacy of the selected compounds

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 on their own as a baseline (Table 2.4). In the investigation

against the yeasts, the MIC values ranged between 0.50 - 2.00 mg/mL (*C. albicans*) and 0.25 - 0.50 mg/mL (*C. neoformans*). Against the Gram-negative bacteria, MIC values ranged between 1.00 - 2.00 mg/mL (*P. aeruginosa*) and 1.00 - 4.00 mg/mL (*K. pneumoniae*). Against the Gram-positive bacteria, MIC values of the selected compounds ranged between 0.50 - 4.00 mg/mL (*S. aureus*) and 1.00 - 4.00 mg/mL (*E. faecium*). 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 - 1.00 mg/mL. Menthol and α-Terpineol displayed noteworthy inhibitory activity against the fungal and Gram-negative pathogens, with MIC values ranging between 0.50 - 1.00 mg/mL.

As was observed with the enantiomers investigated independently, the yeast pathogens were most susceptible, particularly *C. neoformans*, followed by the Gram-negative bacterial strains. Reviews have highlighted the antimicrobial efficacy of the majority of the selected compounds. Studies include that for Camphene (Hachlafi *et al*., 2021), β-Caryophyllene (Francomano *et al*., 2019), Eucalyptol (Campos and Berteina-Raboin, 2022; Martínez-Pabón and Ortega-Cuadros, 2020), Menthol (Kamatou *et al*., 2013), *p*-Cymene (Marchese *et al*., 2017a; Balahbib *et al*., 2021) and α-Terpineol (Sales, 2020). A review conducted by Ahmad *et al.* (2021) highlighted the broad-spectrum antimicrobial activity of Eugenol and Isoeugenol, confirming what was observed in the current investigation. Reviews have highlighted the antimicrobial properties of Eugenol (Kamatou *et al*., 2012; Marchese *et al.*, 2017b; Mak *et al.*, 2018; Eleleemy *et al.*, 2020; Nisar *et al*., 2021; Ulanowska and Olas, 2021) Konuk and Ergüden (2020) attributed the broad-spectrum activity of these volatile phenolics to the different positioning of the hydroxyl group attached to the benzene ring. Their characteristic free hydroxyl groups which cause proton exchange with the microbial cell-membrane, disturbing the membrane gradient leading to cell rupture. Hyldgaard *et al.* (2015) proposed that Isoeugenol interacts with membranes in a reversible and non-disruptive detergent-like manner, which causes membrane destabilization, while also increasing membrane fluidity. The results of the current study, with regards to the antimicrobial inhibitory activity of Eugenol (Carrasco *et al*., 2012; Khan *et al*., 2013; Martins *et al*., 2016; Dąbrowska *et al*., 2021) and Isoeugenol (Pinheiro *et al*., 2017; Rosa *et al*., 2019; Medeiros *et al*., 2020) are in agreement with several previous studies. Reviews have highlighted the biological properties of Geraniol, including the antimicrobial properties (Chen and Viljoen, 2010; Lei *et al.*, 2018; Hadian *et al*., 2020; Maczka *et al*., 2020). Another review conducted by de Lira *et al*. (2020) highlighted the broad-spectrum antimicrobial inhibitory activity of Geraniol, as was observed in the current study.

Table 2.4: The mean MIC (mg/mL) for the selected compounds with standard deviation (SD) (in parentheses)

Selected compound	**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
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)

n = 2 replicates**; 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; positive control (Ciprofloxacin against bacterial pathogens, Amphotericin B against yeast pathogens) = 6.25 x 10^{-3} (±0.00) mg/mL (*C. neoformans* and *C. albicans*), 6.25 x 10^{-4} (±0.00) mg/mL (*P. aeruginosa* and *S. aureus*), 7.8 x 10^{-5} (±0.00) mg/mL (*K. pneumoniae*), 1.25 x 10^{-3} (±0.00) mg/mL (*E. faecium*); negative control (acetone) = > 4.00 (±0.00) mg/mL (*C. albicans, P. aeruginosa, K. pneumoniae, S. aureus* and *E. faecium*), 1.00 (±0.00) mg/mL (*C. neoformans*).

The findings of the current study, in terms of the antimicrobial inhibitory activity of Geraniol, are also in line with previous studies (Miron *et al.*, 2014; Leite *et al.*, 2015; Sharma *et al*., 2016; Singulani *et al.*, 2018).

2.3.3 The interactive efficacy of the equal ratio (1:1) combinations

2.3.3.1 The interactive efficacy of the combinations against *C. neoformans*

The results of the MIC and ΣFIC evaluation of the combinations, against *C. neoformans*, are given in Table 2.5. The MIC values of the combinations ranged between 0.13 - 1.00 mg/mL. *C. neoformans* was highly susceptible, with a total of 92.35% of the combinations having MIC values < 1.00 mg/mL. This was observed with all the enantiomers in combination, with the exception of combinations with Citronellal and (–)-Limonene, which demonstrated MIC values ≥ 1.00 mg/mL. Variations in MIC values between enantiomeric pairs in combination (greater than one well-dilution difference), was observed in 4.08% of the combinations. The greatest variation between enantiomeric pairs in combination was observed with the enantiomers of Limonene in combination with β-Caryophyllene, where (+)-Limonene in combination had an MIC value of 1.00 mg/mL, whereas (–)-Limonene in combination had an MIC value of 0.13 mg/mL.

A total of 12.76% of the combinations were synergistic, 41.84% were additive, while the rest (45.41%) were non-interactive. No antagonism was observed. (+)-Citronellal, (–)-Menthone and both enantiomers of; Borneol, Limonene, α-Pinene and β-Pinene, were involved in the synergy observed against *C. neoformans*, with ΣFIC values ranging between 0.38 - 0.50. The most prevalent type of variation observed between the enantiomeric pairs was ‘additive versus non-interactive’. In addition, the variation ‘synergy versus non-interactive’, which is the greatest variation in interactive efficacy that can be observed, was frequently observed against *C. neoformans*. This was evident with the enantiomers of Limonene, Menthone, α-Pinene and β-Pinene, and particularly evident with the enantiomers of Borneol. In combination with either Estragole, Geraniol, Menthol or β-Caryophyllene, (–)-Borneol interacted synergistically with ΣFIC values ranging between 0.38 - 0.50. However, (+)-Borneol was non-interactive, with ΣFIC values ranging between 1.46 - 2.42. In fact, (+)-Borneol was non-interactive in combination with all of the selected compounds, with the exception of Isoeugenol (additive, ΣFIC = 0.73). In contrast, (–)-Borneol interacted either synergistically or additively in combination with 11 of the 14 selected compounds.

Table 2.5: The mean MIC (mg/mL) with standard deviation and ΣFIC with interaction classification (in parentheses) for the 1:1 combinations, against *C. neoformans*

Selected compound	**Enantiomers**													
	(+)-Borneol	(–)-Borneol	(+)-Camphor	(–)-Camphor	(+)-Citronellal	(–)-Citronellal	(+)-Limonene	(–)-Limonene	(+)-Menthone	(–)-Menthone	(+)-α-Pinene	(–)-α-Pinene	(+)-β-Pinene	(–)-β-Pinene
Camphene	**0.25** ±0.00 **(1.21; Ind)**	**0.25** ±0.00 **(0.75; Add)**	**0.50** ±0.00 **(1.50; Ind)**	**0.25** ±0.00 **(0.58; Add)**	1.00 ±0.00 (1.50; Ind)	1.00 ±0.00 (1.50; Ind)	**0.25** ±0.00 **(0.50; Syn)**	**0.25** ±0.00 **(0.75; Add)**	**0.25** ±0.00 **(0.75; Add)**	**0.50** ±0.00 **(1.50; Ind)**	**0.25** ±0.00 (0.75; Add)	**0.25** ±0.00 (0.58; Add)	**0.25** ±0.00 (0.75; Add)	**0.25** ±0.00 (0.75; Add)
β-Caryophyllene	**0.25** ±0.00 **(1.46; Ind)**	**0.13** ±0.00 **(0.50; Syn)**	**0.25** ±0.00 **(1.00; Add)**	**0.50** ±0.00 **(1.66; Ind)**	1.00 ±0.00 (2.50; Ind)	**0.50** ±0.00 (1.25; Ind)	*1.00 ±0.00* **(3.00; Ind)**	***0.13*** *±0.00* **(0.50; Syn)**	**0.50** ±0.00 **(2.00; Ind)**	0.25 ±0.00 **(1.00; Add)**	**0.25** ±0.00 **(1.00; Add)**	**0.50** ±0.00 **(1.66; Ind)**	**0.25** ±0.00 (1.00; Add)	**0.25** ±0.00 (1.00; Add)
p-Cymene	**0.25** ±0.00 (1.21; Ind)	**0.50** ±0.00 (1.50; Ind)	**0.50** ±0.00 (1.50; Ind)	**0.50** ±0.00 (1.16; Ind)	1.00 ±0.00 (1.50; Ind)	1.00 ±0.00 (1.50; Ind)	***0.50*** *±0.00* **(1.00; Add)**	***0.13*** *±0.00* **(0.38; Syn)**	**0.25** ±0.00 (0.75; Add)	**0.25** ±0.00 (0.75; Add)	**0.25** ±0.00 **(0.75; Add)**	**0.50** ±0.00 **(1.16; Ind)**	**0.25** ±0.00 **(0.75; Add)**	**0.13** ±0.00 **(0.38; Syn)**
Estragole	***0.50*** *±0.00* **(2.42; Ind)**	***0.13*** *±0.00* **(0.38; Syn)**	**0.50** ±0.00 (1.50; Ind)	**0.50** ±0.00 (1.16; Ind)	1.00 ±0.00 (1.50; Ind)	1.00 ±0.00 (1.50; Ind)	**0.19** ±0.09 **(0.38; Syn)**	**0.25** ±0.00 **(0.75; Add)**	**0.25** ±0.00 (0.75; Add)	**0.25** ±0.00 (0.75; Add)	**0.50** ±0.00 **(1.50; Ind)**	**0.25** ±0.00 **(0.58; Add)**	**0.13** ±0.00 (0.38; Syn)	**0.13** ±0.00 (0.38; Syn)
Eucalyptol	**0.50** ±0.00 (2.42; Ind)	**0.50** ±0.00 (1.50; Ind)	**0.25** ±0.00 **(0.75; Add)**	**0.50** ±0.00 **(1.16; Ind)**	1.00 ±0.00 (1.50; Ind)	1.00 ±0.00 (1.50; Ind)	**0.75** ±0.35 **(1.50; Ind)**	**0.25** ±0.00 **(0.75; Add)**	***0.50*** *±0.00* **(1.50; Ind)**	***0.13*** *±0.00* **(0.38; Syn)**	**0.25** ±0.00 **(0.75; Add)**	**0.50** ±0.00 **(1.16; Ind)**	**0.25** ±0.00 (0.75; Add)	**0.25** ±0.00 (0.75; Add)
Eugenol	**0.25** ±0.00 **(1.46; Ind)**	**0.25** ±0.00 **(1.00; Add)**	**0.25** ±0.00 (1.00; Add)	**0.25** ±0.00 (0.83; Add)	**0.50** ±0.00 (1.25; Ind)	**0.50** ±0.00 (1.25; Ind)	**0.50** ±0.00 **(1.50; Ind)**	**0.25** ±0.00 **(1.00; Add)**	**0.50** ±0.00 **(2.00; Ind)**	**0.25** ±0.00 **(1.00; Add)**	**0.13** ±0.00 (0.50; Syn)	**0.13** ±0.00 (0.41; Syn)	**0.13** ±0.00 (0.50; Syn)	**0.13** ±0.00 (0.50; Syn)
Geraniol	**0.25** ±0.00 **(1.46; Ind)**	**0.13** ±0.00 **(0.50; Syn)**	**0.25** ±0.00 (1.00; Add)	**0.25** ±0.00 (0.83; Add)	**0.25** ±0.00 **(0.63; Add)**	**0.50** ±0.00 **(1.25; Ind)**	**0.25** ±0.00 (0.75; Add)	**0.25** ±0.00 (1.00; Add)	**0.50** ±0.00 **(2.00; Ind)**	**0.25** ±0.00 **(1.00; Add)**	**0.25** ±0.00 (1.00; Add)	**0.25** ±0.00 (0.83; Add)	**0.25** ±0.00 (1.00; Add)	**0.25** ±0.00 (1.00; Add)
Isoeugenol	**0.13** ±0.00 **(0.73; Add)**	**0.13** ±0.00 **(0.50; Syn)**	**0.25** ±0.00 (1.00; Add)	**0.25** ±0.00 (0.83; Add)	**0.50** ±0.00 (1.25; Ind)	**0.50** ±0.00 (1.25; Ind)	**0.25** ±0.00 (0.75; Add)	**0.25** ±0.00 (1.00; Add)	**0.50** ±0.00 **(2.00; Ind)**	**0.25** ±0.00 **(1.00; Add)**	**0.13** ±0.00 (0.50; Syn)	**0.13** ±0.00 (0.41; Syn)	***0.50*** *±0.00* **(2.00; Ind)**	***0.13*** *±0.00* **(0.50; Syn)**
Linalyl acetate	***0.50*** *±0.00* **(2.92; Ind)**	***0.19*** *±0.09* **(0.75; Add)**	**0.50** ±0.00 (2.00; Ind)	**0.50** ±0.00 (1.66; Ind)	1.00 ±0.00 (2.50; Ind)	1.00 ±0.00 (2.50; Ind)	*1.00 ±0.00* **(3.00; Ind)**	***0.25*** *±0.00* **(1.00; Add)**	**0.50** ±0.00 **(2.00; Ind)**	**0.25** ±0.00 **(1.00; Add)**	**0.50** ±0.00 (2.00; Ind)	**0.50** ±0.00 (1.66; Ind)	**0.25** ±0.00 **(1.00; Add)**	**0.50** ±0.00 **(2.00; Ind)**
Menthol	**0.25** ±0.00 **(1.21; Ind)**	**0.13** ±0.00 **(0.38; Syn)**	**0.50** ±0.00 (1.50; Ind)	**0.50** ±0.00 (1.16; Ind)	**0.50** ±0.00 (0.75; Add)	**0.50** ±0.00 (0.75; Add)	**0.50** ±0.00 **(1.00; Add)**	**0.50** ±0.00 **(1.50; Ind)**	**0.25** ±0.00 **(0.75; Add)**	**0.50** ±0.00 **(1.50; Ind)**	**0.25** ±0.00 (0.75; Add)	**0.25** ±0.00 (0.58; Add)	**0.25** ±0.00 **(0.75; Add)**	**0.50** ±0.00 **(1.50; Ind)**
Ocimene	**0.25** ±0.00 **(1.46; Ind)**	**0.25** ±0.00 **(1.00; Add)**	**0.50** ±0.00 **(2.00; Ind)**	**0.25** ±0.00 **(0.83; Add)**	**0.50** ±0.00 (1.25; Ind)	1.00 ±0.00 (2.50; Ind)	**0.25** ±0.00 **(0.75; Add)**	**0.13** ±0.00 **(0.50; Syn)**	**0.25** ±0.00 **(1.00; Add)**	**0.50** ±0.00 **(2.00; Ind)**	***0.13*** *±0.00* **(0.50; Syn)**	***0.50*** *±0.00* **(1.66; Ind)**	**0.25** ±0.00 (1.00; Add)	**0.25** ±0.00 (1.00; Add)
Sabinene hydrate	**0.25** ±0.00 (1.46; Ind)	**0.50** ±0.00 (2.00; Ind)	**0.25** ±0.00 (1.00; Add)	**0.19** ±0.09 (0.62; Add)	**0.75** ±0.35 (1.88; Ind)	**0.50** ±0.00 (1.25; Ind)	**0.19** ±0.09 (0.56; Add)	**0.25** ±0.00 (1.00; Add)	**0.50** ±0.00 **(2.00; Ind)**	**0.25** ±0.00 **(1.00; Add)**	**0.38** ±0.18 **(1.52; Ind)**	**0.25** ±0.00 **(0.83; Add)**	**0.25** ±0.00 (1.00; Add)	**0.25** ±0.00 (1.00; Add)
γ-Terpinene	**0.38** ±0.18 **(2.19; Ind)**	**0.25** ±0.00 **(1.00; Add)**	**0.50** ±0.00 (2.00; Ind)	**0.50** ±0.00 (1.66; Ind)	**0.75** ±0.35 (1.88; Ind)	1.00 ±0.00 (2.50; Ind)	**0.50** ±0.00 **(1.50; Ind)**	**0.25** ±0.00 **(1.00; Add)**	**0.50** ±0.00 (2.00; Ind)	**0.50** ±0.00 (2.00; Ind)	***0.13*** *±0.00* **(0.50; Syn)**	***0.50*** *±0.00* **(1.66; Ind)**	**0.50** ±0.00 **(2.00; Ind)**	**0.25** ±0.00 **(1.00; Add)**
α-Terpineol	**0.25** ±0.00 **(1.21; Ind)**	**0.25** ±0.00 **(0.75; Add)**	**0.50** ±0.00 (1.50; Ind)	**0.50** ±0.00 (1.16; Ind)	**0.25** ±0.00 **(0.38; Syn)**	**0.50** ±0.00 **(0.75; Add)**	**0.38** ±0.18 (0.75; Add)	**0.25** ±0.00 (0.75; Add)	**0.50** ±0.00 (1.50; Ind)	**0.50** ±0.00 (1.50; Ind)	**0.25** ±0.00 (0.75; Add)	**0.25** ±0.00 (0.58; Add)	***0.13*** *±0.00* **(0.38; Syn)**	***0.50*** *±0.00* **(1.50; Ind)**

n = 2 replicates, with third consecutive replicate to confirm variations between enantiomers**; bold** = noteworthy activity < 1.00 mg/mL; *italics* = variations in MIC values between enantiomers in combination, that is more than one well-dilution difference; **red bold** = variations in interactive efficacy between enantiomers in combination; Syn = synergy, Add = additive, Ind = non-interactive; positive control (Amphotericin B) = 6.25 x 10^{-3} (±0.00) mg/mL; negative control (acetone) = 1.00 (±0.00) mg/mL.

This indicates the possible stereoselectivity of (–)-Borneol in combination, against *C. neoformans*. Overall, the combination of the enantiomers of Limonene with β-Caryophyllene had the greatest variation observed in terms of the interactive efficacy and hence elaborated on graphically (Figure 2.2). (+)-Limonene in combination with β-Caryophyllene was non-interactive (ΣFIC = 3.00), and (–)-Limonene in combination with β-Caryophyllene interacted synergistically (ΣFIC = 0.50).

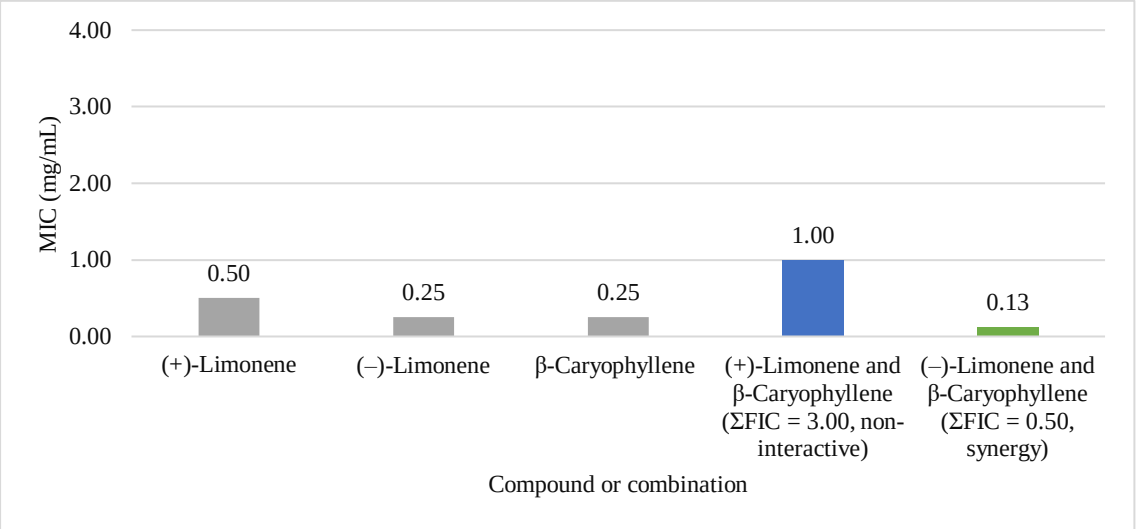


Figure 2.2: The mean MIC of the enantiomers of Limonene and of β-Caryophyllene, independently and in combination, against *C. neoformans.*

Combination studies of essential oil compounds, particularly enantiomers, in combination against *C. neoformans* were limited. Van Vuuren and Viljoen (2007) evaluated the combined inhibitory activity of the enantiomers of Limonene with 1,8-Cineole (Eucalyptol) against *C. neoformans* and reported additivity for (–)-Limonene in combination with 1,8-Cineole (Eucalyptol), which correlates with the findings of the current investigation (ΣFIC = 0.75). Additivity was also reported for (+)-Limonene in combination with 1,8-Cineole (Eucalyptol), whereas the current investigation reports it to be non-interactive with a close-to-additive ΣFIC value of 1.50. Da Silva *et al*. (2012) reported that both (+)-α-Pinene and (+)-β-Pinene were synergistic in combination with Amphotericin B, against *C. neoformans*. Although the enantiomers were not combined with antimicrobials in the current investigation, the favourable combined activity of both (+)-α-Pinene and (+)-β-Pinene were observed against *C. neoformans*, as 78.57% of the combinations involving (+)-α-Pinene, and 85.71% of the combinations involving (+)-β-Pinene, were either additive or synergistic. The type of variations observed

between the enantiomers of α-Pinene in combination, were 'additive versus non-interactive' or 'synergy versus non-interactive', in which (+)-α-Pinene most often interacted either additively or synergistically, when compared to (–)-α-Pinene. Scalas *et al.* (2018) also reported that (+)-α-Pinene interacted additively with Itraconazole against *C. neoformans*, however, the effect of (–)-α-Pinene in combination was not evaluated. In addition, de Rapper *et al.* (2021) identified α-Pinene (racemate) as an active antimicrobial compound against *C. neoformans*, through chemometric analysis. Borneol has previously been described as being a permeation enhancing adjuvant (Kulkarni *et al.*, 2021), which was evident in the current investigation with (–)-Borneol, as the enantiomer often interacted synergistically or additively in combination against *C. neoformans*. In contrast, (+)-Borneol was non-interactive in combination. This further highlights the importance of considering the enantiomeric configuration of essential oil compounds.

2.3.3.2 The interactive efficacy of the combinations against *C. albicans*

The results of the MIC and ΣFIC evaluation of the combinations, against *C. albicans*, are given in Table 2.6. The MIC values ranged between 0.50 - 2.00 mg/mL. *C. albicans* was highly susceptible to the combinations of the enantiomers with the selected compounds, with 83.84% of the combinations having MIC values ≤ 1.00 mg/mL. This was particularly evident with the enantiomers of Borneol, Camphor and β-Pinene in combination. The MIC values of the enantiomers in combination were mostly equivalent. In terms of the variations in the combined MIC values, it was observed that (–)-Limonene often displayed moderately stronger inhibitory activity, when compared to (+)-Limonene, however, the variation in MIC values is not appreciable. In terms of the interactive efficacy of the combinations, 9.69% resulted in synergy, 65.31% were additive, while the rest (25.00%) of the combinations were non-interactive. No antagonism was observed. The enantiomers of Menthone and (+)-β-Pinene were involved in most of the synergistic interactions observed against *C. albicans*, with ΣFIC ranging between 0.38 - 0.50. It is interesting to note that many of the enantiomers in combination with Menthol, interacted additively against *C. albicans* with ΣFIC ranging between 0.58 - 1.00. In addition, the two enantiomers of α-Pinene and (+)-β-Pinene interacted synergistically with Menthol, with ΣFIC ranging between 0.38 - 0.50. The most prevalent type of variation in interactive activity that was observed between the chiral enantiomers in combination was 'additive versus non-interactive'. This was particularly evident with the enantiomer of Borneol in combination,

Table 2.6: The mean MIC (mg/mL) with standard deviation and ΣFIC with interaction classification (in parentheses) for the 1:1 combinations, against *C. albicans*

Selected compound	Enantiomers													
	(+)-Borneol	(–)-Borneol	(+)-Camphor	(–)-Camphor	(+)-Citronellal	(–)-Citronellal	(+)-Limonene	(–)-Limonene	(+)-Menthone	(–)-Menthone	(+)-α-Pinene	(–)-α-Pinene	(+)-β-Pinene	(–)-β-Pinene
Camphene	**0.50** ± 0.00 (0.63; Add)	**0.75** ± 0.35 (0.69; Add)	**1.00** ± 0.00 **(0.75; Add)**	**0.50** ± 0.00 **(0.38; Syn)**	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.75; Add)	2.00 ± 0.00 (1.00; Add)	**1.00** ± 0.00 (0.75; Add)	2.00 ± 0.00 **(1.00; Add)**	**1.00** ± 0.00 **(0.50; Syn)**	1.50 ± 0.71 **(1.13; Ind)**	**1.00** ± 0.00 **(0.58; Add)**	**1.00** ± 0.00 **(0.50; Syn)**	**1.00** ± 0.00 **(0.75; Add)**
β-Caryophyllene	**1.00** ± 0.00 **(1.25; Ind)**	**1.00** ± 0.00 **(0.92; Add)**	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.75; Add)	2.00 ± 0.00 **(1.50; Ind)**	**1.00** ± 0.00 **(0.75; Add)**	2.00 ± 0.00 (1.00; Add)	**1.00** ± 0.00 (0.75; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.50; Ind)	2.00 ± 0.00 (1.17; Ind)	**1.00** ± 0.00 **(0.50; Syn)**	2.00 ± 0.00 **(1.50; Ind)**
p-Cymene	**1.00** ± 0.00 (1.50; Ind)	**1.00** ± 0.00 (1.17; Ind)	**1.00** ± 0.00 (1.00; Add)	**1.00** ± 0.00 (1.00; Add)	**1.00** ± 0.00 (1.00; Add)	**1.00** ± 0.00 (1.00; Add)	2.00 ± 0.00 **(1.50; Ind)**	**1.00** ± 0.00 **(1.00; Add)**	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.75; Add)	2.00 ± 0.00 **(2.00; Ind)**	**1.00** ± 0.00 **(0.83; Add)**	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (1.00; Add)
Estragole	**1.00** ± 0.00 **(1.25; Ind)**	**1.00** ± 0.00 **(0.92; Add)**	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.75; Add)	2.00 ± 0.00 (1.00; Add)	**1.00** ± 0.00 (0.75; Add)	2.00 ± 0.00 **(1.00; Add)**	**1.00** ± 0.00 **(0.50; Syn)**	2.00 ± 0.00 **(1.50; Ind)**	**1.00** ± 0.00 **(0.58; Add)**	**1.00** ± 0.00 **(0.50; Syn)**	**1.00** ± 0.00 **(0.75; Add)**
Eucalyptol	**1.00** ± 0.00 **(1.25; Ind)**	**1.00** ± 0.00 **(0.92; Add)**	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.75; Add)	2.00 ± 0.00 (1.00; Add)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 **(0.50; Syn)**	2.00 ± 0.00 **(1.00; Add)**	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.58; Add)	**1.00** ± 0.00 **(0.50; Syn)**	**1.00** ± 0.00 **(0.75; Add)**
Eugenol	**0.50** ± 0.00 (1.00; Add)	**0.50** ± 0.00 (0.83; Add)	**0.50** ± 0.00 **(0.75; Add)**	**1.00** ± 0.00 **(1.50; Ind)**	**1.00** ± 0.00 (1.50; Ind)	**1.00** ± 0.00 (1.50; Ind)	**1.00** ± 0.00 (1.25; Ind)	**1.00** ± 0.00 (1.50; Ind)	**1.00** ± 0.00 (1.25; Ind)	**1.00** ± 0.00 (1.25; Ind)	**0.50** ± 0.00 (0.75; Add)	**0.50** ± 0.00 (0.67; Add)	**0.50** ± 0.00 (0.63; Add)	**0.50** ± 0.00 (0.75; Add)
Geraniol	**0.50** ± 0.00 (1.00; Add)	**0.50** ± 0.00 (0.83; Add)	**0.50** ± 0.00 **(0.75; Add)**	**1.00** ± 0.00 **(1.50; Ind)**	**1.00** ± 0.00 (1.50; Ind)	**1.00** ± 0.00 (1.50; Ind)	**0.50** ± 0.00 (0.63; Add)	**0.50** ± 0.00 (0.75; Add)	**0.50** ± 0.00 **(0.63; Add)**	**1.00** ± 0.00 **(1.25; Ind)**	**0.50** ± 0.00 (0.75; Add)	**0.50** ± 0.00 (0.67; Add)	**0.50** ± 0.00 (0.63; Add)	**0.50** ± 0.00 (0.75; Add)
Isoeugenol	**0.50** ± 0.00 (1.00; Add)	**0.50** ± 0.00 (0.83; Add)	**0.50** ± 0.00 (0.75; Add)	**0.50** ± 0.00 (0.75; Add)	**1.00** ± 0.00 **(1.50; Ind)**	**0.50** ± 0.00 **(0.75; Add)**	**0.50** ± 0.00 (0.63; Add)	**0.50** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (1.25; Ind)	**1.00** ± 0.00 (1.25; Ind)	**0.50** ± 0.00 (0.75; Add)	**0.50** ± 0.00 (0.67; Add)	**0.50** ± 0.00 (0.63; Add)	**0.50** ± 0.00 (0.75; Add)
Linalyl acetate	**1.00** ± 0.00 **(1.25; Ind)**	**1.00** ± 0.00 **(0.92; Add)**	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.75; Add)	2.00 ± 0.00 **(1.50; Ind)**	**1.00** ± 0.00 **(0.75; Add)**	2.00 ± 0.00 **(1.50; Ind)**	**1.00** ± 0.00 **(0.75; Add)**	**1.00** ± 0.00 **(0.50; Syn)**	2.00 ± 0.00 **(1.00; Add)**	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.58; Add)	**1.00** ± 0.00 **(0.50; Syn)**	**1.00** ± 0.00 **(0.75; Add)**
Menthol	**0.50** ± 0.00 (0.75; Add)	**0.50** ± 0.00 (0.58; Add)	**1.00** ± 0.00 (1.00; Add)	**1.00** ± 0.00 (1.00; Add)	**1.00** ± 0.00 (1.00; Add)	**1.00** ± 0.00 (1.00; Add)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (1.00; Add)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.75; Add)	**0.50** ± 0.00 (0.50; Syn)	**0.50** ± 0.00 (0.42; Syn)	**0.50** ± 0.00 **(0.38; Syn)**	**1.00** ± 0.00 **(1.00; Add)**
Ocimene	**1.00** ± 0.00 **(1.25; Ind)**	**1.00** ± 0.00 **(0.92; Add)**	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.75; Add)	2.00 ± 0.00 **(1.50; Ind)**	**1.00** ± 0.00 **(0.75; Add)**	2.00 ± 0.00 (1.00; Add)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.50; Syn)	**1.00** ± 0.00 (0.50; Syn)	2.00 ± 0.00 **(1.50; Ind)**	**1.00** ± 0.00 **(0.58; Add)**	**1.00** ± 0.00 **(0.50; Syn)**	**1.00** ± 0.00 **(0.75; Add)**
Sabinene hydrate	**1.00** ± 0.00 **(1.50; Ind)**	**0.50** ± 0.00 **(0.58; Add)**	**1.00** ± 0.00 (1.00; Add)	**1.00** ± 0.00 (1.00; Add)	**1.00** ± 0.00 (1.00; Add)	**1.00** ± 0.00 (1.00; Add)	2.00 ± 0.00 **(1.50; Ind)**	**1.00** ± 0.00 **(1.00; Add)**	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (1.00; Add)	**1.00** ± 0.00 (0.83; Add)	**0.50** ± 0.00 **(0.38; Syn)**	**0.75** ± 0.35 **(0.75; Add)**
γ-Terpinene	**1.00** ± 0.00 **(1.25; Ind)**	**0.75** ± 0.35 **(0.69; Add)**	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.75; Add)	2.00 ± 0.00 **(1.50; Ind)**	**1.00** ± 0.00 **(0.75; Add)**	2.00 ± 0.00 (1.00; Add)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.50; Syn)	**1.00** ± 0.00 (0.50; Syn)	2.00 ± 0.00 (1.50; Ind)	2.00 ± 0.00 (1.17; Ind)	2.00 ± 0.00 **(1.00; Add)**	2.00 ± 0.00 **(1.50; Ind)**
α-Terpineol	**0.50** ± 0.00 (1.00; Add)	**0.50** ± 0.00 (0.83; Add)	**0.50** ± 0.00 (0.75; Add)	**0.50** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (1.50; Ind)	**1.00** ± 0.00 (1.50; Ind)	**0.50** ± 0.00 **(0.75; Add)**	**1.00** ± 0.00 **(1.50; Ind)**	**1.00** ± 0.00 (1.25; Ind)	**1.00** ± 0.00 (1.25; Ind)	**1.00** ± 0.00 (1.50; Ind)	**1.00** ± 0.00 (1.33; Ind)	**1.00** ± 0.00 (1.25; Ind)	**1.00** ± 0.00 (1.50; Ind)

n = 2 replicates, with third consecutive replicate to confirm variations between enantiomers; **bold** = noteworthy activity ≤ 1.00 mg/mL; **red bold** = variations in interactive efficacy between enantiomers in combination; Syn = synergy, Add = additive, Ind = non-interactive; positive control (Amphotericin B) = 6.25 x 10^{-3} (±0.00) mg/mL; negative control (acetone) = > 4.00 (±0.00) mg/mL.

where (–)-Borneol displayed additivity in combination with either β-Caryophyllene, Linalyl acetate, Eucalyptol, γ-Terpinene, Ocimene, Estragole or Sabinene hydrate; with ΣFIC values ranging between 0.58 - 0.92. However, (+)-Borneol was non-interactive in combination with the same selected compounds, having ΣFIC values ranging between 1.25 - 1.50. Similarly, (–)-Citronellal displayed additivity in combination with either β-Caryophyllene, Linalyl acetate, Isoeugenol, γ-Terpinene or Ocimene; with ΣFIC values of 0.75. However, (+)-Citronellal was non-interactive in combination with those selected compounds, with ΣFIC values of 1.50. (–)-α-Pinene in combination with either Ocimene, *p*-Cymene, Estragole or Camphene was additive, with ΣFIC values ranging between 0.58 - 0.83. However, (+)-α-Pinene was non-interactive in combination with the same selected compounds, (ΣFIC values ranging between 1.50 - 2.00). This indicates that stereoselectivity exists for (–)-Borneol, (–)-Citronellal and (–)-α-Pinene, when combined with the selected compounds and tested against *C. albicans*. The greatest variation was seen between the combination of the enantiomer of β-Pinene with β-Caryophyllene, where (+)-β-Pinene in combination was synergistic (ΣFIC = 0.50), whereas (–)-β-Pinene in combination was non-interactive (ΣFIC = 1.50). This is visually represented in Figure 2.3.

Previous combination studies in which essential oil compounds in their enantiomeric form were investigated against *C. albicans*, are limited. Van Zyl *et al*. (2010) reported the interaction between (+)-β-Pinene with Eucalyptol to be synergistic (ΣFIC = 0.35) against *C. albicans*, which is in line with the findings of the current study (ΣFIC = 0.50). Viljoen *et al*. (2003) reported that (–)-Camphor with 1,8-Cineole (Eucalyptol) interacted synergistically, which is similar to the findings of the current investigation, which reports that the interaction between both (+)- and (–)-Camphor, in combination with Eucalyptol were additive with ΣFIC values of 0.75, each. Ahmad *et al*. (2014) reported that Borneol (racemate) in combination with *p*-Cymene was non-interactive (ΣFIC = 2.13), which correlates to the findings of the current investigation for both enantiomers of Borneol (ΣFIC values between 1.17 - 1.50). It was also reported that Borneol (racemate) in combination with γ-Terpinene was synergistic (ΣFIC = 0.50), and the current investigation found that (–)-Borneol was additive in combination with γ-Terpinene (ΣFIC value of 0.69). However, (+)-Borneol was non-interactive (ΣFIC = 1.25) with γ-Terpinene. This suggests that the sample of Borneol evaluated by Ahmad *et al*. (2014) may have had a greater enantiomeric distribution of (–)-Borneol.

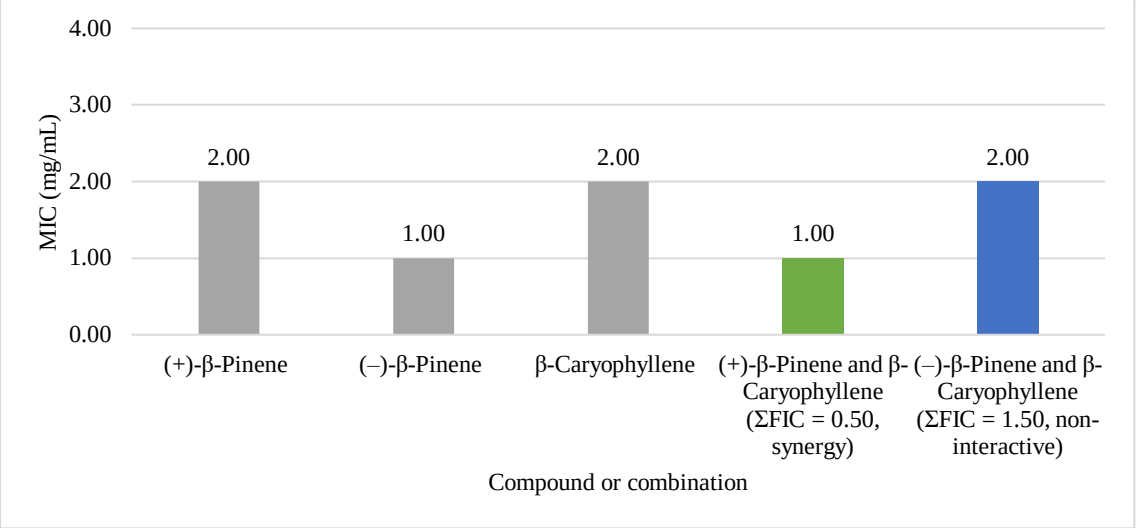


Figure 2.3: The mean MIC of the enantiomers of β-Pinene and of β-Caryophyllene, independently and in combination, against *C. albicans.*

Other investigations looked at the combined interactive efficacy of the enantiomers with antifungals, against *C. albicans*. Da Silva *et al*. (2012) found both (+)-α-Pinene and (+)-β-Pinene to be synergistic in combination with Amphotericin B against *C. albicans*. While the current investigation did not evaluate the combined activity of the enantiomers with standard antifungals, 50.00% of the combinations involving (+)-α-Pinene and 92.86% of the combinations involving (+)-β-Pinene, were either additive or synergistic. Maree *et al.* (2014) reported that Borneol and Camphor were active antimicrobial compounds (putative biomarkers) in the essential oils in which they constitute, against *C. albicans*. This was determined through chemometric analysis. The current study found, through MIC determination, that the enantiomers of Borneol interacted favourably in combination with the selected compounds, where 67.86% of the combinations involving the two enantiomers of Borneol, and 89.29% of the combinations involving the two enantiomers of Camphor, in combination were additive. In addition, 3.57% of the combinations involving Camphor were synergistic. Maree *et al.* (2014) also reported that there was a correlation between essential oils with MIC values > 2.00 mg/mL and the presence of Limonene in those oils, against *C. albicans*. The current investigation found that although the majority of the combinations had MIC values ≤ 1.00 mg/mL against *C. albicans*, the combinations that had MIC values > 1.00 mg/mL were mostly observed with Limonene, particularly (+)-Limonene. Orchard *et al.* (2017) identified Eugenol as a putative biomarker against *C. albicans* and the current investigation found that Eugenol had MIC values ranging between 0.50 - 1.00 mg/mL in combination with the enantiomers, and 50.00% of the combinations were additive.

2.3.3.3 The interactive efficacy of the combinations against *P. aeruginosa*

The results of the MIC and ΣFIC evaluation of the combinations, against *P. aeruginosa* are given in Table 2.7. The MIC values ranged between 0.50 - 4.00 mg/mL. A total of 45.41% combinations had MIC values ≤ 1.00 mg/mL. This was particularly evident with the enantiomers of Citronellal in combination. The MIC values were mostly equivalent between the enantiomers in combination, however, a variation in MIC values was observed with the enantiomers of α-Pinene in combination with Linalyl acetate, where (+)-α-Pinene had a combined MIC value of 4.00 mg/mL, whereas (–)-α-Pinene had a combined MIC of 1.00 mg/mL. Similarly, this was seen with the enantiomers of α-Pinene in combination with β-Caryophyllene, where (+)-α-Pinene had a combined MIC value of 3.00 mg/mL, whereas (–)-α-Pinene had a combined MIC value of 1.00 mg/mL. (–)-α-Pinene displayed stronger inhibitory activity in combination with both of the selected compounds, as compared to (+)-α-Pinene.

A total of 3.06% of the combinations were synergistic, 46.94% were additive, and 50.00% were non-interactive. No antagonism was observed. The synergistic interactions were seen with (+)-Citronellal in combination with either *p*-Cymene, Eugenol, Isoeugenol, Ocimene or γ-Terpinene (ΣFIC values ranging between 0.42 - 0.50). Synergy was also observed between (–)-α-Pinene in combination with Eucalyptol (ΣFIC = 0.50), whereas (+)-α-Pinene was non-interactive with Eucalyptol (ΣFIC = 1.50). Interestingly, all enantiomers combined with Camphene resulted in additivity, with ΣFIC values ranging between 0.63 - 1.00.

The most prevalent type of variation in interactive efficacy that was observed between the enantiomers in combination was ‘additive versus non-interactive’. This was evident with the two enantiomers of Limonene, Menthone, and (+)-Borneol, (–)-α-Pinene and (–)-Camphor. Of particular interest was (–)-α-Pinene, which was either synergistic or additive in combination with either β-Caryophyllene, Eucalyptol, Eugenol, Geraniol, Isoeugenol or Linalyl acetate, with ΣFIC values ranging between 0.50 - 0.75. However, (+)-α-Pinene was non-interactive in combination with those selected compounds, with ΣFIC values ranging between 1.50 - 3.33. This suggests the possible pathogen-specific nature of (–)-α-Pinene against *P. aeruginosa*.

Table 2.7: The mean MIC (mg/mL) with standard deviation and ΣFIC with interaction classification (in parentheses) for the 1:1 combinations, against *P. aeruginosa*

Selected compound	**Enantiomers**													
	(+)-Borneol	(–)-Borneol	(+)-Camphor	(–)-Camphor	(+)-Citronellal	(–)-Citronellal	(+)-Limonene	(–)-Limonene	(+)-Menthone	(–)-Menthone	(+)-α-Pinene	(–)-α-Pinene	(+)-β-Pinene	(–)-β-Pinene
Camphene	**1.00** ± 0.00 (1.00; Add)	**1.00** ± 0.00 (1.00; Add)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.63; Add)	**1.00** ± 0.00 (1.00; 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 (0.83; Add)	**1.00** ± 0.00 (0.83; Add)	**1.00** ± 0.00 (1.00; Add)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.83; Add)
β-Caryophyllene	2.00 ± 0.00 (1.67; Ind)	2.00 ± 0.00 (1.67; Ind)	2.00 ± 0.00 **(1.17; Ind)**	1.50 ± 0.71 **(0.69; Add)**	**1.00** ± 0.00 (0.83; Add)	**1.00** ± 0.00 (0.83; Add)	1.50 ± 0.71 **(1.00; Add)**	2.00 ± 0.00 **(1.67; Ind)**	2.00 ± 0.00 (1.33; Ind)	2.00 ± 0.00 (1.33; Ind)	*3.00* ± 1.41 **(2.50; Ind)**	***1.00*** ± 0.00 **(0.58; Add)**	2.00 ± 0.00 (1.17; Ind)	2.00 ± 0.00 (1.33; Ind)
p-Cymene	2.00 ± 0.00 (2.00; Ind)	2.00 ± 0.00 (2.00; Ind)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.63; Add)	**0.50** ± 0.00 **(0.50; Syn)**	**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 (1.67; 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 (1.67; Ind)
Estragole	**1.00** ± 0.00 **(1.00; Add)**	2.00 ± 0.00 **(2.00; Ind)**	1.50 ± 0.71 (1.13; Ind)	2.00 ± 0.00 (1.25; Ind)	**1.00** ± 0.00 (1.00; Add)	**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 (1.67; 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 (1.67; Ind)
Eucalyptol	2.00 ± 0.00 (1.50; Ind)	2.00 ± 0.00 (1.50; Ind)	1.50 ± 0.71 (0.75; Add)	2.00 ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.75; 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 (1.17; Ind)	3.00 ± 1.41 (1.75; Ind)	2.00 ± 0.00 **(1.50; Ind)**	**1.00** ± 0.00 **(0.50; Syn)**	2.00 ± 0.00 **(1.00; Add)**	2.00 ± 0.00 **(1.17; Ind)**
Eugenol	**1.00** ± 0.00 (1.00; Add)	**1.00** ± 0.00 (1.00; Add)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.63; Add)	**0.50** ± 0.00 **(0.50; Syn)**	**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 **(0.83; Add)**	2.00 ± 0.00 **(1.67; Ind)**	2.00 ± 0.00 **(2.00; Ind)**	**1.00** ± 0.00 **(0.75; Add)**	2.00 ± 0.00 (1.50; Ind)	2.00 ± 0.00 (1.67; Ind)
Geraniol	2.00 ± 0.00 (2.00; Ind)	2.00 ± 0.00 (2.00; Ind)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.63; Add)	**1.00** ± 0.00 (1.00; Add)	**1.00** ± 0.00 (1.00; Add)	**1.00** ± 0.00 **(0.83; Add)**	2.00 ± 0.00 **(2.00; Ind)**	**1.00** ± 0.00 **(0.83; Add)**	2.00 ± 0.00 **(1.67; Ind)**	2.00 ± 0.00 **(2.00; Ind)**	**1.00** ± 0.00 **(0.75; Add)**	2.00 ± 0.00 (1.50; Ind)	2.00 ± 0.00 (1.67; Ind)
Isoeugenol	**1.00** ± 0.00 (1.00; Add)	**1.00** ± 0.00 (1.00; Add)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.63; Add)	**0.50** ± 0.00 **(0.50; Syn)**	**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 **(0.83; Add)**	2.00 ± 0.00 **(1.67; Ind)**	2.00 ± 0.00 **(2.00; Ind)**	**1.00** ± 0.00 **(0.75; Add)**	2.00 ± 0.00 (1.50; Ind)	2.00 ± 0.00 (1.67; Ind)
Linalyl acetate	2.00 ± 0.00 (1.67; Ind)	2.00 ± 0.00 (1.67; Ind)	2.00 ± 0.00 **(1.17; Ind)**	1.50 ± 0.71 **(0.69; Add)**	**1.00** ± 0.00 (0.83; Add)	**1.00** ± 0.00 (0.83; Add)	2.00 ± 0.00 (1.33; Ind)	1.50 ± 0.71 (1.25; Ind)	**1.00** ± 0.00 **(0.67; Add)**	2.00 ± 0.00 **(1.33; Ind)**	*4.00* ± 0.00 **(3.33; Ind)**	***1.00*** ± 0.00 **(0.58; Add)**	2.00 ± 0.00 (1.17; Ind)	2.00 ± 0.00 (1.33; Ind)
Menthol	**1.00** ± 0.00 (1.00; Add)	**1.00** ± 0.00 (1.00; Add)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.63; Add)	**1.00** ± 0.00 (1.00; 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 (0.83; 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 (1.67; Ind)
Ocimene	2.00 ± 0.00 (2.00; Ind)	2.00 ± 0.00 (2.00; Ind)	1.50 ± 0.71 **(1.13; Ind)**	1.50 ± 0.71 **(0.94; Add)**	**0.50** ± 0.00 **(0.50; Syn)**	**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 (1.67; 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 (1.67; Ind)
Sabinene hydrate	2.00 ± 0.00 (2.00; Ind)	2.00 ± 0.00 (2.00; Ind)	1.50 ± 0.71 **(1.13; Ind)**	**1.00** ± 0.00 **(0.63; Add)**	**1.00** ± 0.00 (1.00; Add)	**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 **(1.67; Ind)**	**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 (1.67; Ind)
γ-Terpinene	2.00 ± 0.00 (1.67; Ind)	2.00 ± 0.00 (1.67; Ind)	1.50 ± 0.71 (0.88; Add)	2.00 ± 0.00 (0.92; Add)	**0.50** ± 0.00 **(0.42; Syn)**	**1.00** ± 0.00 **(0.83; Add)**	**1.00** ± 0.00 **(0.67; Add)**	2.00 ± 0.00 **(1.67; Ind)**	2.00 ± 0.00 (1.33; Ind)	2.00 ± 0.00 (1.33; Ind)	2.00 ± 0.00 (1.67; Ind)	3.00 ± 1.41 (1.75; Ind)	2.00 ± 0.00 (1.17; Ind)	2.00 ± 0.00 (1.33; Ind)
α-Terpineol	2.00 ± 0.00 **(2.00; Ind)**	**1.00** ± 0.00 **(1.00; Add)**	2.00 ± 0.00 (1.50; Ind)	2.00 ± 0.00 (1.25; Ind)	**1.00** ± 0.00 (1.00; Add)	**1.00** ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.67; Ind)	2.00 ± 0.00 (2.00; Ind)	2.00 ± 0.00 (1.67; Ind)	2.00 ± 0.00 (1.67; Ind)	**1.00** ± 0.00 (1.00; Add)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.83; Add)

n = 2 replicates, with third consecutive replicate to confirm variations between enantiomers; **bold** = noteworthy activity ≤ 1.00 mg/mL; *italics* = variations in MIC values between enantiomers in combination, that is more than one well dilution difference; **red bold** = variations in interactive efficacy between enantiomers in combination; Syn = synergy, Add = additive, Ind = non-interactive; positive control (Ciprofloxacin) = 6.25 x 10^{-4} (±0.00) mg/mL; negative control (acetone) = > 4.00 (±0.00) mg/mL.

The greatest variation observed in terms of interactive efficacy was observed between the enantiomers of α-Pinene in combination with Linalyl acetate. (+)-α-Pinene in combination with Linalyl acetate was non-interactive (ΣFIC = 3.33), whereas (–)-α-Pinene in combination with Linalyl acetate was additive (ΣFIC = 0.58). This is visually represented in Figure 2.4.

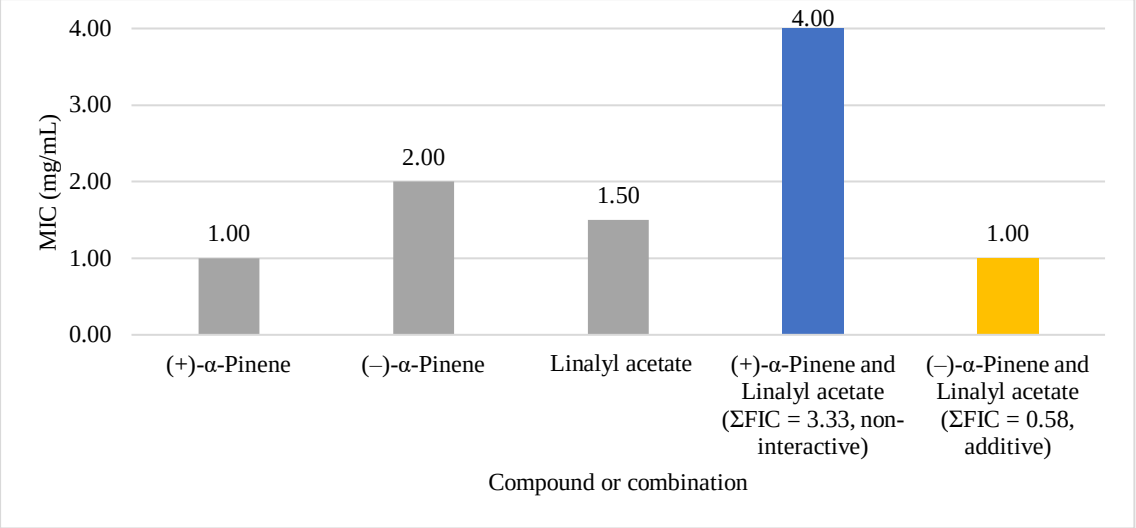


Figure 2.4: The mean MIC of the enantiomers of α-Pinene and of Linalyl acetate, independently and in combination, against *P. aeruginosa*.

Van Vuuren and Viljoen (2007) reported that (+)-Limonene and (–)-Limonene were each additive in combination with 1,8-Cineole (Eucalyptol). This correlates with the findings of the current investigation, where (–)-Limonene had a ΣFIC value of 0.75 (additive), however, (+)-Limonene had a ΣFIC value of 1.17 (non-interactive), when combined with Eucalyptol. Orchard *et al.* (2017) identified Eugenol as a putative biomarker against *P. aeruginosa*, through chemometric analysis. The current investigation found this to be true even for combinations, as Eugenol had MIC values ranging between 0.50 - 1.00 mg/mL in combination with the enantiomers of Borneol, Camphor Citronellal and Limonene, and 71.43% of the combinations involving Eugenol were additive or synergistic.

2.3.3.4 The interactive efficacy of the combinations against *K. pneumoniae*

The results of the MIC and ΣFIC evaluation of the combinations, against *K. pneumoniae* are given in Table 2.8. The MIC values of the combinations ranged between 0.50 - 4.00 mg/mL. A total of 21.43% of the combinations had MIC values ≤ 1.00 mg/mL. This was particularly

evident with the enantiomers of Borneol, Camphor, Menthone and α-Pinene in combination with the selected compounds, particularly Eugenol, Geraniol or Isoeugenol. A total of 75.00% of the combinations were additive, 2.04% were synergistic and 22.96% were non-interactive. No antagonism was observed. Synergy was observed with (+)-Borneol in combination with Estragole or with Sabinene hydrate (ΣFIC = 0.50). (–)-Borneol combined with Menthol also resulted in synergy (ΣFIC = 0.38), as well as (–)-α-Pinene in combination with Camphene (ΣFIC = 0.50). The most prevalent type of variation observed was 'additive versus non-interactive', the majority of which was seen with the enantiomers of β-Pinene in combination with either β-Caryophyllene, Linalyl acetate, Eucalyptol, Isoeugenol, γ-Terpinene, Ocimene or *p*-Cymene. (+)-β-Pinene interacted additively in combination with the aforementioned selected compounds, with ΣFIC values of 0.75, whereas (–)-β-Pinene was non-interactive, with ΣFIC values of 1.50. This indicates that (+)-β-Pinene is likely pathogen-specific in combination, against *K. pneumoniae*. One of the greatest variation in interactive activity was seen with the enantiomers of α-Pinene in combination with Geraniol, where (+)-α-Pinene was additive in combination (ΣFIC = 0.75), whereas (–)-α-Pinene was non-interactive (ΣFIC = 1.88) (Figure 2.5).

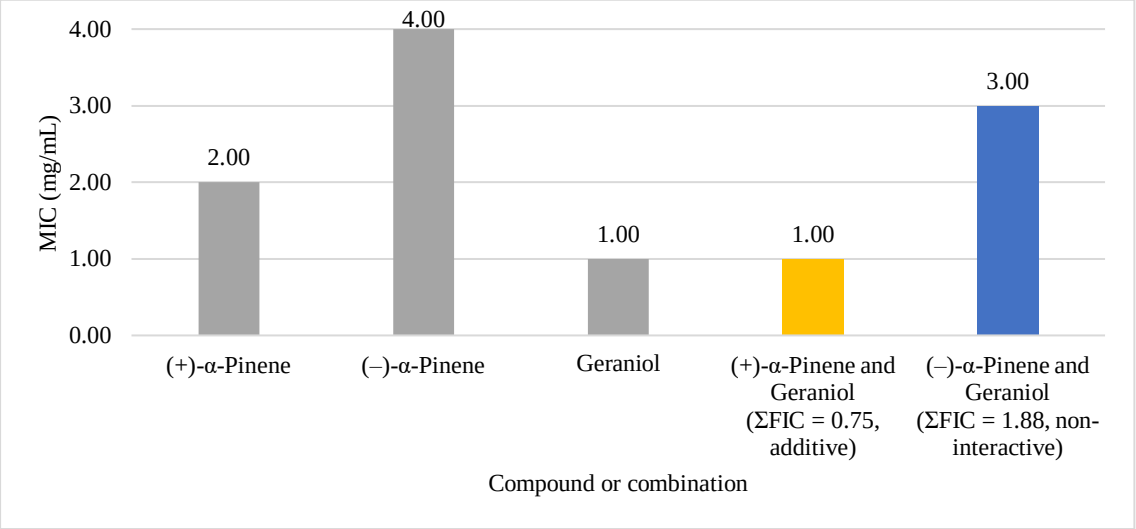


Figure 2.5: The mean MIC of the enantiomers of α-Pinene and of Geraniol, independently and in combination, against *K. pneumoniae*.

Table 2.8: The mean MIC (mg/mL) with standard deviation and ΣFIC with interaction classification (in parentheses) for the 1:1 combinations, against *K. pneumoniae*

Selected compound	Enantiomers													
	(+)-Borneol	(–)-Borneol	(+)-Camphor	(–)-Camphor	(+)-Citronellal	(–)-Citronellal	(+)-Limonene	(–)-Limonene	(+)-Menthone	(–)-Menthone	(+)-α-Pinene	(–)-α-Pinene	(+)-β-Pinene	(–)-β-Pinene
Camphene	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)	2.00 ± 0.00 (0.75; Add)	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)	2.00 ± 0.00 (0.75; Add)	2.00 ± 0.00 **(0.75; Add)**	2.00 ± 0.00 **(0.50; Syn)**	2.00 ± 0.00 (0.75; Add)	2.00 ± 0.00 (0.75; Add)
β-Caryophyllene	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 **(1.00; Add)**	4.00 ± 0.00 **(1.50; Ind)**	2.00 ± 0.00 **(1.00; Add)**	4.00 ± 0.00 **(2.00; Ind)**
p-Cymene	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 **(2.00; Ind)**	2.00 ± 0.00 **(0.75; Add)**	2.00 ± 0.00 **(1.00; Add)**	4.00 ± 0.00 **(2.00; Ind)**
Estragole	**1.00** ± 0.00 **(0.50; Syn)**	2.00 ± 0.00 **(1.00; Add)**	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	4.00 ± 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 (1.00; Add)	2.00 ± 0.00 (1.00; Add)
Eucalyptol	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	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 **(1.00; Add)**	4.00 ± 0.00 **(2.00; Ind)**
Eugenol	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.75)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 **(0.75; Add)**	1.50 ± 0.71 **(1.13; Ind)**	2.00 ± 0.00 **(1.50; Ind)**	**1.00** ± 0.00 **(0.75; Add)**	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 **(0.75; Add)**	2.00 ± 0.00 **(1.25; Ind)**	2.00 ± 0.00 (1.50; Ind)	2.00 ± 0.00 (1.50; Ind)
Geraniol	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 **(0.75; Add)**	2.00 ± 0.00 **(1.50; Ind)**	2.00 ± 0.00 (1.50; Ind)	2.00 ± 0.00 (1.50; Ind)	2.00 ± 0.00 (1.50; Ind)	2.00 ± 0.00 (1.50; Ind)	***1.00*** ± 0.00 **(0.75; Add)**	*3.00* ± 1.41 **(1.88; Ind)**	2.00 ± 0.00 (1.50; Ind)	2.00 ± 0.00 (1.50; Ind)
Isoeugenol	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.75; Add)	2.00 ± 0.00 **(1.50; Ind)**	**1.00** ± 0.00 **(0.75; Add)**	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 **(0.75; Add)**	2.00 ± 0.00 **(1.25; Ind)**	**1.00** ± 0.00 **(0.75; Add)**	2.00 ± 0.00 **(1.50; Ind)**
Linalyl acetate	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 **(1.00; Add)**	3.00 ± 1.41 **(1.50; Ind)**	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	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 **(1.00; Add)**	4.00 ± 0.00 **(2.00; Ind)**
Menthol	**1.00** ± 0.00 **(0.75; Add)**	**0.50** ± 0.00 **(0.38; Syn)**	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.75; Add)	2.00 ± 0.00 (1.50; Ind)	2.00 ± 0.00 (1.50; Ind)	**1.00** ± 0.00 **(0.75; Add)**	2.00 ± 0.00 **(1.50; Ind)**	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 **(0.75; Add)**	2.00 ± 0.00 **(1.25; Ind)**	2.00 ± 0.00 (1.50; Ind)	2.00 ± 0.00 (1.50; Ind)
Ocimene	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 **(2.00; Ind)**	2.00 ± 0.00 **(0.75; Add)**	2.00 ± 0.00 **(1.00; Add)**	4.00 ± 0.00 **(2.00; Ind)**
Sabinene hydrate	**1.00** ± 0.00 (0.50; Syn)	1.50 ± 0.71 **(0.75; Add)**	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	4.00 ± 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 (1.00; Add)	2.00 ± 0.00 (1.00; Add)
γ-Terpinene	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 **(2.00; Ind)**	2.00 ± 0.00 **(0.75; Add)**	2.00 ± 0.00 **(1.00 ; Add)**	4.00 ± 0.00 **(2.00; Ind)**
α-Terpineol	**1.00** ± 0.00 **(0.75; Add)**	2.00 ± 0.00 **(1.50; Ind)**	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.75; Add)	2.00 ± 0.00 (1.50; Ind)	2.00 ± 0.00 (1.50; Ind)	2.00 ± 0.00 (1.50; Ind)	2.00 ± 0.00 (1.50; Ind)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.75; Add)	2.00 ± 0.00 (1.50; Ind)	2.00 ± 0.00 (1.25; Ind)	2.00 ± 0.00 (1.50; Ind)	2.00 ± 0.00 (1.50; Ind)

n = 2 replicates, with third consecutive replicate to confirm variations between enantiomers; **bold** = noteworthy activity ≤ 1.00 mg/mL; **red bold** = variations in interactive efficacy between enantiomers in combination; Syn = synergy, Add = additive, Ind = non-interactive; positive control (Ciprofloxacin) = 7.80 x 10^{-5} (±0.00) mg/mL; negative control (acetone) = > 4.00 (±0.00) mg/mL.

Van Vuuren and Viljoen (2007) reported that (+)-Limonene and (–)-Limonene were both antagonistic in combination with 1,8-Cineole (Eucalyptol), with ΣFIC values of 4.60 and 4.00, respectively. However, the current investigation found that (+)-Limonene and (–)-Limonene were both additive in combination with 1,8-Cineole (Eucalyptol), with a ΣFIC value of 1.00. This discrepancy in interactive efficacy may be as a result of the use of different ATCC strains studied. Another study, conducted by Filipowicz *et al.* (2003), evaluated two samples of *Juniperus communis* L. essential oils against *K. pneumoniae*, in which one sample had a greater enantiomeric distribution of (+)-α-Pinene (42.73%) and the other had a greater enantiomeric distribution of (–)-α-Pinene (60.99%), as their major compounds. The sample with the greater enantiomeric distribution of (+)-α-Pinene had inhibitory activity, whereas the sample with the greater enantiomeric distribution of (–)-α-Pinene did not have any inhibitory activity. The current investigation found that (+)-α-Pinene interacted more favourably in combination, in terms of being either synergistic or additive in combination with the selected compounds, while (–)-α-Pinene was often non-interactive. Therefore, the inhibitory activity of the sample with a greater enantiomeric distribution of (+)-α-Pinene may have been as a result of the combined activity with other major or minor compounds present. For example, Geraniol was present as a minor compound (0.62 - 1.50%), and the current investigation found that the combination of (+)-α-Pinene with Geraniol was additive (ΣFIC = 0.75), whereas (–)-α-Pinene with Geraniol was non-interactive (ΣFIC = 1.88).

2.3.3.5 The interactive efficacy of the combinations against *S. aureus*

The results of the MIC and ΣFIC evaluation of the combinations, against *S. aureus*, are given in Table 2.9. The MIC values ranged between 1.00 - 4.00 mg/mL. A total of 15.31% of the combinations had MIC values ≤ 1.00 mg/mL, which was particularly evident with the enantiomers of Citronellal in combination. The greatest variation in terms of MIC values were seen with the enantiomers of Borneol in combination with β-Caryophyllene, where (+)-Borneol had a combined MIC value of 1.00 mg/mL, whereas (–)-Borneol had a combined MIC of 4.00 mg/mL. The enantiomers of Limonene in combination with γ-Terpinene displayed a similar variation, where (+)-Limonene had a combined MIC value of 1.00 mg/mL, whereas (–)-Limonene had a combined MIC value of 4.00 mg/mL. A total of 6.12% of the combinations were synergistic, 44.39% were additive, and 49.49% were non-interactive. No antagonism was observed. Synergy was observed with the enantiomers of Borneol, Limonene, (+)-Camphor

Table 2.9: The mean MIC (mg/mL) with standard deviation and ΣFIC with interaction classification (in parentheses) for the 1:1 combinations, against *S. aureus*

Selected compound	**Enantiomers**													
	(+)-Borneol	(–)-Borneol	(+)-Camphor	(–)-Camphor	(+)-Citronellal	(–)-Citronellal	(+)-Limonene	(–)-Limonene	(+)-Menthone	(–)-Menthone	(+)-α-Pinene	(–)-α-Pinene	(+)-β-Pinene	(–)-β-Pinene
Camphene	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 (1.00; Add)	2.00 ± 0.00 (1.50; Ind)	2.00 ± 0.00 (1.50; Ind)	4.00 ± 0.00 (1.50; Ind)	4.00 ± 0.00 (1.50; Ind)	4.00 ± 0.00 (1.50; Ind)	4.00 ± 0.00 (1.50; Ind)	2.00 ± 0.00 **(1.00; Add)**	4.00 ± 0.00 **(1.50; Ind)**	4.00 ± 0.00 (2.00; Ind)	4.00 ± 0.00 (2.00; Ind)
β-Caryophyllene	***1.00*** *± 0.00* **(0.38; Syn)**	*4.00 ± 0.00* **(1.50; Ind)**	2.00 ± 0.00 **(0.50; Syn)**	4.00 ± 0.00 **(1.50; Ind)**	2.00 ± 0.00 (1.25; Ind)	2.00 ± 0.00 (1.25; Ind)	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)	4.00 ± 0.00 **(1.50; Ind)**	2.00 ± 0.00 **(0.50; Syn)**	2.00 ± 0.00 **(0.75; Add)**	4.00 ± 0.00 **(1.50; Ind)**
p-Cymene	4.00 ± 0.00 (1.50; Ind)	4.00 ± 0.00 (1.50; Ind)	4.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (0.75; Add)	2.00 ± 0.00 (1.25; Ind)	2.00 ± 0.00 (1.25; Ind)	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)	4.00 ± 0.00 **(1.50; Ind)**	3.00 ± 1.41 **(0.75; Add)**	4.00 ± 0.00 (1.50; Ind)	4.00 ± 0.00 (1.50; Ind)
Estragole	4.00 ± 0.00 **(1.50; Ind)**	2.00 ± 0.00 **(0.75; Add)**	2.00 ± 0.00 **(0.50; Syn)**	4.00 ± 0.00 **(1.50; Ind)**	**1.00** ± 0.00 (0.63; Add)	**1.00** ± 0.00 (0.63; Add)	4.00 ± 0.00 **(1.00; Add)**	2.00 ± 0.00 **(0.50; Syn)**	4.00 ± 0.00 (1.00; Ind)	4.00 ± 0.00 (1.00; Ind)	4.00 ± 0.00 **(1.50; Ind)**	2.00 ± 0.00 **(0.50; Syn)**	2.00 ± 0.00 (0.75; Add)	2.00 ± 0.00 (0.75; Add)
Eucalyptol	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 **(1.00; Add)**	2.00 ± 0.00 **(1.50; Ind)**	**1.00** ± 0.00 **(0.75; Add)**	4.00 ± 0.00 **(1.50; Ind)**	2.00 ± 0.00 **(0.75; Add)**	4.00 ± 0.00 (1.50; Ind)	4.00 ± 0.00 (1.50; Ind)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (0.75; Add)	3.00 ± 1.41 (1.50; Ind)	3.00 ± 1.41 (1.50; Ind)
Eugenol	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 (1.50; Ind)	**1.00** ± 0.00 (1.00; Add)	**1.00** ± 0.00 (1.00; 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)	2.00 ± 0.00 **(1.50; Ind)**	**1.00** ± 0.00 **(0.63; Add)**	**1.00** ± 0.00 **(0.75; Add)**	2.00 ± 0.00 **(1.50; Ind)**
Geraniol	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 (1.50; Ind)	**1.00** ± 0.00 (1.00; Add)	**1.00** ± 0.00 (1.00; 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)	2.00 ± 0.00 **(1.50; Ind)**	**1.00** ± 0.00 **(0.63; Add)**	**1.00** ± 0.00 **(0.75; Add)**	2.00 ± 0.00 **(1.50; Ind)**
Isoeugenol	**1.00** ± 0.00 (1.25; Ind)	2.00 ± 0.00 (2.50; Ind)	2.00 ± 0.00 (2.25; Ind)	2.00 ± 0.00 (2.50; Ind)	**1.00** ± 0.00 (1.50; Ind)	**1.00** ± 0.00 (1.50; Ind)	**1.00** ± 0.00 (1.13; Ind)	**1.00** ± 0.00 (1.13; Ind)	2.00 ± 0.00 (2.25; Ind)	2.00 ± 0.00 (2.25; Ind)	2.00 ± 0.00 (2.50; Ind)	**1.00** ± 0.00 (1.13; Ind)	**1.00** ± 0.00 (1.25; Ind)	2.00 ± 0.00 (2.50; Ind)
Linalyl acetate	2.00 ± 0.00 **(1.00; Add)**	4.00 ± 0.00 **(2.00; Ind)**	2.00 ± 0.00 **(0.75; Add)**	4.00 ± 0.00 **(2.00; Ind)**	2.00 ± 0.00 **(1.50; Ind)**	**1.00** ± 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 (1.50; Ind)	4.00 ± 0.00 **(2.00; Ind)**	2.00 ± 0.00 **(0.75; Add)**	3.00 ± 1.41 (1.50; Ind)	4.00 ± 0.00 (2.00; Ind)
Menthol	**1.00** ± 0.00 (0.50; Syn)	**1.00** ± 0.00 (0.50; Syn)	2.00 ± 0.00 (0.75; Add)	2.00 ± 0.00 (1.00; Add)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 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)	2.00 ± 0.00 (0.75; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (0.75; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)
Ocimene	4.00 ± 0.00 (1.50; Ind)	4.00 ± 0.00 (1.50; Ind)	4.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (0.75; Add)	2.00 ± 0.00 (1.25; Ind)	2.00 ± 0.00 (1.25; Ind)	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)	4.00 ± 0.00 **(1.50; Ind)**	2.00 ± 0.00 **(0.50; Syn)**	2.00 ± 0.00 **(0.75; Add)**	4.00 ± 0.00 **(1.50; Ind)**
Sabinene hydrate	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 **(0.75; Add)**	4.00 ± 0.00 **(2.00; Ind)**	2.00 ± 0.00 **(1.50; Ind)**	**1.00** ± 0.00 **(0.75; 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)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (0.75; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)
γ-Terpinene	**1.00** ± 0.00 **(0.50; Syn)**	2.00 ± 0.00 **(1.00; Add)**	4.00 ± 0.00 **(1.50; Ind)**	2.00 ± 0.00 **(1.00; Add)**	2.00 ± 0.00 (1.50; Ind)	2.00 ± 0.00 (1.50; Ind)	***1.00*** ± 0.00 **(0.38; Syn)**	*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 (2.00; Ind)	4.00 ± 0.00 (1.50; Ind)	4.00 ± 0.00 (2.00; Ind)	4.00 ± 0.00 (2.00; Ind)
α-Terpineol	**1.00** ± 0.00 **(0.50; Syn)**	2.00 ± 0.00 **(1.00; Add)**	2.00 ± 0.00 (0.75; Add)	2.00 ± 0.00 (1.00; Add)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 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)	2.00 ± 0.00 (0.75; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (0.75; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (1.00; Add)

n = 2 replicates, with third consecutive replicate to confirm variations between enantiomers; **bold** = noteworthy activity ≤ 1.00 mg/mL; *italics* = variations in MIC values between enantiomers in combination, that is more than one well dilution difference; **red bold** = variations in interactive efficacy between enantiomers in combination; Syn = synergy, Add = additive, Ind = non-interactive; positive control (Ciprofloxacin) = 6.25 x 10^{-4} (±0.00) mg/mL; negative control (acetone) = > 4.00 (±0.00) mg/mL.

and (–)-α-Pinene in combination with certain selected compounds, with ΣFIC values ranging between 0.38 - 0.50. It was interesting to note that Menthol and α-Terpineol, displayed either additivity or synergy in combination with all the enantiomers against *S. aureus*.

The most prevalent type of variation between the enantiomeric pairs was 'additive versus non-interactive'. This was particularly evident with the enantiomers of α-Pinene and β-Pinene in combination, where (–)-α-Pinene and (+)-β-Pinene were often observed as additive (ΣFIC values of 0.63 - 0.75), and their enantiomeric counterparts having non-interactive efficacies, with ΣFIC values ranging between 1.50 - 2.00. The variation 'synergy versus non-interactive', which is the biggest variation in interactive efficacy that can be observed, was frequently observed against *S. aureus*. This was observed with the enantiomers of Borneol, Camphor and α-Pinene. In the case of α-Pinene, (–)-α-Pinene interacted synergistically with either β-Caryophyllene, Ocimene or Estragole (ΣFIC = 0.50), whereas (+)-α-Pinene was non-interactive (ΣFIC = 1.50). The greatest variation in interactive efficacy was observed with the enantiomers of Limonene in combination with γ-Terpinene, where (+)-Limonene in combination with γ-Terpinene was synergistic (ΣFIC = 0.38), while (–)-Limonene in combination with γ-Terpinene was non-interactive (ΣFIC = 1.50) (Figure 2.6).

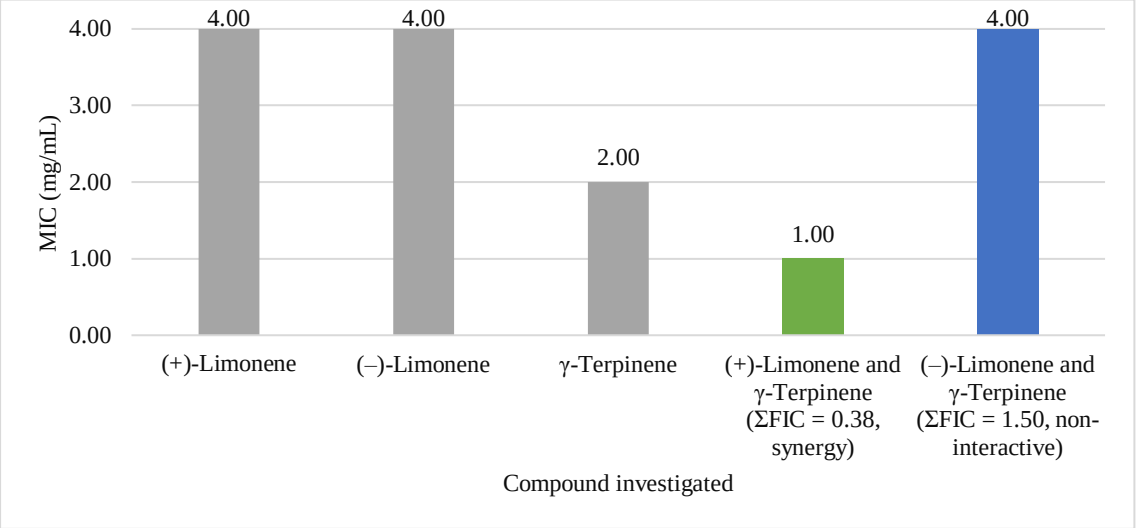


Figure 2.6: The mean MIC of the enantiomers of Limonene and of γ-Terpinene, independently and in combination, against *S. aureus*.

Van Vuuren and Viljoen (2007) reported that in combination with 1,8-Cineole (Eucalyptol), (–)-Limonene was antagonistic, whereas (+)-Limonene was synergistic against *S. aureus*. However, the current study found that (+)-Limonene combined with Eucalyptol was non-

interactive (ΣFIC = 1.50) and (–)-Limonene combined with Eucalyptol was additive (ΣFIC = 0.75). Ahmad *et al*., (2014) reported that the combination of Borneol (racemate) with *p*-Cymene was synergistic against *S. aureus* (ΣFIC = 0.38). However, the current investigation found that (+)-Borneol and (–)-Borneol were both non-interactive with *p*-Cymene (ΣFIC = 1.50). These discrepancies may be as a result of the use of different ATCC strains of *S. aureus*. Ahmad *et al*., (2014) also reported that the combination of Borneol (racemate) with γ-Terpinene was additive against *S. aureus* (ΣFIC = 1.00). Similarly, the current investigation found that (+)-Borneol was synergistic (ΣFIC = 0.50) and (–)-Borneol was additive (ΣFIC = 1.00), when combined with γ-Terpinene. This suggests that the sample of Borneol investigated by Ahmad *et al*., (2014) may have had a greater enantiomeric distribution of (+)-Borneol.

2.3.3.6 The interactive efficacy of the combinations against *E. faecium*

The results of the MIC and ΣFIC evaluation of the combinations, against *E. faecium* are given in Table 2.10. The MIC values ranged between 1.00 - 4.00 mg/mL. A total of 5.10% of the combinations had MIC values ≤ 1.00 mg/mL, which was particularly evident with the enantiomers of Borneol. The selected compounds involved in the noteworthy activity observed were Geraniol, Eugenol and Isoeugenol. A total of 65.31% of the combinations were additive, and the rest (34.69%) were non-interactive. No synergy or antagonism was observed with the combinations against *E. faecium*. The additive combinations had ΣFIC values ranging between 0.63 - 1.00. The only exception was the enantiomers of Citronellal, which was mostly non-interactive in combination (ΣFIC ranging between 1.50 - 2.00). Both enantiomers of Borneol had additive interactions in combination with the majority of the selected compounds.

The only type of variation seen between the enantiomers in combination was 'additive versus non-interactive'. This was particularly evident with the enantiomers of α-Pinene and of β-Pinene. (–)-α-Pinene was additive in combination with either β-Caryophyllene, Linalyl acetate, γ-Terpinene, Ocimene, *p*-Cymene, Estragole or Camphene (ΣFIC = 1.00); whereas (+)-α-Pinene was non-interactive (ΣFIC = 1.50). Similarly, (+)-β-Pinene was additive in combination with either β-Caryophyllene, Linalyl acetate, γ-Terpinene, Ocimene, *p*-Cymene, Estragole, Camphene or Menthol (ΣFIC values ranging between 0.75 -1.00); whereas (–)-β-Pinene was non-interactive (ΣFIC values ranging between 1.50 -2.00). One of the greatest variations was seen with the enantiomers of Camphor in combination with Isoeugenol, where (+)-Camphor

interacted additively (ΣFIC = 0.63), whereas (–)-Camphor in combination with Isoeugenol was non-interactive (ΣFIC = 1.25) (Figure 2.7).

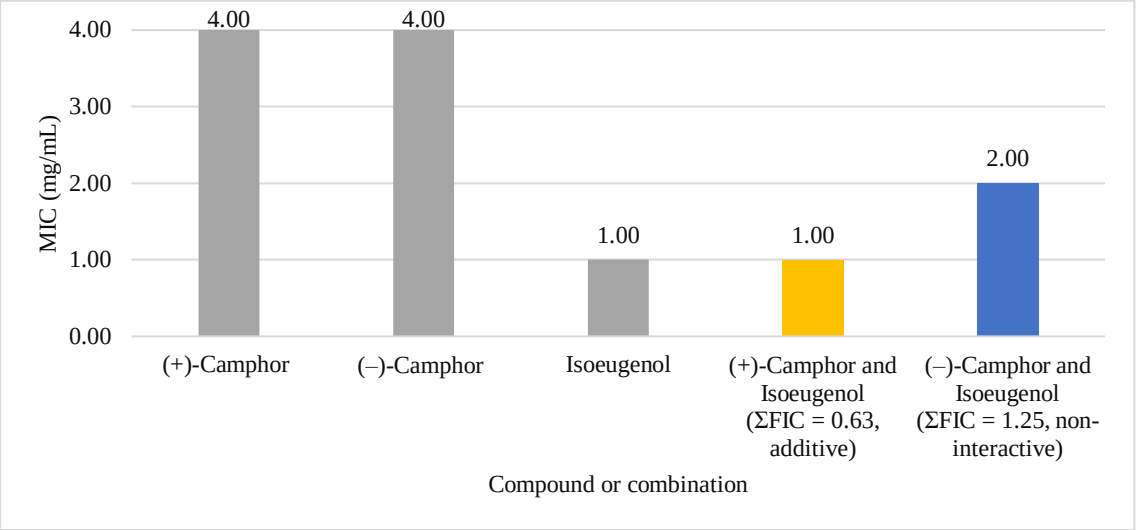


Figure 2.7: The mean MIC of the enantiomers of Camphor and of Isoeugenol, independently and in combination, against *E. faecium*.

The investigation of essential oils and essential oil compounds against *E. faecium* were notably scarce. This is interesting as the World Health Organisation (WHO) classified this pathogen as 'high priority' in terms of finding alternative treatment options (WHO, 2017). A review conducted by Tariq *et al.* (2019) provides an overview of studies involving the antimicrobial inhibitory activity of essential oils and essential oil compounds, in which the scarcity of investigations against *E. faecium* is clear. Another recent review conducted by Yu *et al.* (2020) aimed to highlight recent findings on the antimicrobial inhibitory activity of essential oils against the ESKAPE pathogens, including *E. faecium*, and also highlighted this scarcity. I quote directly from Yu *et al.* (2020): "However, though there are some reports against strains of *E. coli*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, there are very few or no reports against *E. faecium* and *Enterobacter* and this necessitates further investigations in this direction". As such, relating the findings of the current investigation to the literature was difficult. A correlation was observed with Nissen *et al.* (2010), which evaluated the inhibitory activity of *Cannabis sativa* L. essential oils against *E. faecium*. The major constituents of the oil were reported to be α-Pinene (10.90 - 16.99%) and β-Caryophyllene (10.56 - 13.90%). The study reported MIC values ranging between 1.55 - 1.78% (v/v) (approximately 0.02 mg/ml). The current investigation found that in combination with β-Caryophyllene, (+)-α-Pinene was non-interactive, whereas (–)-α-Pinene was additive in combination.

Table 2.10: The mean MIC (mg/mL) with standard deviation and ΣFIC with interaction classification (in parentheses) for the 1:1 combinations, against *E. faecium*

Selected compound	Enantiomers													
	(+)-Borneol	(–)-Borneol	(+)-Camphor	(–)-Camphor	(+)-Citronellal	(–)-Citronellal	(+)-Limonene	(–)-Limonene	(+)-Menthone	(–)-Menthone	(+)-α-Pinene	(–)-α-Pinene	(+)-β-Pinene	(–)-β-Pinene
Camphene	2.00 ± 0.00 (0.75; Add)	2.00 ± 0.00 (0.75; Add)	4.00 ± 0.00 (1.00; Add)	4.00 ± 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)	4.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 **(1.50; Ind)**	4.00 ± 0.00 **(1.00; Add)**	4.00 ± 0.00 **(1.00; Add)**	4.00 ± 0.00 **(1.50; Ind)**
β-Caryophyllene	2.00 ± 0.00 (0.75; Add)	2.00 ± 0.00 (0.75; Add)	4.00 ± 0.00 (1.00; Add)	4.00 ± 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)	4.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 **(1.50; Ind)**	4.00 ± 0.00 **(1.00; Add)**	4.00 ± 0.00 **(1.00; Add)**	4.00 ± 0.00 **(1.50; Ind)**
p-Cymene	2.00 ± 0.00 (0.75; Add)	2.00 ± 0.00 (0.75; Add)	4.00 ± 0.00 (1.00; Add)	4.00 ± 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)	4.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 **(1.50; Ind)**	4.00 ± 0.00 **(1.00; Add)**	4.00 ± 0.00 **(1.00; Add)**	4.00 ± 0.00 **(1.50; Ind)**
Estragole	2.00 ± 0.00 (0.75; Add)	2.00 ± 0.00 (0.75; Add)	4.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 **(0.75; Add)**	4.00 ± 0.00 **(1.50; Ind)**	4.00 ± 0.00 (1.00; 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 **(1.50; Ind)**	4.00 ± 0.00 **(1.00; Add)**	4.00 ± 0.00 **(1.00; Add)**	4.00 ± 0.00 **(1.50; Ind)**
Eucalyptol	2.00 ± 0.00 (0.75; Add)	2.00 ± 0.00 (0.75; Add)	4.00 ± 0.00 (1.00; Add)	4.00 ± 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)	4.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (0.75; Add)	4.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (0.75; Add)
Eugenol	**1.00** ± 0.00 (0.75; Add)	**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 (1.50; 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)	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 (1.50; Ind)
Geraniol	**1.00** ± 0.00 (0.75; Add)	**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 (1.50; 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)	**1.00** ± 0.00 **(0.75; Add)**	2.00 ± 0.00 **(1.25; Ind)**	2.00 ± 0.00 **(1.25; Ind)**	**1.00** ± 0.00 **(0.75; Add)**
Isoeugenol	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 (0.75; Add)	**1.00** ± 0.00 **(0.63; Add)**	2.00 ± 0.00 **(1.25; 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)	**1.00** ± 0.00 **(0.75; Add)**	2.00 ± 0.00 **(1.25; Ind)**	2.00 ± 0.00 **(1.25; Ind)**	**1.00** ± 0.00 **(0.75; Add)**
Linalyl acetate	2.00 ± 0.00 (0.75; Add)	2.00 ± 0.00 (0.75; Add)	4.00 ± 0.00 (1.00; Add)	4.00 ± 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)	4.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 **(1.50; Ind)**	4.00 ± 0.00 **(1.00; Add)**	4.00 ± 0.00 **(1.00; Add)**	4.00 ± 0.00 **(1.50; Ind)**
Menthol	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)	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)	2.00 ± 0.00 (0.75; Add)	2.00 ± 0.00 (0.75; 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 **(2.00; Ind)**
Ocimene	2.00 ± 0.00 (0.75; Add)	2.00 ± 0.00 (0.75; Add)	4.00 ± 0.00 (1.00; Add)	4.00 ± 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)	4.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 **(1.50; Ind)**	4.00 ± 0.00 **(1.00; Add)**	4.00 ± 0.00 **(1.00; Add)**	4.00 ± 0.00 **(1.50; Ind)**
Sabinene hydrate	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)	2.00 ± 0.00 **(1.00; Add)**	4.00 ± 0.00 **(2.00; Ind)**	4.00 ± 0.00 (1.50; Ind)	4.00 ± 0.00 (1.50; Ind)	2.00 ± 0.00 **(0.75; Add)**	4.00 ± 0.00 **(1.50; Ind)**	2.00 ± 0.00 **(1.00; Add)**	4.00 ± 0.00 **(1.50; Ind)**	2.00 ± 0.00 (0.75; Add)	2.00 ± 0.00 (1.00; Add)
γ-Terpinene	2.00 ± 0.00 **(0.75; Add)**	3.00 ± 1.41 **(1.13; Ind)**	4.00 ± 0.00 (1.00; Add)	4.00 ± 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)	4.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 (1.00; Add)	4.00 ± 0.00 **(1.50; Ind)**	4.00 ± 0.00 **(1.00; Add)**	4.00 ± 0.00 **(1.00; Add)**	4.00 ± 0.00 **(1.50; Ind)**
α-Terpineol	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)	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)**	2.00 ± 0.00 (0.75; Add)	2.00 ± 0.00 (0.75; Add)	2.00 ± 0.00 (1.00; Add)	2.00 ± 0.00 (0.75; Add)	2.00 ± 0.00 (0.75; Add)	2.00 ± 0.00 (1.00; Add)

n = 2 replicates, with third consecutive replicate to confirm variations between enantiomers; **bold** = noteworthy activity ≤ 1.00 mg/mL; **red bold** = variations in interactive efficacy between enantiomers in combination; Add = additive, Ind = non-interactive; positive control (Ciprofloxacin) = 1.25 x 10^{-3} (±0.00) mg/mL; negative control (acetone) = > 4.00 (±0.00) mg/mL.

The noteworthy MIC values displayed by the *C. sativa* essential oils is likely due to the enantiomeric distribution of (–)-α-Pinene being greater than (+)-α-Pinene. However, one needs to consider the influence of other compounds within the neat essential oils.

2.3.4 Summary of the interactive efficacy studies

The ΣFIC studies revealed that the majority of the combinations were additive, followed by non-interactive, and synergistic. No antagonism was observed amongst all the combinations (n = 1176) investigated against all six pathogens. The overall interactive profiles between the (+)- and (–)-enantiomers in combinations were mostly similar (Figure 2.8).

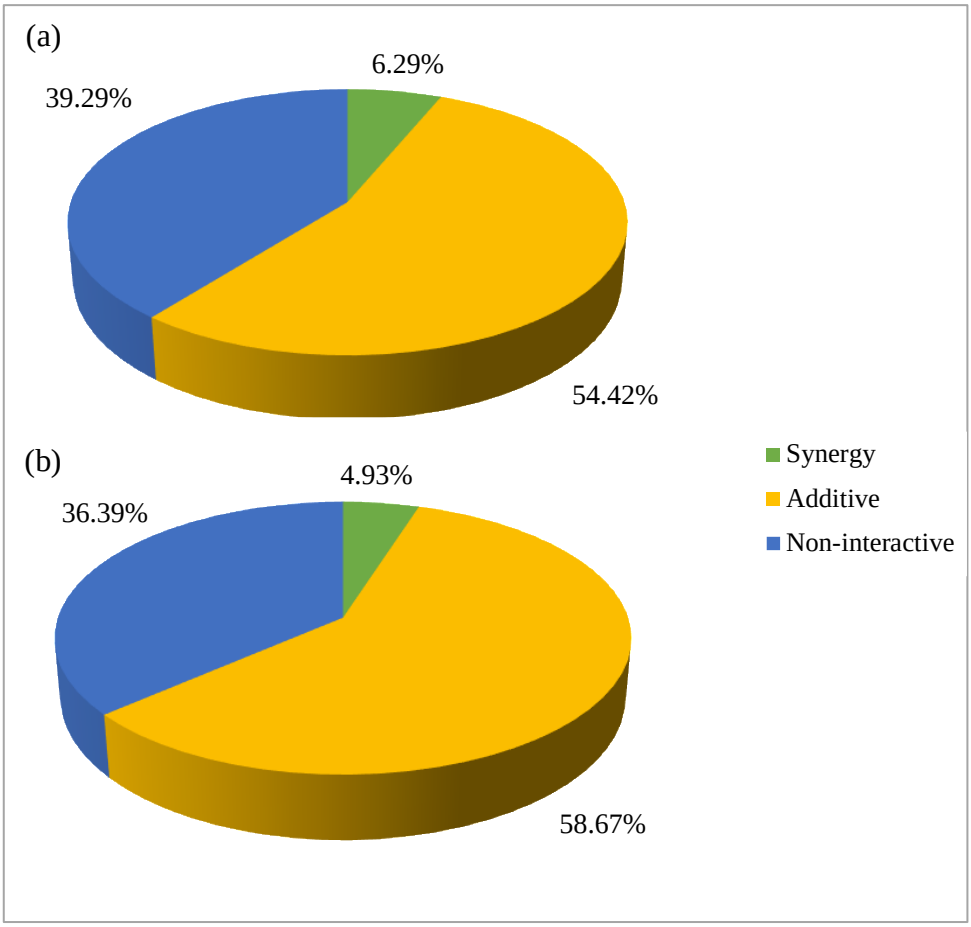


Figure 2.8: Summary of the ΣFIC interactive profiles observed with the (a) (+)-enantiomers (b) and (–)-enantiomers, in combination.

While the interactions between the enantiomeric pairs were similar overall, a few important observations were made. Firstly, the majority of the synergistic combinations were against the two yeast pathogens, *C. neoformans* and *C. albicans*, followed by *S. aureus*. The most

synergistic of these combinations (20 interactions) had ΣFIC values of between 0.38 - 0.48 against all the pathogens, except *E. faecium*, where no synergy was observed. The majority of the additivity observed was against *K. pneumoniae* and *E. faecium*. Overall, the combination of (–)-Borneol with Menthol was the most interesting of all combinations studied, demonstrating synergy against *S. aureus*, *K. pneumoniae* and *C. neoformans*, with ΣFIC values ranging between 0.38 - 0.50. In addition, additivity was observed against the remaining pathogens, namely: *E. faecium*, *P. aeruginosa* and *C. albicans*, with ΣFIC values ranging between 0.58 - 1.00. A total of 17.18% of the combinations displayed variations in terms of the ΣFIC interactive efficacy between the (+) and (–)-enantiomers. The enantiomers of Borneol, Limonene, α-Pinene and β-Pinene varied the most in terms of interactive efficacy, where the (–)-enantiomers often interacted more favourably in those combinations, when compared to the (+)-enantiomers. This means that where variations were observed, the (–)-enantiomers of Borneol, Limonene, α-Pinene and β-Pinene often interacted in a way that reduced the effective concentration required to inhibit the pathogen, whereas the (+)-enantiomers did not. However, in the case of β-Pinene, (+)-β-Pinene interacted more favourably in combination than (–)-β-Pinene (Figure 2.9).

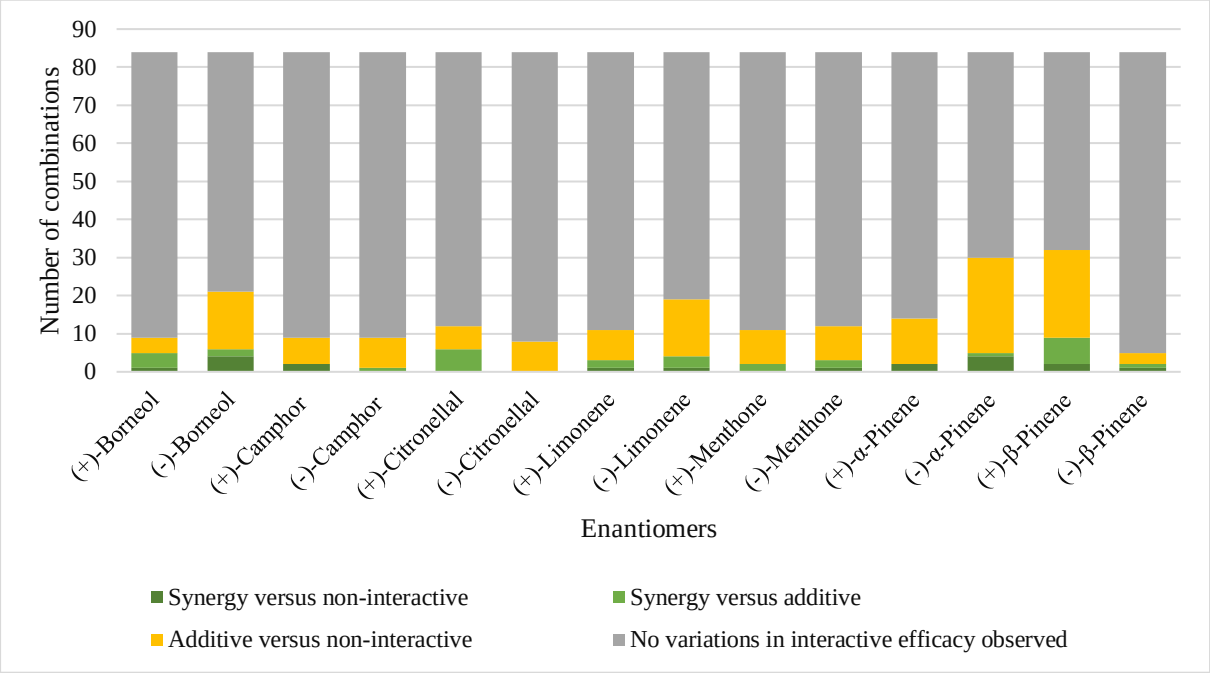


Figure 2.9: Summary of the types of variations seen between the enantiomers in combination, against all the pathogens investigated.

The overall greatest variations in interactive efficacy that were observed with the enantiomers in combination with the selected compounds are given in Table 2.11. It was observed that (+)-

β-Pinene in combination with β-Caryophyllene, and (–)-α-Pinene in combination with Estragole interacted either additively or synergistically against the majority of the micro-organisms tested, when compared to their enantiomeric counterparts. While the same trend was not observed for the enantiomers of Limonene in combination with γ-Terpinene, where there were variations (against *P. aeruginosa* and *S. aureus*). These were amongst the greatest variations observed. (+)-Limonene with γ-Terpinene interacted either additively or synergistically, with ΣFIC values ranging between 0.38 - 0.67, whereas (–)-Limonene with γ-Terpinene was non-interactive with ΣFIC values ranging between 1.50 - 1.67.

Table 2.11: The overall biggest variations in interactive efficacy that were observed with the combinations

Enantiomer	**Selected compound**	**Micro-organism**					
		C. neoformans	*C. albicans*	*K. pneumoniae*	*P. aeruginosa*	*S. aureus*	*E. faecium*
		ΣFIC (Interaction)					
(+)-β-Pinene	β-Caryophyllene	**1.00 (Add)**	**0.50 (Syn)**	**1.00 (Add)**	1.17 (Ind)	**0.75 (Add)**	**1.00 (Add)**
(–)-β-Pinene		**1.00 (Add)**	1.50 (Ind)	2.00 (Ind)	1.33 (Ind)	1.50 (Ind)	1.50 (Ind)
(+)-α-Pinene	Estragole	1.50 (Ind)	1.50 (Ind)	**1.00 (Add)**	2.00 (Ind)	1.50 (Ind)	1.50 (Ind)
(–)-α-Pinene		**0.58 (Add)**	**0.58 (Add)**	**0.75 (Add)**	1.50 (Ind)	**0.50 (Syn)**	**1.00 (Add)**
(+)-Limonene	γ-Terpinene	1.50 (Ind)	**1.00 (Add)**	**1.00 (Add)**	**0.67 (Add)**	**0.38 (Syn)**	**1.00 (Add)**
(–)-Limonene		**1.00 (Add)**	**0.75 (Add)**	**1.00 (Add)**	1.67 (Ind)	1.50 (Ind)	**1.00 (Add)**

Bold = ΣFIC values ≤ 1.00; Syn = synergy, Add = Additivity, Ind = non-interactive.

2.4 Summary

- The MIC values of the enantiomers ranged between 0.13 - 4.00 mg/mL. The most noteworthy inhibitory activity of the enantiomers was observed against *C. neoformans*. This was observed with (+)-Borneol, with an MIC of 0.13 mg/mL.
- The variations in the inhibitory activity displayed by the enantiomers investigated independently were mostly equivalent.
- (+)-α-Pinene showed moderately stronger inhibitory activity than (–)-α-Pinene against all the pathogens investigated.
- (–)-Limonene displayed moderately stronger inhibitory activity against the yeast pathogens and with *P. aeruginosa*, when compared to (+)-Limonene. This was also observed with (+)-Camphor, when compared to (–)-Camphor.
- (–)-β-Pinene displayed moderately stronger inhibitory activity against *C. albicans* and *P. aeruginosa*, when compared to (+)-β-Pinene.

- (+)-Borneol displayed moderately stronger inhibitory activity against the yeast pathogens, when compared to (–)-Borneol.
- The MIC values of the selected compounds ranged between 0.25 - 4.00 mg/mL. Eugenol, Geraniol and Isoeugenol displayed the most noteworthy and broad-spectrum inhibitory activity against all six pathogens investigated. Menthol and α-Terpineol displayed noteworthy inhibitory activity against the fungal and Gram-negative pathogens.
- The results of the 1:1 combination study revealed that the most prevalent interaction observed was additivity (56.46%), followed by non-interactive (37.93%), and synergy (5.61%). No antagonism was observed.
- The combination of (–)-Borneol with Menthol was the most interesting, demonstrating synergy against *S. aureus*, *K. pneumoniae* and *C. neoformans*, with ΣFIC values ranging between 0.38 - 0.50. In addition, additivity was observed against the remaining pathogens, namely: *E. faecium*, *P. aeruginosa* and *C. albicans*, with ΣFIC values ranging between 0.58 - 1.00.
- Where variations in terms of ΣFIC were observed, (–)-Borneol, (–)-Limonene, (–)-α-Pinene, (+)-β-Pinene often interacted synergistically or additively in combination, when compared to their enantiomeric counterparts, which were often non-interactive.
- The enantiomers of β-Pinene in combination with β-Caryophyllene demonstrated the most variability. (+)-β-Pinene with β-Caryophyllene was either synergistic or additive in combination against *S. aureus*, *E. faecium*, *K. pneumoniae*, *C. albicans* and *C. neoformans*, with ΣFIC values ranging between 0.50 - 1.00; and was only non-interactive against *P. aeruginosa* (ΣFIC = 1.17). However, (+)-β-Pinene with β-Caryophyllene was only additive against *C. neoformans* (ΣFIC = 1.00), and was non-interactive against *S. aureus*, *E. faecium*, *P. aeruginosa*, *K. pneumoniae* and *C. albicans* (ΣFIC values ranging between 1.33 - 2.00).

Chapter 3 - Anti-quorum sensing studies on enantiomers and combinations with the selected compounds

3.1 Introduction

The stereoselective antimicrobial activity, using the minimum inhibitory concentration (MIC) assay, of the enantiomers on planktonic cells was the focus in Chapter 2. However, one needs to consider that it is just as important to evaluate the antimicrobial activity in terms of curbing factors that aid in virulence, in addition to the inhibitory activity. Bacterial communication, known as quorum sensing (QS), is an important factor in promoting virulence and the development of resistant adaptations in bacteria. Thus, the aim of the current chapter was to examine the enantiomeric variability in terms of the inhibition of violacein production in *Chromobacterium violaceum*, a biosensor strain. This was achieved through the determination of the minimum quorum sensing inhibitory concentration (MQSIC) and determination of the extent of percentage violacein inhibition. In addition, the influence of the enantiomeric form of the enantiomers in combination with the selected compounds were also investigated, through interactive efficacy studies. The selected compounds were therefore initially investigated independently in order to determine a baseline when comparing the combined anti-QS activity.

3.2 Materials and methods

3.2.1 Preparation of the compounds and controls

The compounds and controls were prepared as described in Chapter 2, Section 2.2.1. The only modification was that dimethyl sulfoxide (DMSO) (Riedel-de-Haën) was used as the solvent instead of acetone. The compounds were made up to stock concentrations of 32.00 mg/mL (final concentration of DMSO tested at 0.20 - 1.60%). To ensure that the solvent was not responsible for anti-QS activity, DMSO was used as a negative control at 32.00 mg/mL. Vanillin (Merck) was used as a positive control to ensure that the strain of *C. violaceum* used was susceptible to a known anti-QS agent (Chenia, 2013). The Vanillin was made up to a stock

concentration of 32.00 mg/mL and tested in the same manner as the compounds. Untreated *C. violaceum* was added as a comparative culture control and acted as a baseline from which to measure violacein inhibition, and to ensure that the broth used was capable of supporting growth of *C. violaceum*.

3.2.2 Preparation of *C. violaceum*

In order to monitor for QS activity, *C. violaceum* (ATCC 12472) was used as a biosensor strain. A waiver for the use of this micro-organism was obtained from the University of the Witwatersrand Human Research Ethics Committee (reference number W-CP-201028-2, Appendix C). To prepare the culture, *C. violaceum* was incubated, under aerobic conditions, in Luria Bertani broth (LBB) at 30 °C for 24 hrs 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 hrs 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 hrs under constant agitation (140 rpm). After incubation, this culture contained approximately 5 x 10^6 CFU/mL. This suspension was then diluted by a factor of ten using LBB as a diluent, to achieve a culture suspension of 5 x 10^5 CFU/mL, which was then used for the anti-QS assay.

3.2.3 The anti-quorum sensing (QS) analysis

3.2.3.1 The macro-dilution assay

Anti-QS studies were undertaken using the methods described by Ahmad *et al*. (2015). The enantiomers and selected compounds were assayed at concentrations of 0.50, 0.25, 0.13 and 0.06 mg/mL, in 5.00 mL of LBB. The volume of the compound needed to achieve these concentrations were determined using Equation 3.1 and are outlined in Table 3.1.

$$C_1V_1 = C_2V_2$$

Where C_1 = concentration of the stock suspension, i.e.: 32.00 mg/mL;

C_2 = the concentration of the compound that is required;

V_1 = the volume of compound to add; and

V_2= the volume of LBB used (5.00 mL).

Equation 3.1

Table 3.1: The amount of sample to add to 5.00 mL of LBB, in order to achieve the desired concentration

Dilutions	Conc. required (mg/mL)	Vol. to add for individual compounds (mL)*	Vol. to add for individual compounds (µL)	Vol. to add for 1:1 combinations (µL)
1	0.50	0.08	78.13	39.06
2	0.25	0.04	39.06	19.53
3	0.13	0.02	19.53	9.77
4	0.06	0.01	9.77	4.89

*From a stock concentration of 32.00 mg/mL.

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, as prepared in Section 3.2.2. This was then incubated at 30 °C for 24 hrs under constant agitation (140 rpm) in an orbital shaker incubator (Labcon). Following the 24 hrs incubation, the test tubes were vortexed, and the results were observed macroscopically. The anti-QS activity of the compounds was indicated by the absence of violacein. 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 hrs (Figure 3.1). 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.

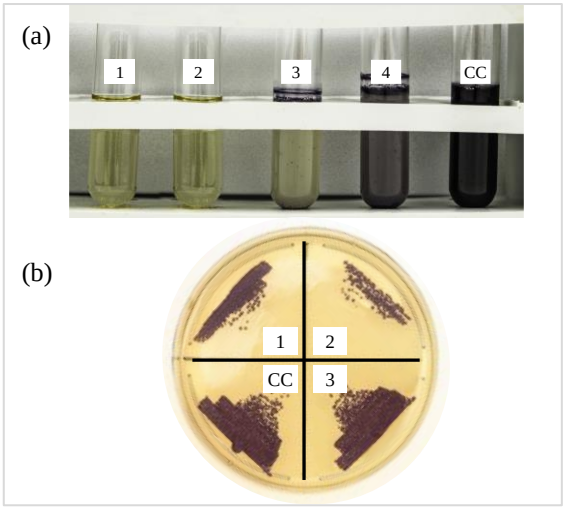


Figure 3.1: Visual representation of the results of the macro-dilution assay of the anti-QS analysis at the varying concentrations: (1) 0.50 mg/mL, (2) 0.25 mg/mL, (3) 0.13 mg/mL, (4) 0.06 mg/mL and (CC) the culture control.

3.2.3.2 Violacein quantification and determination of the minimum quorum sensing inhibitory concentration (MQSIC)

After observing the compounds macroscopically, it was determined that they did in fact display anti-QS activity at the concentrations investigated. Therefore, the next step was to quantify the extent to which QS was inhibited. This was achieved by determining the optical density (O.D.) of the sample at each concentration and thereafter determining the percentage of violacein inhibition relative to the O.D. of the culture control. In order to do this, 1.00 mL aliquots of the compounds at each concentration (after the overnight incubation) were transferred into corresponding 1.50 mL Eppendorf microtubes (Thermo Fisher Scientific) and were centrifuged at 15000 rpm for 10 min in an LLG-uniCFUGE 5 centrifuge. This allowed for the bacteria to form pellets, which settled to the bottom and retained the colour of the *C. violaceum*. The clear supernatant was thereafter discarded. As violacein is insoluble in water and poorly soluble in acetone, DMSO was used as the vehicle of choice (Mahumane, 2016). The pellets were then suspended in 1.00 mL of 100.00% DMSO solution and each sample was vortexed for 20 sec, in order to solubilize the violacein. Following this step, the samples were then further centrifuged at 15000 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 (Figure 3.2). The absorbance of the supernatant in each well was then read at an optical density (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 Equation 3.2.

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

Equation 3.2

Once the percentage of violacein inhibition was determined quantitatively through O.D. evaluation, the MQSIC was then designated as the effective concentration of the compound at which 50.00% or greater of the QS activity was inhibited (Ahmad *et al*., 2015). This means that violacein production that is inhibited by 50.00% or greater means that the compound investigated has anti-QS activity worth noting, whereas violacein inhibition that is less than 50.00% means that the anti-QS activity of the compound is not appreciable.

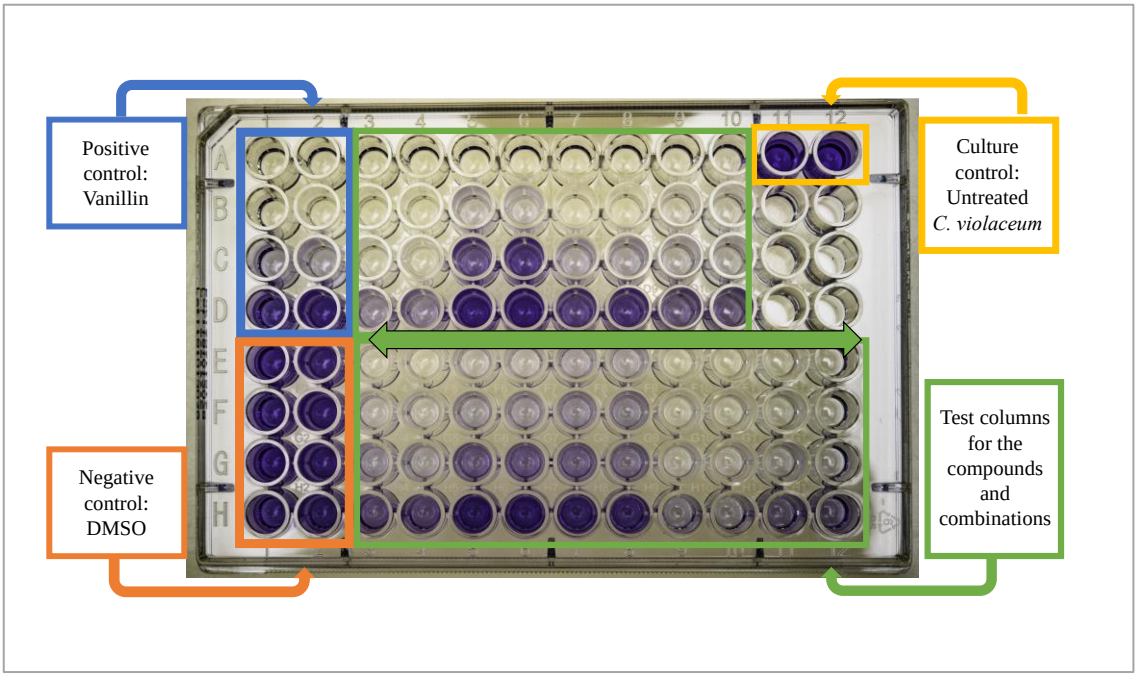


Figure 3.2: Visual representation of the results of the final plate used for O.D. analysis of the anti-QS analysis.

3.2.3.3 The interactive efficacy studies

The 1:1 combinations were achieved by the addition of equal amounts of the enantiomers and selected compounds at half the original volumes, as described in Table 3.1, in order to achieve the same concentrations of 0.50, 0.25, 0.13 and 0.06 mg/mL. To determine the types of interactions between compounds in terms of the effective concentration required to disrupt bacterial communication, the fractional quorum sensing inhibitory concentration (ΣFQSIC) was determined, as adapted from van Vuuren and Viljoen (2011) and Ramulondi (2017). This was calculated using Equation 3.3.

$$FQSIC^{(i)} = \frac{\text{MQSIC (a) in combination with (b)}}{\text{MQSIC (a) independently}}$$

$$FQSIC^{(ii)} = \frac{\text{MQSIC (b) in combination with (a)}}{\text{MQSIC (b) independently}}$$

Where (a) is the MQSIC of the one compound in the combination and (b) is the MQSIC of the other compound used in the combination.

Equation 3.3

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

$$\Sigma FQSIC = FQSIC^{(i)} + FQSIC^{(ii)}$$

Equation 3.4

The ΣFQSIC was interpreted as either synergistic, additive, non-interactive or antagonistic. This is described in detail in Table 3.2.

Table 3.2: Interaction classification based on the ΣFQSIC values

ΣFQSIC	**Interaction classification**	**Description**
≤ 0.50	Synergy	The effective concentration required to disrupt bacterial communication of the two compounds combined, was markedly lower when combined than when tested for each of the two compounds independently.
> 0.50 - 1.00	Additive	There was a decrease in the effective concentration required to disrupt bacterial communication of the two compounds in combination, compared to the two compounds tested independently. However, not to the extent seen with the synergistic combinations.
> 1.0 ≤ 4.0	Non-interactive	The two compounds had no effect on bacterial communication when tested in combination, compared to the two compounds tested independently.
> 4.00	Antagonism	The effective concentration of the two compounds when combined, was markedly higher than when the compounds were tested independently, resulting in a reduced ability to disrupt bacterial communication.

The first step was to determine the effect of the interactions of the enantiomers with the selected compounds on 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) values. In order to calculate this, the percentage of

violacein production that was reduced was calculated from the percentage violacein inhibition values, using Equation 3.5:

Percentage violacein reduction = 100.00% - Percentage violacein inhibition

Equation 3.5

Once this was determined for the compounds individually and in combination, Equations 3.3 and 3.4 were used to calculate the ΣFPVR. As with the ΣFQSIC interpretation, the ΣFPVR was interpreted as either synergistic, additive, non-interactive or antagonistic. This is described in detail in Table 3.3.

Table 3.3: Interaction classification based on the ΣFPVR values

ΣFPVR	**Interaction classification**	**Description**
≤ 0.50	Synergy	There was a marked increase in the extent of the disruption of bacterial communication of the two compounds combined, compared to the compounds when tested independently.
> 0.50 - 1.00	Additive	There was an increase in the extent of the disruption of bacterial communication of the two compounds when combined, compared to the compounds when tested independently. However, not to the extent seen with the synergistic combinations.
> 1.0 ≤ 4.0	Non-interactive	The two compounds had no effect on bacterial communication in combination. This means that there was no increase or decrease in the disruption to bacterial communication, compared to when the two compounds were tested independently.
> 4.00	Antagonism	The extent of the disruption to bacterial communication of the two compounds combined, was markedly less compared to when studying the compounds independently, resulting in an unfavorable interaction.

Once the interactive efficacy of the enantiomers in combination with the selected compounds were determined, the variations between the enantiomeric pairs in combination were classified as described in Table 2.2 (Chapter 2)

3.3 Results and discussion

3.3.1 The anti-quorum sensing (QS) activity of the enantiomers

The MQSIC values of the enantiomers, are given in Table 3.4. The enantiomers investigated independently had MQSIC values ranging between 0.13 - 0.50 mg/mL. The enantiomers of Borneol had the lowest MQSIC values of the enantiomers investigated, of 0.13 mg/mL each. The enantiomeric pairs mostly displayed equivalent MQSIC values. However, (–)-α-Pinene and (–)-β-Pinene did not display violacein inhibition of 50.00% or greater at the highest concentration investigated, and therefore the MQSIC could not be determined; while (+)-α-Pinene and (+)-β-Pinene had MQSIC values of 0.38 and 0.50 mg/mL, respectively. This means that the (+)-enantiomers of α-Pinene and β-Pinene considerably inhibited bacterial communication at concentrations of 0.38 and 0.50 mg/mL, respectively, whereas the (–)-enantiomers did not, at the concentrations investigated. These were the only variations observed in terms of MQSIC values.

The percentage of violacein that was inhibited at the MQSIC was also determined for each of the enantiomers (Table 3.4). The percentage violacein values of the enantiomers ranged between 3.84 - 90.68%. Variations were observed in terms of the percentage violacein inhibition at certain concentrations, as highlighted in Table 3.4. This was evident with the enantiomers of Camphor, α-Pinene and β-Pinene. For example, (+)-α-Pinene, which had a percentage violacein inhibition of 67.02% at the highest concentration investigated, while that of (–)-α-Pinene was 30.72%. Similarly, the percentage violacein inhibition of (+)-β-Pinene at the highest concentration investigated was 57.32%, while that of (–)-β-Pinene was 22.78%. This was the greatest variation observed in terms of the percentage violacein inhibition. This means that at the same concentration, the enantiomeric pairs disrupted bacterial communication to varying degrees, where the (+)-enantiomers of α-Pinene and β-Pinene inhibited violacein considerably from concentrations of 0.25 and 0.50 mg/mL, respectively, whereas the (–)-enantiomers did not have notable violacein inhibition at the concentrations investigated.

Table 3.4: The mean MQSIC and percentage violacein inhibition of the enantiomers

Conc. mg/mL	Enantiomers			
	Percentage Violacein inhibition (%)	**MQSIC (mg/mL) ± (SD)**	**Percentage Violacein inhibition (%)**	**MQSIC (mg/mL) ± (SD)**
	(+)-Borneol		(–)-Borneol	
0.50	86.98	0.13 ± (0.00)	86.58	0.13 ± (0.00)
0.25	80.62		86.58	
0.13	**56.75**		**69.61**	
0.06	18.33		15.74	
	(+)-Camphor		(–)-Camphor	
0.50	**86.31**	0.50 ± (0.00)	**53.88**	0.50 ± (0.00)
0.25	23.95		48.47	
0.13	18.97		22.82	
0.06	7.66		6.12	
	(+)-Citronellal		(–)-Citronellal	
0.50	90.57	0.25 ± (0.00)	88.39	0.25 ± (0.00)
0.25	**90.68**		**88.21**	
0.13	35.69		39.08	
0.06	6.72		9.68	
	(+)-Limonene		(–)-Limonene	
0.50	**53.71**	0.50 ± (0.00)	**53.71**	0.38 ± (0.18)
0.25	43.36		44.73	
0.13	21.09		31.25	
0.06	15.63		21.29	
	(+)-Menthone		(–)-Menthone	
0.50	89.80	0.25 ± (0.00)	89.72	0.25 ± (0.00)
0.25	**62.80**		**82.09**	
0.13	35.20		24.77	
0.06	36.40		21.59	
	(+)-α-Pinene		(–)-α-Pinene	
0.50	67.02	*0.38 ± (0.18)*	30.72	*> 0.50*
0.25	**50.30**		31.53	
0.13	36.34		24.37	
0.06	13.15		8.57	
	(+)-β-Pinene		(–)-β-Pinene	
0.50	**57.32**	*0.50 ± (0.00)*	22.78	*> 0.50*
0.25	40.22		28.78	
0.13	22.68		3.96	
0.06	19.28		3.84	

n = 2 replicates, with third consecutive replicate to confirm variations between enantiomers; **bold** = mean percentage violacein inhibition that is ≥ 50.00%; *italics* = variation in the MQSIC values greater than one well-dilution difference, red = difference in percentage violacein inhibition that is ≥ 20.00%; 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).

Upon review of the literature, it was evident that there is a scarcity of investigations in which the enantiomeric forms of essential oil compounds are considered when evaluating anti-QS activity. A further in-depth analysis for each set of enantiomers are summarised below and to the best of my knowledge, these were the only studies in which the anti-quorum sensing activity of the enantiomeric compounds were evaluated and compared.

3.3.1.1 Borneol

One other study investigated and compared the anti-QS effects of (+)-Borneol and (–)-Borneol (Ahmad *et al*. 2015). The study reported that (+)-Borneol promoted the production of violacein by 100.00%, whereas (–)-Borneol inhibited the production of violacein by 70.00%, at an MQSIC of 0.50 mg/mL. The percentage inhibition of violacein for (–)-Borneol is consistent with the finding of the current investigation, however, the current investigation found that both enantiomers of Borneol had concentration-dependent inhibition of violacein, with MQSIC values of 0.13 mg/mL each, and a percentage violacein inhibition of 56.75% and 69.61% for (+)-Borneol and (–)-Borneol, respectively. The lack of studies related to the anti-QS effects of Borneol is surprising as the current investigation demonstrates that the anti-QS effects of both enantiomers of Borneol are appreciable, as they required the lowest effective dose (0.13 mg/mL) to achieve considerable violacein inhibition, when compared to the other enantiomers investigated.

3.3.1.2 Camphor

Ahmad *et al*. (2015) reported the anti-QS activity of Camphor (racemate), with an MQSIC value of 0.25 mg/mL, and approximately 70.00% of violacein was inhibited at this concentration. This is consistent with the findings of the current investigation for both enantiomeric forms of Camphor.

3.3.1.3 Citronellal

Patil *et al*. (2017) reported that the glycol-derivative of Citronellal inhibited violacein production in *C. violaceum* by 100.00% at a concentration of 0.50 mg/mL. While the investigation did not examine Citronellal in either enantiomeric form, the results correlate with those of the current investigation, which reports that at a concentration of 0.50 mg/mL, both

enantiomers of Citronellal inhibited violacein production by 90.57% and 88.39%, respectively. While studies that evaluated the anti-QS activity of Citronellal against *C. violaceum* were limited, one study carried out the investigation against a different biosensor strain (Zheng *et al.*, 2020). The study investigated the QS inhibitory activity of the racemate of Citronellal against the Gram-negative *Vibrio campbelli*, at concentrations of 0.001% (1×10^{-5} mg/mL) and 0.0001% (1×10^{-6} mg/mL) and found that it inhibited bioluminescence in *V. campbelli*. This correlates to the findings of the current study in terms of both enantiomers of Citronellal inhibiting QS, albeit at higher concentrations against a different biosensor strain and QS-regulated factor (violacein production in *C. violaceum*).

3.3.1.4 Limonene

Ngenge *et al.* (2021) reported an MQSIC value of 0.25 mg/mL against *C. violaceum*, for *Citrus sinensis* L. essential oil, of which Limonene is the major compound (71.20%). Similarly, the current investigation reported that (+)-Limonene has an MQSIC 0.25 mg/mL, and (–)-Limonene has an MQSIC of 0.50 mg/mL. This suggests that the anti-QS activity reported by Ngenge *et al.* (2021) for *C. sinensis* essential oil may be attributed to the major compound, Limonene, irrespective of the enantiomeric distribution. However, the effects of the other major and minor compounds need to also be considered. Kerekes *et al.* (2015) investigated the anti-QS activity of Limonene (racemate) using the disc diffusion assay against *C. violaceum*, at concentrations of 1.00, 2.00 and 3.00 µL/disc. The study reported that Limonene had no, or minimal inhibitory effect on the production of violacein. The concentrations and method of investigation differed to those of the current study, whereby the method employed by the current study allowed for a more quantitative analysis of the effects of the enantiomers of Limonene on violacein production, and reports that at a concentration of 0.50 mg/mL, both (+)-Limonene and (–)-Limonene inhibited violacein by 53.71%. In terms of the investigation of the anti-QS activity of the enantiomers of Limonene, only one other study could be found that used *C. violaceum* as a biosensor strain (Ahmad *et al.*, 2015). The study reported a variation in the anti-QS activity between the two 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 0.50 mg/mL for both enantiomeric forms.

While studies that evaluated the anti-QS activity of the enantiomers of Limonene against *C. violaceum* were limited, a few studies carried out the investigation against other biosensor strains. Ahmad *et al*. (2015) investigated the anti-QS activity of the enantiomers of Limonene against the production of pyocyanin in *P. aeruginosa*, which is a blue-green pigment produced by the micro-organism. The study found that the pyocyanin inhibitory profile of the compounds investigated differed to that of the violacein inhibitory profile, where (+)-Limonene promoted pyocyanin production by approximately 50.00%, whereas (–)-Limonene inhibited pyocyanin production by approximately 20.00%, at a concentration of 0.50 mg/mL. The study concluded that the patterns in inhibitory activity of pyocyanin and violacein were similar, however, pyocyanin was inhibited to a lesser extent than violacein. This may be due to the QS system responsible for the production of pyocyanin in *P. aeruginosa* being controlled by the *Pseudomonas* quinolone signal molecule, while the production of violacein is controlled by the acyl homoserine lactone (AHL) signal molecule (Zhou *et al*., 2013). Zheng *et al.* (2020) investigated the QS inhibitory activity of (+)-Limonene and (–)-Limonene against the Gram-negative biosensor strain *V. campbelli*, at concentrations of 0.001% (1 x 10^{-5} mg/mL) and 0.0001% (1 x 10^{-6} mg/mL) and found that both enantiomeric forms inhibited bioluminescence in *V. campbelli*. This corresponds to the findings of the current investigation in terms of both enantiomeric forms of Limonene inhibiting QS-regulated violacein production even when tested on a different biosensor strain (*C. violaceum*). The findings of the current investigation are also similar to Wang *et al.* (2018), who investigated the effect of a *d*-Limonene emulsion against the QS-regulated properties of the Gram-negative *E. coli* and found that it interfered with the auto-inducer 2 (AI-2) communication, thereby inhibiting virulence factors such as biofilm formation, extracellular polymeric substance formation, motility and differential expression of genes at concentrations as low as 1.25 - 2.50% (v/v). However, the effects of (–)-Limonene were not investigated. The findings of the investigation correspond the findings of the current study, in terms of (+)-Limonene inhibiting QS-regulated factors (violacein production).

3.3.1.5 Menthone

Ahmad *et al*. (2015) investigated the anti-QS activity of Menthone (racemate) and reported an MQSIC of 0.13 mg/mL with approximately 75.00% violacein inhibition at this concentration, which is consistent with the findings of the current investigation for both enantiomeric forms of Menthone.

3.3.1.6 α-Pinene and β-Pinene

Kerekes *et al.* (2015) investigated the anti-QS activity of α-Pinene (racemate) using the disc diffusion assay against *C. violaceum*, at concentrations of 1.00, 2.00 and 3.00 µL/disc, and found that α-Pinene had equivalent inhibition of violacein at all three concentrations. As mentioned previously, the MIC assay conducted in the current investigation is more accurate than the disc diffusion assay. The current investigation found that QS inhibition was observed by (+)-α-Pinene from a concentration of 0.38 mg/mL (percentage violacein inhibition = 50.30%), while the inhibition displayed by (–)-α-Pinene was not, up to the highest concentration tested (percentage violacein inhibition = 30.72%). In terms of the investigation of the anti-QS activity of the enantiomers of α-Pinene, only two other studies could be found that used *C. violaceum* as a biosensor strain. Ramirez-Rueda and Salvador (2020) reported that at a concentration of 2.48 mg/mL, (+)-α-Pinene inhibited violacein by 71.37%. The current study did not investigate concentrations higher than 0.50 mg/mL and reports a 67.02% inhibition of violacein at this concentration. Ahmad *et al*. (2015) reported that (+)-α-Pinene and (–)-α-Pinene promoted the production of violacein by approximately 25.00% and 150.00% for (+)-α-Pinene and (–)-α-Pinene, respectively. However, the current study reports that (+)-α-Pinene had an MQSIC of 0.38 mg/mL, whereas (–)-α-Pinene did not have appreciable anti-QS activity up to the highest concentration investigated. The same study also investigated the anti-QS activity of β-Pinene (racemate) and reported that it promoted the production of violacein by 20.00%. However, the current study reports that (+)-β-Pinene had anti-QS activity at an MQSIC of 0.50 mg/mL, whereas (–)-β-Pinene did not have appreciable anti-QS activity at the concentrations investigated, with a 22.78% inhibition of violacein at a concentration of 0.50 mg/mL.

While studies that evaluated the anti-QS activity of the enantiomers of α-Pinene against *C. violaceum* were limited, a few studies carried out the investigation against other biosensor strains. Zheng *et al.* (2020) investigated the QS inhibitory activity of α-Pinene (racemate) and (–)-β-Pinene against the Gram-negative *V. campbelli*, at concentrations of 0.001% (1×10^{-5} mg/mL) and 0.0001% (1×10^{-6} mg/mL) and found that both compounds inhibited bioluminescence. This correlates with the inhibition observed by the enantiomers of α-Pinene and β-Pinene in this present investigation, albeit different biosensor strains. Šimunović *et al.* (2020) used a different method and biosensor strain and reported that (–)-α-Pinene had inhibited anti-QS activity at a concentration of 0.13 mg/mL, by 85.00%. However, the current

study found that against *C. violaceum*, (–)-α-Pinene inhibited violacein production by 24.37% and 8.57% at concentrations of 0.13 mg/mL and 0.06 mg/mL, respectively. This variation between the current study and Šimunović *et al.* (2020) is likely due to the use of different biosensor strains.

3.3.2 The anti-quorum sensing (QS) activity of the selected compounds

The results of the percentage violacein inhibition at the varying concentrations, and the MQSIC values of the selected compounds, are given in Table 3.5. The selected compounds investigated independently had MQSIC values ranging between 0.06 - 0.50 mg/mL, with the exception of Camphene, which did not display violacein inhibition of 50.00% or greater at the highest concentration investigated, and therefore the MQSIC could not be determined. Eugenol, Isoeugenol, Menthol, Ocimene, *p*-Cymene, α-Terpineol and γ-Terpinene had the lowest MQSIC values of 0.06 mg/mL, of the selected compounds tested. This means that these compounds inhibited bacterial communication to a considerable extent (≥ 50.00%) at the lowest concentration investigated. The percentage violacein inhibition values of the selected compounds ranged between 26.75 - 76.92%. Overall, the greatest percentage violacein inhibition at the lowest concentration investigated (0.06 mg/mL) was seen with Isoeugenol (74.65%), followed by *p*-Cymene (72.08%). This means that, at lowest effective dose, Isoeugenol and *p*-Cymene inhibited violacein production to a considerable extent (≥ 50.00%).

Table 3.5: The anti-QS activity of the selected compounds investigated independently

Conc. mg/mL	**Selected compound**	
	Percentage Violacein inhibition (%)	**MQSIC (mg/mL) ± (SD)**
	Camphene	
0.50	36.86	> 0.50
0.25	**54.33**	
0.13	32.27	
0.06	26.75	
	β-Caryophyllene	
0.50	**57.05**	0.50 ± (0.00)
0.25	39.90	
0.13	34.25	

Conc. mg/mL	Selected compound	
	Percentage Violacein inhibition (%)	**MQSIC (mg/mL) ± (SD)**
0.06	27.84	0.50 ± (0.00)
	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	76.60	0.13 ± (0.00)
0.25	76.28	
0.13	**76.28**	
0.06	44.40	
	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	75.80	
0.13	73.26	

Conc. mg/mL	Selected compound	
	Percentage Violacein inhibition (%)	**MQSIC (mg/mL) ± (SD)**
0.06	**74.65**	0.06 ± (0.00)
	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	76.60	
0.06	**56.42**	
	Ocimene	
0.50	76.92	0.06 ± (0.00)
0.25	76.60	
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	76.60	

Conc. mg/mL	Selected compound	
	Percentage Violacein inhibition (%)	**MQSIC (mg/mL) ± (SD)**
0.06	**56.42**	0.06 ± (0.00)

n = 2 replicates; **bold** = mean percentage violacein inhibition at lowest concentration to inhibit ≥ 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).

Ahmad *et al*. (2015) investigated the inhibitory and anti-QS activity of α-Terpineol, Camphene, Estragole and Isoeugenol, the findings of which were in line with those of the current investigation in terms of MQSIC values. Mokhetho *et al.* (2018) 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. The current study evaluated the anti-QS activity of pure Eugenol, Geraniol and Menthol, and found that they did in fact have considerable anti-QS activity. Eugenol and Menthol inhibited over 50.00% of violacein production at the lowest concentration investigated (0.06 mg/mL), with percentage violacein inhibition values of 66.87 and 56.42%, respectively; and Geraniol inhibited violacein production by 76.44% at a concentration of 0.25 mg/mL. Ramirez-Rueda and Salvador (2020) reported that at a concentration of 0.06 mg/mL, Geraniol inhibited violacein by 54.12%. Similarly, the current investigation reports a 46.70% inhibition of violacein at 0.06 mg/mL. Husain *et al.* (2015) reported that Menthol inhibited violacein production in *C. violaceum* in a concentration-dependent manner, with a violacein inhibition of 85.00% at a concentration of 0.40 mg/mL and a violacein inhibition of 26.00% at 0.05 mg/mL. The current study reports that Menthol inhibited violacein production by 76.60 - 76.76% at concentrations of 0.13 - 0.50 mg/mL, and a 56.42% inhibition at a concentration of 0.06 mg/mL. Bound *et al.* (2020) evaluated the anti-QS activity of Menthol against *C. violaceum* and found that at a concentration of 0.75 µmol/mL (0.12 mg/mL), violacein production was inhibited by 65.00 - 79.00%, which corresponds to the findings of the current investigation, which reports that violacein was inhibited by 76.60% at a concentration of 0.13 mg/mL. Zhou *et al.* (2013) reported that Eugenol inhibited violacein production in *C. violaceum* by 48.00 - 56.50% at a concentration of 0.15 - 0.20 mM (0.03 mg/mL), and the current study reported a 66.87% inhibition of violacein at a concentration of 0.06 mg/mL. Al-Shabib *et al.* (2017) reported that Eugenol inhibited QS-regulated violacein production in *C. violaceum* in a concentration-dependent manner, with 80.00% of violacein inhibited at the highest concentration investigated (0.10 mg/mL). The current study reported a

69.23% inhibition of violacein production at a concentration of 0.13 mg/mL, which is approximately a difference of 10.77%, in terms of violacein inhibition.

3.3.3 The anti-quorum sensing (QS) activity of the equal ratio (1:1) combinations

The anti-QS activity of the combinations of the enantiomers with the selected compounds were carried out. First, the percentage violacein inhibition of the combinations at the varying concentrations are discussed. Thereafter, the MQSIC values, and the interactive efficacy of the combinations are evaluated. The interactive efficacy (ΣFQSIC) in terms of the effect of the combinations on the effective concentration to inhibit QS was then determined. This is followed by the discussion on the interactive efficacy in terms of the extent to which QS is inhibited (ΣFPVR). Lastly, the findings of the ΣFQSIC and ΣFPVR are compared and discussed.

3.3.3.1 The percentage violacein inhibition of the equal ratio (1:1) combinations at the varying concentrations

The percentage violacein inhibition of the combinations at varying concentrations investigated, are given in Table 3.6. The variations in the percentage violacein inhibition that were 20.00% or greater between enantiomeric pairs are highlighted. This was observed in 19.90% of the combinations and was particularly evident with the enantiomers of β-Pinene. Of the 19.90%, a total of 4.37% of those combinations had variations that were 40.00% or greater. For example, when the enantiomers of β-Pinene were combined with *p*-Cymene at a concentration of 0.06 mg/mL, (+)-β-Pinene inhibited 47.10% of violacein production, whereas (–)-β-Pinene only inhibited 2.16% (difference of 44.94%).

The percentage violacein inhibition at the MQSIC (i.e., the lowest concentration to inhibit ≥ 50.00 of violacein production) is highlighted for each of the combinations in Table 3.6. A total of 6.12% of the combinations did not inhibit violacein by 50.00% or greater at the concentrations investigated, and therefore had MQSIC values greater than 0.50 mg/mL. This was observed when the enantiomers of Limonene were combined with either Camphene, β-Caryophyllene or Linalyl acetate; and when the enantiomers of β-Pinene were combined with either Camphene or Ocimene, and the enantiomers of Menthone with β-Caryophyllene.

Table 3.6: The percentage violacein inhibition (%) of the 1:1 combinations of the enantiomers and selected compounds at the varying concentrations

Selected compound	Conc. mg/mL	Percentage violacein inhibition (%)													
		Enantiomer													
		(+)-Borneol	(–)-Borneol	(+)-Camphor	(–)-Camphor	(+)-Citronellal	(–)-Citronellal	(+)-Limonene	(–)-Limonene	(+)-Menthone	(–)-Menthone	(+)-α-Pinene	(–)-α-Pinene	(+)-β-Pinene	(–)-β-Pinene
Camphene	0.50	86.58	86.58	**72.86**	**71.58**	90.70	83.72	49.12	**53.52**	86.63	89.80	**61.76**	90.55	18.91	-8.15
	0.25	**63.37**	**74.39**	40.06	32.10	85.48	83.00	40.82	38.67	**62.70**	**80.20**	39.09	**52.76**	17.25	15.95
	0.13	43.03	37.84	36.18	20.31	**51.39**	**74.25**	25.10	39.45	43.25	42.40	20.46	43.41	35.65	20.50
	0.06	15.81	19.81	35.33	13.84	20.53	24.69	24.41	31.64	11.26	30.85	25.45	36.08	7.90	14.51
β-Caryophyllene	0.50	83.82	**80.18**	45.14	**54.77**	**87.48**	**83.96**	32.23	19.14	35.70	29.74	**70.15**	**59.21**	-35.94	-48.44
	0.25	**65.27**	22.80	21.73	8.17	43.52	46.93	13.77	16.60	28.80	-10.94	29.77	30.66	18.55	-35.49
	0.13	44.35	22.16	6.64	4.83	17.10	29.60	13.18	4.00	6.13	-17.19	25.04	30.13	15.07	-23.98
	0.06	43.19	1.55	-4.07	-2.33	16.41	-4.01	9.67	7.71	3.57	-5.72	3.49	15.47	15.94	-26.38
p-Cymene	0.50	86.58	86.78	87.53	86.80	90.70	90.82	67.09	**76.37**	88.98	89.73	81.04	62.64	70.51	**57.67**
	0.25	82.21	82.16	76.16	**84.43**	90.65	**90.70**	**64.26**	47.07	74.80	85.61	**67.25**	**54.91**	**52.17**	45.08
	0.13	**50.54**	**70.37**	**74.51**	36.58	**79.96**	43.79	49.02	22.85	**55.35**	**64.60**	37.47	36.63	31.67	27.58
	0.06	37.94	13.64	21.75	37.67	2.83	-0.09	40.72	0.98	31.04	35.35	22.07	26.79	19.13	-23.50
Estragole	0.50	86.58	86.87	87.74	87.32	88.15	90.70	86.72	87.11	84.27	89.80	90.82	91.03	90.14	83.93
	0.25	86.58	86.58	87.43	87.24	88.39	90.79	86.72	86.82	**84.15**	89.50	90.89	90.96	90.14	83.93
	0.13	**56.31**	**54.57**	**78.00**	**57.10**	**52.86**	**57.44**	77.64	**81.93**	46.71	**71.65**	**73.21**	**77.15**	**74.49**	**75.54**
	0.06	24.15	20.26	10.19	38.30	19.07	31.12	**70.31**	48.34	25.49	6.70	31.26	35.42	47.10	2.16
Eucalyptol	0.50	**86.56**	86.58	66.21	37.47	90.82	89.15	66.11	**72.85**	**89.60**	**77.42**	**62.14**	**53.18**	**66.01**	35.13
	0.25	45.73	**69.60**	**50.89**	30.74	64.06	**80.23**	**50.49**	31.74	46.30	37.72	49.98	38.79	37.10	0.48
	0.13	35.05	35.93	19.86	25.47	**52.39**	8.35	46.09	37.21	30.43	19.91	31.90	20.32	27.39	9.23
	0.06	32.03	4.85	24.10	6.74	19.92	6.11	35.16	20.90	27.34	16.75	9.65	6.54	15.65	-25.78
Eugenol	0.50	86.69	86.67	87.53	87.14	90.70	84.47	86.82	86.91	89.88	89.64	91.03	90.95	90.07	83.69

Selected compound	Conc. mg/mL	Percentage violacein inhibition (%)													
		Enantiomer													
		(+)-Borneol	(–)-Borneol	(+)-Camphor	(–)-Camphor	(+)-Citronellal	(–)-Citronellal	(+)-Limonene	(–)-Limonene	(+)-Menthone	(–)-Menthone	(+)-α-Pinene	(–)-α-Pinene	(+)-β-Pinene	(–)-β-Pinene
Eugenol	0.25	86.67	85.14	87.43	86.47	90.76	84.00	86.91	86.62	89.66	88.84	90.82	88.95	89.93	83.69
	0.13	**70.40**	72.88	**54.97**	**79.65**	**72.59**	**71.76**	82.91	81.54	80.80	**57.55**	60.17	66.32	**64.13**	**54.20**
	0.06	44.52	**62.62**	41.54	47.33	46.09	49.18	**79.00**	**64.45**	**67.54**	33.55	**59.23**	**52.48**	23.41	31.29
Geraniol	0.50	86.98	86.58	87.45	87.38	88.04	88.56	87.01	86.72	89.81	89.87	90.88	91.09	90.22	83.57
	0.25	86.58	85.49	87.43	87.27	88.21	88.39	86.13	86.82	89.96	89.65	88.99	89.20	89.64	82.25
	0.13	**53.79**	**65.49**	**52.56**	**75.06**	**68.36**	**59.09**	78.91	**75.68**	**81.17**	**69.10**	**66.44**	**81.36**	**58.55**	**66.91**
	0.06	25.77	15.59	24.02	47.74	26.80	32.10	**60.16**	45.90	44.70	48.82	26.26	30.51	42.68	8.15
Isoeugenol	0.50	86.87	86.58	87.53	87.32	90.70	90.79	86.72	86.82	83.86	89.64	90.82	90.82	90.22	83.69
	0.25	78.75	83.78	**87.53**	**86.03**	90.79	90.73	86.72	87.01	**83.27**	**89.50**	81.95	90.10	90.00	**83.69**
	0.13	**55.18**	**57.84**	42.66	43.45	**78.43**	**58.36**	74.41	**67.97**	37.57	37.11	**60.20**	**65.07**	71.81	31.06
	0.06	31.20	43.01	40.09	43.33	40.98	36.60	**64.55**	41.11	30.94	29.98	37.99	37.36	**54.35**	-2.52
Linalyl acetate	0.50	83.12	86.58	**68.58**	73.58	90.49	90.70	28.32	40.23	89.73	89.72	59.58	**56.55**	**72.97**	**56.46**
	0.25	**80.75**	**72.58**	20.75	**51.85**	71.28	**81.55**	22.66	27.34	**65.41**	**58.15**	**52.19**	45.24	37.61	35.04
	0.13	41.69	25.50	33.42	17.50	**59.95**	30.81	24.12	24.61	33.04	14.82	33.38	24.60	26.88	36.45
	0.06	22.23	24.36	19.92	-3.99	35.78	30.32	26.17	16.80	26.96	10.27	33.90	18.47	24.49	23.47
Menthol	0.50	86.58	86.58	87.53	84.70	88.50	86.75	86.72	86.72	89.80	89.80	91.15	90.96	90.29	84.05
	0.25	86.58	86.47	87.38	84.48	88.27	86.63	87.11	86.82	89.58	89.48	91.09	90.88	86.96	83.93
	0.13	**58.28**	**54.11**	**58.01**	**75.44**	**62.29**	**72.64**	74.80	**72.27**	**59.02**	**55.04**	**72.48**	**62.48**	**59.28**	**55.40**
	0.06	38.23	26.99	28.86	47.47	23.72	26.89	**54.00**	38.87	34.70	27.00	41.40	41.67	27.61	17.63
Ocimene	0.50	86.49	86.78	85.20	**86.13**	90.70	90.87	86.82	86.82	89.58	89.65	90.82	53.74	0.45	24.58
	0.25	76.94	**71.11**	**73.60**	46.77	87.15	**88.85**	**58.20**	**50.29**	66.70	74.39	**65.29**	**50.03**	7.61	-29.50
	0.13	**51.09**	37.91	18.16	0.73	**57.17**	47.82	49.41	32.62	**55.15**	86.05	41.61	25.79	32.61	-35.97
	0.06	27.01	10.45	10.82	0.63	16.80	-3.29	47.46	25.98	30.22	**60.10**	8.57	27.90	11.88	-37.41
Sabinene hydrate	0.50	86.58	86.87	87.22	87.35	90.70	90.70	86.82	86.91	89.72	89.73	90.88	90.88	90.14	**84.05**

Selected compound	Conc. mg/mL	Percentage violacein inhibition (%)													
		Enantiomer													
		(+)-Borneol	(–)-Borneol	(+)-Camphor	(–)-Camphor	(+)-Citronellal	(–)-Citronellal	(+)-Limonene	(–)-Limonene	(+)-Menthone	(–)-Menthone	(+)-α-Pinene	(–)-α-Pinene	(+)-β-Pinene	(–)-β-Pinene
Sabinene hydrate	0.25	86.58	86.36	**57.88**	**54.10**	**87.36**	87.66	71.58	74.32	87.66	82.27	88.63	90.28	**78.48**	37.77
	0.13	**67.83**	**59.61**	35.36	25.22	22.62	**56.78**	**51.46**	**51.37**	**62.95**	**61.59**	**59.50**	**58.20**	40.58	3.12
	0.06	39.95	15.22	21.99	5.67	20.89	29.32	36.33	34.47	36.40	9.10	31.22	9.78	29.20	-8.27
γ-Terpinene	0.50	86.78	85.54	87.22	87.38	90.54	90.87	75.59	68.95	89.66	89.94	71.61	**52.75**	45.72	-23.86
	0.25	78.86	79.57	**66.17**	70.80	**90.21**	**90.01**	56.54	**59.47**	88.33	**86.02**	**55.48**	40.99	12.54	-45.80
	0.13	**59.66**	**65.85**	15.03	**57.46**	33.13	36.36	**55.37**	37.89	**67.49**	33.17	39.31	21.53	12.03	-11.63
	0.06	40.80	16.63	11.55	41.09	25.07	30.01	34.28	17.38	33.39	11.80	24.37	4.34	31.09	-73.38
α-Terpineol	0.50	86.47	86.67	87.53	87.14	90.70	86.51	86.72	87.01	84.04	89.72	91.00	90.88	90.43	84.05
	0.25	86.36	85.93	87.01	87.22	90.70	86.32	86.91	86.91	82.63	89.09	86.67	89.94	90.36	83.45
	0.13	**65.17**	66.71	**62.53**	**83.87**	**76.54**	**76.18**	77.54	**77.34**	**73.70**	**64.37**	82.98	**79.56**	76.38	**50.12**
	0.06	39.41	**50.31**	4.45	27.62	34.52	44.10	**66.02**	45.61	17.14	34.30	**65.42**	30.33	**65.00**	30.94

n = 2 replicates, with third consecutive replicate to confirm variations between enantiomers; **bold** = mean percentage violacein inhibition at the MQSIC; red = difference in percentage violacein inhibition that is ≥ 20.00%; 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).

In combination with certain selected compounds, (+)-β-Pinene inhibited violacein production, whereas (–)-β-Pinene promoted the production of violacein. It was also observed that (–)-β-Pinene in combination with β-Caryophyllene or γ-Terpinene promoted the production of violacein, by 48.44% and 23.86% at the highest concentration investigated (0.50 mg/mL), respectively. However, (+)-β-Pinene inhibited violacein production in combination with β-Caryophyllene or γ-Terpinene (by less than 50.00%).

3.3.3.2 The MQSIC values and interactive efficacy of the equal ratio (1:1) combinations

The MQSIC values and interactive efficacy profiles of the enantiomers in combination with the selected compounds were determined (Tables 3.8 - 3.14). To the best of my knowledge, no investigations could be found where pure compounds, or the enantiomeric forms of the compounds, were combined at fixed ratios and investigated for their anti-QS activity. Reviews conducted by Silva *et al.* (2016), Khan *et al*. (2018) and Reichling (2020) highlight the research conducted on essential oil compounds in terms of their effect on various QS-regulated virulence factors, however, no evidence of interactive QS efficacy of the essential oil compounds could be found. Vasudevan *et al.* (2018) reports on the synergism between natural QS inhibitors and antibiotics. For example, Baicalein, a compound from Thyme leaf extract, was shown to interfere with the transcriptional activator protein (TraR) of the *P. aeruginosa* QS system and was synergistic with ampicillin (Zeng *et al*., 2008). While this demonstrates some evidence of a positive interaction, the study acutely focused on extracts and not essential oil compounds. A detailed analysis of the anti-QS activity of the enantiomers in combination are discussed as follows and to the best of my knowledge, no other investigations in which the enantiomers, in its racemic or enantiomeric forms, were evaluated for their combined anti-QS activity.

3.3.3.2.1 The interactive efficacy of the enantiomers of Borneol in combination

The mean MQSIC and interactive efficacy (ΣFQSIC and ΣFPVR) of the enantiomers of Borneol in combination are given in Table 3.7. The MQSIC values ranged between 0.06 - 0.25 mg/mL and were equivalent between the enantiomers in combination. In terms of the ΣFQSIC values, 25.00% of the combinations were additive, 67.86% were non-interactive and 7.14% were undefined. This means that due to the MQSIC value of either the independent compounds or the combination, being greater than the highest concentration investigated (0.50 mg/mL), the MQSIC could not be determined and hence the interactive efficacy could not be defined

evaluated. No synergy or antagonism was observed. Only one combination demonstrated a difference in the type of interaction between the enantiomers, which was 'additive versus non-interactive'. (+)-Borneol was non-interactive in combination with Eugenol (ΣFQSIC = 1.50), whereas (–)-Borneol interacted additively (ΣFQSIC = 0.75).

In terms of the ΣFPVR, 7.14% of the combinations were synergistic, 39.28% were additive, 46.43% were non-interactive, and 7.14% were undefined. No antagonism was observed. Synergy was observed with (+)-Borneol in combination with Eucalyptol or Linalyl acetate, with ΣFPVR values of 0.31 and 0.49, respectively. The types of variations observed were 'synergy versus additive', or 'additive versus non-interactive', the latter being more prevalent. In combination with Ocimene, (–)-Borneol interacted additively (ΣFPVR = 0.83), whereas (+)-Borneol was non-interactive (ΣFPVR = 0.16). However, in combination with either Eugenol, Menthol, Sabinene hydrate or α-Terpineol, (+)-Borneol interacted additively (ΣFPVR values ranging between 0.81 - 0.98), whereas (–)-Borneol was non-interactive (ΣFPVR values ranging between 1.20 - 1.64).

Although the enantiomers of Borneol mostly interacted similarly in terms of the combined effective concentration to inhibit violacein production (ΣFQSIC), they varied more in terms of the extent of violacein that was inhibited (ΣFPVR) in combination. Overall, where variations were observed in terms of the ΣFPVR, (+)-Borneol interacted more favourably to inhibit the extent of bacterial communication in combination, when compared to (–)-Borneol.

Table 3.7: The mean MQSIC, interactive efficacy (ΣFQSIC and ΣFPVR) and interaction classification of the enantiomers of Borneol in combination

Selected compound	**Mean MQSIC (mg/mL)**		**ΣFQSIC**		**ΣFPVR**	
	(+)-Borneol	(–)-Borneol	(+)-Borneol	(–)-Borneol	(+)-Borneol	(–)-Borneol
Camphene	0.25 ± 0.00	0.25 ± 0.00	nd; nd	nd; nd	nd; nd	nd; nd
β-Caryophyllene	0.25 ± 0.00	0.25 ± 0.00	1.25; Ind	1.25; Ind	0.81; Add	0.56; Add
p-Cymene	0.13 ± 0.00	0.13 ± 0.00	1.50; Ind	1.50; Ind	1.46; Ind	1.02; Ind
Estragole	0.13 ± 0.00	0.13 ± 0.00	1.00; Add	1.00; Add	1.43; Ind	1.71; Ind
Eucalyptol	0.50 ± 0.00	0.25 ± 0.00	2.67; Ind	1.33; Ind	**0.31; Syn**	**0.85; Add**
Eugenol	0.13 ± 0.00	0.06 ± 0.00	**1.50; Ind**	**0.75; Add**	**0.81; Add**	**1.20; Ind**

Selected compound	Mean MQSIC (mg/mL)		ΣFQSIC		ΣFPVR	
	(+)-Borneol	(–)-Borneol	(+)-Borneol	(–)-Borneol	(+)-Borneol	(–)-Borneol
Geraniol	0.13 ± 0.00	0.13 ± 0.00	0.75; Add	0.75; Add	1.51; Ind	1.30; Ind
Isoeugenol	0.13 ± 0.00	0.13 ± 0.00	1.50; Ind	1.50; Ind	1.40; Ind	1.53; Ind
Linalyl acetate	0.25 ± 0.00	0.25 ± 0.00	1.25; Ind	1.25; Ind	**0.49; Syn**	**0.83; Add**
Menthol	0.13 ± 0.00	0.13 ± 0.00	1.50; Ind	1.50; Ind	**0.96; Add**	**1.28; Ind**
Ocimene	0.13 ± 0.00	0.25 ± 0.00	1.50; Ind	3.00; Ind	**1.16; Ind**	**0.83; Add**
Sabinene hydrate	0.13 ± 0.00	0.13 ± 0.00	1.00; Add	1.00; Add	**0.92; Add**	**1.35; Ind**
γ-Terpinene	0.13 ± 0.00	0.13 ± 0.00	1.50; Ind	1.50; Ind	0.91; Add	0.94; Add
α-Terpineol	0.13 ± 0.00	0.13 ± 0.00	1.50; Ind	1.50; Ind	**0.98; Add**	**1.64; Ind**

n = 2 replicates, with third consecutive replicate to confirm variations between enantiomers; **red bold** = variation in interactive efficacy (ΣFQSIC or ΣFPVR); Syn = synergy, Add = additive, Ind = non-interactive, 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).

3.3.3.2.2 The interactive efficacy of the enantiomers of Camphor in combination

The mean MQSIC and interactive efficacy (ΣFQSIC and ΣFPVR) of the enantiomers of Camphor in combination are given in Table 3.8. The MQSIC values ranged between 0.13 - 0.50 mg/mL. However, a few combinations had MQSIC values > 0.50 mg/mL. This was evident with (+)-Camphor in combination with β-Caryophyllene, and (–)-Camphor in combination with Eucalyptol. The MQSIC values between the enantiomers of Camphor in combination were mostly equivalent, with one exception. This was observed when combined with Eucalyptol, where (+)-Camphor had a combined MQSIC of 0.25 mg/mL, whereas (–)-Camphor had a combined MQSIC that was > 0.50 mg/mL.

In terms of ΣFQSIC, 10.71% of the combinations were synergistic, 21.43% were additive, 53.57% were non-interactive, and 14.29% were undefined. No antagonism was observed. Synergy was observed with both enantiomers of Camphor in combination with Geraniol (ΣFQSIC = 0.38), as well as (–)-Camphor in combination with Linalyl acetate (ΣFQSIC = 0.50). Only two combinations demonstrated a difference in the type of interaction, which were ‘synergy versus additive’ and ‘additive versus non-interactive’. In combination with Linalyl

acetate, (+)-Camphor was additive (ΣFQSIC = 1.00), whereas (–)-Camphor interacted synergistically (ΣFQSIC = 0.50). In combination with Sabinene hydrate, (+)-Camphor was additive (ΣFQSIC of 0.94), whereas (–)-Camphor was non-interactive (ΣFQSIC = 1.25).

In terms of ΣFPVR, 14.29% of the combinations were synergistic, 17.86% were additive, 53.57% were non-interactive, and 14.29% were undefined. No antagonism was observed. Synergy was observed with (–)-Camphor in combination with either *p*-Cymene, Isoeugenol, Ocimene or α-Terpineol, with ΣFPVR values ranging between 0.32 - 0.45. The types of variations observed were 'synergy versus additive', 'synergy versus non-interactive' and 'additive versus non-interactive'. The latter two were the most prevalent. When combined with β-Caryophyllene, Ocimene or α-Terpineol, (–)-Camphor was synergistic with ΣFPVR values ranging between 0.32 - 0.45. However, (+)-Camphor was non-interactive in combination, with ΣFPVR values ranging between 1.29 - 1.99. When combined with either Eugenol, Geraniol, Menthol, or γ-Terpinene, (–)-Camphor was additive, with ΣFPVR ranging between 0.54 - 0.94. However, (+)-Camphor was non-interactive, with ΣFPVR values ranging between 1.61 - 2.74.

What is evident is that although the enantiomers of Camphor mostly interacted similarly in terms of the combined effective concentration to inhibit violacein production (ΣFQSIC), they varied more in terms of the extent of violacein that was inhibited (ΣFPVR) in combination. Overall, where variations were observed in terms of the ΣFPVR, (–)-Camphor interacted more favourably to inhibit the extent of bacterial communication in combination, when compared to (+)-Camphor.

Qaisrani *et al.* (2021) evaluated the anti-QS activity of the essential oil *Seriphidium quett*ense (Podlech) K.Bremer and Humphries, of which the major constituents were found to be Camphor (28.80%) and Eucalyptol (3.80%). The study reported that the *S. quettense* essential oil inhibited violacein production by approximately 60.00% at a concentration of 3.70% (0.04 mg/mL). The current investigation evaluated the combined anti-QS activity of the enantiomers of Camphor in combination with Eucalyptol and found that (+)-Camphor had a combined MQSIC of 0.25 mg/mL, whereas (–)-Camphor in combination did not inhibit more than 50.00% of violacein production at the highest concentration investigated. In addition, (+)-Camphor interacted additively with Eucalyptol in terms of ΣFPVR. This means that there was a decrease in the effective concentration required to disrupt bacterial communication of (+)-Camphor and Eucalyptol in combination, compared to the two compounds tested

independently. The results obtained by Qaisrani *et al.* (2021) in terms of the anti-QS activity of *S. quettense* essential oil may be as a result of the combination of Camphor and Eucalyptol, or other major or minor compounds present. In addition, the enantiomeric distribution of (+)-Camphor may have been greater than (–)-Camphor. However, one needs to consider the influence of other compounds within the neat essential oils investigated.

Table 3.8: The mean MQSIC, interactive efficacy (ΣFQSIC and ΣFPVR) and interaction classification of the enantiomers of Camphor in combination

Selected compound	**Mean MQSIC (mg/mL)**		**ΣFQSIC**		**ΣFPVR**	
	(+)-Camphor	(–)-Camphor	(+)-Camphor	(–)-Camphor	(+)-Camphor	(–)-Camphor
Camphene	0.50 ± 0.00	0.50 ± 0.00	nd; nd	nd; nd	nd; nd	nd; nd
β-Caryophyllene	> 0.50	0.50 ± 0.00	nd; nd	1.00; Add	nd; nd	1.02; Ind
p-Cymene	0.13 ± 0.00	0.25 ± 0.00	1.13; Ind	2.25; Ind	**1.39; Ind**	**0.45; Syn**
Estragole	0.13 ± 0.00	0.13 ± 0.00	0.63; Add	0.63; Add	1.27; Ind	1.37; Ind
Eucalyptol	**0.25 ± 0.00**	**> 0.50**	0.58; Add	nd; nd	2.35; Ind	nd; nd
Eugenol	0.13 ± 0.00	0.13 ± 0.00	1.13; Ind	1.13; Ind	**2.35; Ind**	**0.54; Add**
Geraniol	0.13 ± 0.00	0.13 ± 0.00	0.38; Syn	0.38; Syn	**2.74; Ind**	**0.80; Add**
Isoeugenol	0.25 ± 0.00	0.25 ± 0.00	2.25; Ind	2.25; Ind	**0.70; Add**	**0.43; Syn**
Linalyl acetate	0.50 ± 0.00	0.25 ± 0.00	**1.00; Add**	**0.50; Syn**	1.58; Ind	1.18; Ind
Menthol	0.13 ± 0.00	0.13 ± 0.00	1.13; Ind	1.13; Ind	**2.02; Ind**	**0.55; Add**
Ocimene	0.25 ± 0.00	0.25 ± 0.00	2.25; Ind	2.25; Ind	**1.29; Ind**	**0.32; Syn**
Sabinene hydrate	0.19 ± 0.09	0.25 ± 0.00	**0.94; Add**	**1.25; Ind**	2.26; Ind	1.28; Ind
γ-Terpinene	0.25 ± 0.00	0.13 ± 0.00	2.25; Ind	1.13; Ind	**1.61; Ind**	**0.93; Add**
α-Terpineol	0.13 ± 0.00	0.13 ± 0.00	1.13; Ind	1.13; Ind	**1.99; Ind**	**0.44; Syn**

n = 2 replicates, with third consecutive replicate to confirm variations between enantiomers; **red bold** = variation in interactive efficacy (ΣFQSIC or ΣFPVR); Syn = synergy, Add = additive, Ind = non-interactive, 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).

3.3.3.2.3 The interactive efficacy of the enantiomers of Citronellal in combination

The mean MQSIC and interactive efficacy (ΣFQSIC and ΣFPVR) of the enantiomers of Citronellal in combination are given in Table 3.9. The MQSIC values ranged between 0.09 - 0.50 mg/mL. In terms of the ΣFQSIC, 14.29% of the combinations were synergistic, 14.29% were additive, 64.29% were non-interactive, and 7.14% were undefined. No antagonism was observed. Synergy was noted with (+)-Citronellal in combination with either Eucalyptol or Linalyl acetate, with ΣFQSIC values of 0.42 and 0.38, respectively. Synergy was also observed with both enantiomers of Citronellal in combination with Geraniol (ΣFQSIC = 0.50). The types of variations observed were 'additive versus non-interactive' and 'synergy versus additive'. In combination with Eugenol, (+)-Citronellal interacted additively (ΣFQSIC = 0.94), whereas (–)-Citronellal was non-interactive (ΣFQSIC = 1.25). However, in combination with Sabinene hydrate, (–)-Citronellal interacted additively (ΣFQSIC = 0.75), whereas (–)-Citronellal was non-interactive (ΣFQSIC = 1.50).

In terms of ΣFPVR, 25.00% of the combinations were additive, 67.87% were non-interactive, and 7.14% were undefined. No synergy or antagonism was observed. Only three combinations demonstrated a difference in the type of interaction, which was 'additive versus non-interactive'. In combination with Ocimene or *p*-Cymene, (–)-Citronellal interacted additively, with ΣFPVR values ranging between 0.56 - 0.61. However, (+)-Citronellal in combination was non-interactive, with ΣFPVR values ranging between 1.43 - 2.82. Although, in combination with Sabinene hydrate, it was (+)-Citronellal that interacted additively (ΣFPVR = 0.89), whereas (–)-Citronellal was non-interactive (ΣFPVR = 2.57). Overall, (+)-Citronellal often interacted more favourably than (–)-Citronellal in terms of the ΣFQSIC. In addition, it was interesting to note that in combination with Sabinene hydrate, (–)-Citronellal interacted more favourably in terms of the combined effective concentration to inhibit violacein production (ΣFQSIC), whereas (+)-Citronellal interacted more favourably in terms of the extent of violacein that was inhibited (ΣFPVR) in combination.

Luís *et al.* (2017) evaluated the anti-QS activity of *Eucalyptus citriodora* Hook. essential oil using the disc diffusion method. The study reported that the essential oil had considerable anti-QS activity at a concentration of 10 µL/disc against violacein production. The major constituents of *E. citriodora* were reported to be Citronellal (78.15%) and Eucalyptol (2.05%). The current investigation evaluated the 1:1 combined anti-QS activity of the enantiomers of

Citronellal with Eucalyptol and found that both had MQSIC values as low as 0.13 and 0.25 mg/mL for (+)-Citronellal and (–)-Citronellal, in combination with Eucalyptol, respectively. In addition, (+)-Citronellal interacted synergistically with Eucalyptol (ΣFQSIC = 0.42) and (–)-Citronellal was additive (ΣFQSIC = 0.83). This suggests that the anti-QS activity reported by Luís *et al.* (2017) for *E. citriodora*, may be as a result of the combination of its major constituents. However, the effects of the other major or minor constituents present in the essential oil must also be considered.

Table 3.9: The mean MQSIC, interactive efficacy (ΣFQSIC and ΣFPVR) and interaction classification of the enantiomers of Citronellal in combination

Selected compound	**Mean MQSIC (mg/mL)**		**ΣFQSIC**		**ΣFPR at MQSIC**	
	(+)-Citronellal	(–)-Citronellal	(+)-Citronellal	(–)-Citronellal	(+)-Citronellal	(–)-Citronellal
Camphene	0.19 ± 0.09	0.13 ± 0.00	nd; nd	nd; nd	nd; nd	nd; nd
β-Caryophyllene	0.50 ± 0.00	0.50 ± 0.00	1.50; Ind	1.50; Ind	0.82; Add	0.87; Add
p-Cymene	0.13 ± 0.00	0.19 ± 0.09	1.25; Ind	1.88; Ind	**1.43; Ind**	**0.56; Add**
Estragole	0.19 ± 0.09	0.19 ± 0.09	1.13; Ind	1.13; Ind	3.52; Ind	2.70; Ind
Eucalyptol	0.13 ± 0.00	0.25 ± 0.00	**0.42; Syn**	**0.83; Add**	3.10; Ind	1.06; Ind
Eugenol	0.09 ± 0.05	0.13 ± 0.00	**0.94; Add**	**1.25; Ind**	1.90; Ind	1.64; Ind
Geraniol	0.13 ± 0.00	0.13 ± 0.00	0.50; Syn	0.50; Syn	2.37; Ind	2.60; Ind
Isoeugenol	0.13 ± 0.00	0.13 ± 0.00	1.25; Ind	1.25; Ind	1.58; Ind	2.59; Ind
Linalyl acetate	0.13 ± 0.00	0.25 ± 0.00	**0.38; Syn**	**0.75; Add**	2.70; Ind	1.04; Ind
Menthol	0.13 ± 0.00	0.13 ± 0.00	1.25; Ind	1.25; Ind	2.46; Ind	1.47; Ind
Ocimene	0.19 ± 0.09	0.19 ± 0.09	1.88; Ind	1.88; Ind	**2.82; Ind**	**0.61; Add**
Sabinene hydrate	0.25 ± 0.00	0.13 ± 0.00	**1.50; Ind**	**0.75; Add**	**0.89; Add**	**2.57; Ind**
γ-Terpinene	0.25 ± 0.00	0.25 ± 0.00	2.50; Ind	2.50; Ind	0.63; Add	0.53; Add
α-Terpineol	0.13 ± 0.00	0.13 ± 0.00	1.25; Ind	1.25; Ind	1.65; Ind	1.41; Ind

n = 2 replicates, with third consecutive replicate to confirm variations between enantiomers; **red bold** = variation in interactive efficacy (ΣFQSIC or ΣFPVR); Syn = synergy, Add = additive, Ind = non-interactive, 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).

3.3.3.2.4 The interactive efficacy of the enantiomers of Limonene in combination

The mean MQSIC and interactive efficacy (ΣFQSIC and ΣFPVR) of the enantiomers of Limonene in combination are given in Table 3.10. The MQSIC values ranged between 0.06 - 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 (–)-Limonene in combination with Camphene. The MQSIC values between the enantiomeric pairs in combination were mostly equivalent, with two exceptions. In combination with *p*-Cymene, (+)-Limonene had a combined MQSIC of 0.19 mg/mL, whereas (–)-Limonene had a combined MQSIC of 0.50 mg/mL. In addition, when combined with α-Terpineol, (+)-Limonene had a combined MQSIC of 0.06 mg/mL, whereas (–)-Limonene had a combined MQSIC of 0.25 mg/mL. These were the greatest variations observed in terms of MQSIC values between the enantiomers in combination, and (+)-Limonene had the reduced MQSIC in both combinations.

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 undefined. Antagonism was observed with one combination ((–)-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 (+)-Limonene and (–)-Limonene, respectively. Synergy was also observed with (+)-Limonene in combination with Estragole (ΣFQSIC = 0.31). The types of variation observed were 'synergy versus additive', 'non-interactive versus antagonism', and 'additive versus non-interactive', the latter being the most prevalent. This was observed in combination with either Eucalyptol, Isoeugenol, Menthol or α-Terpineol, where (+)-Limonene interacted additively, with the ΣFQSIC values ranging between 0.56 - 0.88. However, (–)-Limonene was non-interactive in combination, with ΣFQSIC values ranging between 1.17 - 2.33. 'Non-interactive versus antagonism', was the greatest variation that was observed in terms of interactive efficacy. This was seen in combination with *p*-Cymene, where (+)-Limonene was non-interactive (ΣFQSIC of 1.69), whereas (–)-Limonene interacted antagonistically (ΣFQSIC = 4.67).

The ΣFPVR studies revealed that 50.00% of the combinations were additive, 28.57% were non-interactive, and 21.43% were undefined. No synergy or antagonism was observed. The only type of variation observed was 'additive versus non-interactive'. In combination with Ocimene, (+)-Limonene interacted additively (ΣFPVR = 0.96), whereas (–)-Limonene was

non-interactive (ΣFPVR = 1.16). However, (–)-Limonene interacted additively in combination with either *p*-Cymene, Eucalyptol, Geraniol, Isoeugenol or Menthol, with ΣFPVR values ranging between 0.61 - 0.99; whereas (+)-Limonene was non-interactive, with ΣFPVR values ranging between 1.02 - 1.28. Overall, (+)-Limonene interacted more favourably in terms of ΣFQSIC, whereas (–)-Limonene interacted more favourably in terms of ΣFPVR. This means that (+)-Limonene interacted with certain selected compounds in a way that reduced the combined effective concentration to inhibit bacterial communication by more than 50.00%, when compared to (–)-Limonene. However, (–)-Limonene interacted with certain selected compounds in way that increased the extent to which bacterial communication was inhibited, and therefore had increased anti-QS activity, when compared to (+)-Limonene at the same concentration.

Poli *et al.* (2018) evaluated the anti-QS activity of *Citrus limon* L. essential oil against *C. violaceum* and reported the MQSIC value of 0.10 mg/mL, however, the percentage of violacein inhibition was not reported. The major compounds of the essential oil were reported to be Limonene (66.40%) and γ-Terpinene (10.10%). The current investigation evaluated the enantiomers of Limonene in combination with γ-Terpinene in a 1:1 ratio, and found that (+)-Limonene in combination with γ-Terpinene inhibited QS at an MQSIC of 0.13 mg/mL, similar to what Poli *et al.* (2018) reported for *C. limon* essential oil. The current investigation also reported that (–)-Limonene had a combined MQSIC of 0.25 mg/mL with γ-Terpinene. In addition, the current investigation reports that the interaction between the enantiomers of Limonene with γ-Terpinene are additive (ΣFPVR = 0.90 - 0.97). This means that there was an increase in the extent of the disruption of bacterial communication of the two compounds when combined, compared to the compounds when tested independently. Luís *et al.* (2016) evaluated the anti-QS activity of *Eucalyptus radiata* DC. essential oil, of which the major constituents are Limonene (68.51%) and α-Terpineol (8.60%). The study reported that violacein was inhibited by approximately 50.00% at a concentration of 0.10 µL/mL (0.10 mg/mL). The current investigation evaluated the combined anti-QS activity of the enantiomers of Limonene with α-Terpineol, and similarly reports an MQSIC of 0.06 mg/mL for (+)-Limonene in combination with α-Terpineol. (–)-Limonene had a higher combined MQSIC of 0.25 mg/mL. In addition, the interaction between (+)-Limonene with α-Terpineol was found to be additive in terms of the combination having a lower MQSIC and greater extent of violacein being inhibited, than the compounds when tested alone. Therefore, it is likely that the anti-QS activity reported by Luís *et al.* (2016) for *E. radiata* essential oil, and that reported for *C. limon* by Poli

et al. (2018), is as a result of the combined anti-QS activity for the major compounds. In addition, the enantiomeric distribution of (+)-Limonene in the essential oils may have been greater than (–)-Limonene. However, one needs to consider the influence of other compounds within the neat essential oils.

Table 3.10: The mean MQSIC, interactive efficacy (ΣFQSIC and ΣFPVR) and interaction classification of the enantiomers of Limonene in combination

Selected compound	Mean MQSIC (mg/mL)		ΣFQSIC		ΣFPVR at MQSIC	
	(+)-Limonene	(–)-Limonene	(+)-Limonene	(–)-Limonene	(+)-Limonene	(–)-Limonene
Camphene	> 0.50	0.50 ± 0.00	nd; nd	nd; nd	nd; nd	nd; nd
β-Caryophyllene	> 0.50	> 0.50	nd; nd	nd; nd	nd; nd	nd; nd
p-Cymene	**0.19 ± 0.09**	**0.50 ± 0.00**	**1.69; Ind**	**4.67; Ant**	**1.03; Ind**	**0.69; Add**
Estragole	0.06 ± 0.00	0.13 ± 0.00	**0.31; Syn**	**0.67; Add**	0.95; Add	0.58; Add
Eucalyptol	0.38 ± 0.18	0.50 ± 0.00	**0.88; Add**	**1.33; Ind**	**1.10; Ind**	**0.61; Add**
Eugenol	0.06 ± 0.00	0.06 ± 0.00	0.56; Add	0.58; Add	0.56; Add	0.95; Add
Geraniol	0.06 ± 0.00	0.13 ± 0.00	0.19; Syn	0.42; Syn	**1.28; Ind**	**0.79; Add**
Isoeugenol	0.06 ± 0.00	0.13 ± 0.00	**0.56; Add**	**1.17; Ind**	**1.08; Ind**	**0.99; Add**
Linalyl acetate	> 0.50	> 0.50	nd; nd	nd; nd	nd; nd	nd; nd
Menthol	0.06 ± 0.00	0.13 ± 0.00	**0.56; Add**	**1.17; Ind**	**1.02; Ind**	**0.63; Add**
Ocimene	0.25 ± 0.00	0.25 ± 0.00	2.25; Ind	2.33; Ind	**0.96; Add**	**1.16; Ind**
Sabinene hydrate	0.13 ± 0.00	0.13 ± 0.00	0.63; Add	0.67; Add	1.35; Ind	1.37; Ind
γ-Terpinene	0.13 ± 0.00	0.25 ± 0.00	1.13; Ind	2.33; Ind	0.97; Add	0.90; Add
α-Terpineol	**0.06 ± 0.00**	**0.25 ± 0.00**	**0.56; Add**	**2.33; Ind**	0.93; Add	0.63; Add

n = 2 replicates, with third consecutive replicate to confirm variations between enantiomers; **red bold** = variation in interactive efficacy (ΣFQSIC or ΣFPVR); Syn = synergy, Add = additive, Ind = non-interactive, Ant = antagonism, 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).

3.3.3.2.5 The interactive efficacy of the enantiomers of Menthone in combination

The mean MQSIC and interactive efficacy (ΣFQSIC and ΣFPVR) of the enantiomers of Menthone in combination are given in Table 3.11. The MQSIC values ranged between 0.06 -

0.50 mg/mL. However, in combination with β-Caryophyllene, the combined MQSIC values for both (+)-Menthone and (–)-Menthone was > 0.50 mg/mL. The MQSIC values of the enantiomers in combination were equivalent. In terms of ΣFQSIC values, 7.14% of the combinations were synergistic, 25.00% were additive, 53.57% were non-interactive, and 14.29% were undefined. Synergy was observed for both enantiomers of Menthone in combination with Geraniol (ΣFQSIC = 0.38). The only variation between the enantiomers of Menthone in combination was 'additive versus non-interactive'. This was observed in combination with Eugenol, where (+)-Menthone interacted additively (ΣFQSIC = 0.63), whereas (–)-Menthone was non-interactive (ΣFQSIC = 1.25). In combination with Estragole or Ocimene, (–)-Menthone interacted additively, with ΣFQSIC values ranging between 0.63 - 0.75. However, (+)-Menthone was non-interactive, with ΣFQSIC values ranging between 1.25 - 1.50.

In terms of the ΣFPVR, 7.14% of the combinations were synergistic, 32.14% were additive, 46.43% were non-interactive, and 14.29% were undefined. Synergy was observed with (+)-Menthone in combination with Eucalyptol (ΣFPVR = 0.26) and (–)-Menthone in combination with Isoeugenol (ΣFPVR = 0.50). The types of variations observed were 'synergy versus additive', and 'additive versus non-interactive', the latter being the most prevalent. This was observed in combination with either Estragole, Eucalyptol, Eugenol, Geraniol, Linalyl acetate or α-Terpineol. (+)-Menthone interacted additively in combination with the selected compounds, with ΣFPVR values ranging between 0.55 - 0.94. However, (–)-Menthone was non-interactive, with ΣFPVR values ranging between 1.39 - 1.85. What is evident is that although the enantiomers of Menthone mostly interacted similarly in terms of the combined effective concentration to inhibit violacein production (ΣFQSIC), they varied more in terms of the extent of violacein that was inhibited (ΣFPVR) in combination. Overall, where variations were observed in terms of the ΣFPVR, (+)-Menthone often interacted more favourably to inhibit the extent of bacterial communication in combination, when compared to (–)-Menthone.

Husain *et al.* (2015) evaluated the anti-QS activity of *Mentha piperita* Stokes, essential oil, of which the major constituents are Menthol (36.87%) and Menthone (16.44%). The study reported that *M. piperita* inhibited approximately 50.00% of violacein production in *C. violaceum* at a concentration 0.012% (1.2×10^{-4} mg/mL). Yang *et al.* (2018) also evaluated the anti-QS activity of *M. piperita* through evaluation of the inhibition of QS-regulated light production in *Escherichia coli*. The major compounds of *M. piperita* were reported to be

Menthol (41.36%) and *l*-Menthone (17.78%). The study reported that the essential oil significantly ($p < 0.05$) inhibited light production in *E. coli* at a concentration of 0.05% (5 x 10^{-4} mg/mL). The current investigation evaluated the combined anti-QS activity of the enantiomers of Menthone in combination with Menthol and reports MQSIC values of 0.19 and 0.13 mg/mL for (+)-Menthone and (–)-Menthone, in combination with Menthol, respectively. Therefore, the anti-QS activity reported by Husain *et al.* (2015) and Yang *et al.* (2018) for *M. piperita* is likely due to the combination of the major compounds in combination with one or more of the minor compounds present.

Table 3.11: The mean MQSIC, interactive efficacy (ΣFQSIC and ΣFPVR) and interaction classification of the enantiomers of Menthone in combination

Selected compound	**Mean MQSIC (mg/mL)**		**ΣFQSIC**		**ΣFPR at MQSIC**	
	(+)-Menthone	(–)-Menthone	(+)-Menthone	(–)-Menthone	(+)-Menthone	(–)-Menthone
Camphene	0.19 ± 0.09	0.25 ± 0.00	nd; nd	nd; nd	nd; nd	nd; nd
β-Caryophyllene	> 0.50	> 0.50	nd; nd	nd; nd	nd; nd	nd; nd
p-Cymene	0.13 ± 0.00	0.13 ± 0.00	1.25; Ind	1.25; Ind	1.40; Ind	1.62; Ind
Estragole	0.25 ± 0.00	0.13 ± 0.00	**1.50; Ind**	**0.75; Add**	**0.55; Add**	**1.39; Ind**
Eucalyptol	0.50 ± 0.00	0.50 ± 0.00	1.67; Ind	1.67; Ind	**0.26; Syn**	**0.89; Add**
Eugenol	0.06 ± 0.00	0.13 ± 0.00	**0.63; Add**	**1.25; Ind**	**0.94; Add**	**1.85; Ind**
Geraniol	0.09 ± 0.05	0.09 ± 0.05	0.38; Syn	0.38; Syn	**0.65; Add**	**1.52; Ind**
Isoeugenol	0.25 ± 0.00	0.25 ± 0.00	2.50; Ind	2.50; Ind	**0.55; Add**	**0.50; Syn**
Linalyl acetate	0.25 ± 0.00	0.25 ± 0.00	0.75; Add	0.75; Add	**0.94; Add**	**1.74; Ind**
Menthol	0.19 ± 0.09	0.13 ± 0.00	1.88; Ind	1.25; Ind	1.02; Ind	1.77; Ind
Ocimene	0.13 ± 0.00	0.06 ± 0.00	**1.25; Ind**	**0.63; Add**	1.15; Ind	1.60; Ind
Sabinene hydrate	0.13 ± 0.00	0.13 ± 0.00	0.75; Add	0.75; Add	1.13; Ind	1.73; Ind
γ-Terpinene	0.13 ± 0.00	0.25 ± 0.00	1.25; Ind	2.50; Ind	0.80; Add	0.54; Add
α-Terpineol	0.13 ± 0.00	0.13 ± 0.00	1.25; Ind	1.25; Ind	**0.79; Add**	**1.59; Ind**

n = 2 replicates, with third consecutive replicate to confirm variations between enantiomers; **red bold** = variation in interactive efficacy (ΣFQSIC or ΣFPVR); Syn = synergy, Add = additive, Ind = non-interactive, 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).

3.3.3.2.6 The interactive efficacy of the enantiomers of α-Pinene in combination

The mean MQSIC and interactive efficacy (ΣFQSIC and ΣFPVR) of the enantiomers of α-Pinene are given in Table 3.12. The MQSIC values ranged between 0.06 - 0.50 mg/mL. The MQSIC values of the enantiomers in combination were equivalent. In the investigation of the anti-QS activity of the enantiomers individually, (–)-α-Pinene did not display violacein inhibition that was greater than 50.00%, at the highest concentration investigated. As a result, the combined interactive efficacy was undefined for (–)-α-Pinene and could not be compared with (+)-α-Pinene. Therefore, only the interactive efficacy of (+)-α-Pinene is discussed. In terms of the ΣFQSIC of (+)-α-Pinene in combination, 35.71% of the combinations were additive, 50.00% were non-interactive and 7.14% were undefined. Only one combination ((+)-α-Pinene with Geraniol having an ΣFQSIC value of 0.42) was synergistic and no antagonism was observed, 28.57% of the combinations were additive, 64.29% were non-interactive, and 7.14% were undefined.

Poli *et al.* (2018) evaluated the anti-QS activity of *Myrtus communis* L. essential oil against *C. violaceum* and reported and MQSIC value of 0.10 mg/mL, however, the percentage of violacein inhibition was not reported. The major compounds of the essential oil were reported to be α-Pinene (52.90%) and 1,8-Cineole (Eucalyptol) (20.60%) The current investigation evaluated the enantiomers of α-Pinene in combination with Eucalyptol and found that (+)-α-Pinene in combination with Eucalyptol inhibited QS at an MQSIC of 0.38 mg/mL. In addition, the combination was additive (ΣFQSIC = 1.00). (–)-α-Pinene in combination with Eucalyptol inhibited QS at an MQSIC of 0.50 mg/mL and was non-interactive (ΣFQSIC = 1.21). It is likely that the activity reported by Poli *et al.* (2018) is as a result of the combination of the major constituents, and the enantiomeric distribution of (+)-α-Pinene may have greater than (–)-α-Pinene. However, it should be noted that the anti-QS activity is not necessarily as a result of the major constituents, and may be due to the minor constituents, and requires further evaluation. Luís *et al.* (2016) evaluated the anti-QS activity of *Eucalyptus globulus* Labill. essential oil, of which the major constituents are 1,8-Cineole (Eucalyptol) (63.81%) and α-Pinene (16.06%). The study reported that violacein was inhibited by approximately 50.00% at a concentration of 0.25 µL/mL (0.25 mg/mL). The current investigation evaluated the combined anti-QS activity of the enantiomers of α-Pinene with Eucalyptol and similarly reports MQSIC values of 0.38 and 0.50 mg/mL for (+)-α-Pinene and (–)-α-Pinene in combination with Eucalyptol, respectively. In addition, the interaction between (+)-α-Pinene and Eucalyptol was

found to be additive in terms of the combination having a lower MQSIC and greater extent of violacein being inhibited, than the compounds when tested alone. The results of the anti-QS activity of the *E. globulus* essential oil reported by Luís *et al.* (2016) correlate with the findings of this investigation, in which the major constituents of the oil were evaluated in combination. However, one needs to consider the influence of other compounds within the neat essential oils and the ratios at which these compounds occur.

Table 3.12: The mean MQSIC, interactive efficacy (ΣFQSIC and ΣFPVR) and interaction classification of the enantiomers of α-Pinene in combination

Selected compound	Mean MQSIC (mg/mL)		ΣFQSIC		ΣFPVR at MQSIC	
	(+)-α-Pinene	(–)-α-Pinene	(+)-α-Pinene	(–)-α-Pinene	(+)-α-Pinene	(–)-α-Pinene
Camphene	0.50 ± 0.00	0.25 ± 0.00	nd; nd	nd; nd	nd; nd	nd; nd
β-Caryophyllene	0.50 ± 0.00	0.50 ± 0.00	1.17; Ind	nd; nd	0.73; Add	nd; nd
p-Cymene	0.25 ± 0.00	0.25 ± 0.00	2.33; Ind	nd; nd	1.01; Ind	nd; nd
Estragole	0.13 ± 0.00	0.13 ± 0.00	0.67; Add	nd; nd	0.91; Add	nd; nd
Eucalyptol	0.38 ± 0.18	0.50 ± 0.00	1.00; Add	nd; nd	1.21; Ind	nd; nd
Eugenol	0.06 ± 0.00	0.19 ± 0.09	0.58; Add	nd; nd	1.16; Ind	nd; nd
Geraniol	0.13 ± 0.00	0.13 ± 0.00	0.42; Syn	nd; nd	1.14; Ind	nd; nd
Isoeugenol	0.13 ± 0.00	0.13 ± 0.00	1.17; Ind	nd; nd	1.30; Ind	nd; nd
Linalyl acetate	0.50 ± 0.00	0.38 ± 0.18	1.17; Ind	nd; nd	1.27; Ind	nd; nd
Menthol	0.13 ± 0.00	0.09 ± 0.05	1.17; Ind	nd; nd	0.67; Add	nd; nd
Ocimene	0.25 ± 0.00	0.25 ± 0.00	2.33; Ind	nd; nd	0.87; Add	nd; nd
Sabinene hydrate	0.13 ± 0.00	0.13 ± 0.00	0.67; Add	nd; nd	1.21; Ind	nd; nd
γ-Terpinene	0.25 ± 0.00	0.50 ± 0.00	2.33; Ind	nd; nd	1.06; Ind	nd; nd
α-Terpineol	0.06 ± 0.00	0.13 ± 0.00	0.58; Add	nd; nd	1.02; Ind	nd; nd

n = 2 replicates, with third consecutive replicate to confirm variations between enantiomers; Syn = synergy, Add = additive, Ind = non-interactive, 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).

3.3.3.2.7 The interactive efficacy of the enantiomers of β-Pinene in combination

The mean MQSIC and interactive efficacy (ΣFQSIC and ΣFPVR) of the enantiomers of β-Pinene are given in Table 3.13. The MQSIC values ranged between 0.06 - 0.50 mg/mL. The variation in MQSIC values between the enantiomers of β-Pinene in combination were mostly equivalent. However, a few variations were observed. In combination with Isoeugenol or α-Terpineol, (+)-β-Pinene had a combined MQSIC as low as 0.06 mg/mL, while (+)-β-Pinene had a combined MQSIC at 0.25 mg/mL. This was the greatest variation observed in terms of combined MQSIC values. The enantiomers of β-Pinene varied in terms of MQSIC values when combined with either β-Caryophyllene, Isoeugenol, γ-Terpinene or α-Terpineol. (+)-β-Pinene in combination with β-Caryophyllene or γ-Terpinene had MQSIC values > 0.50 mg/mL. In contrast, (–)-β-Pinene in combination with β-Caryophyllene or γ-Terpinene promoted violacein production.

In terms of the ΣFQSIC of (+)-β-Pinene in combination, 21.43% of the combinations were synergistic, 14.29% were additive, 35.71% were non-interactive and 14.29% were undefined. No antagonism was observed. The synergistic interactions were observed between (+)-β-Pinene with Geraniol, Estragole or α-Terpineol, having ΣFQSIC values ranging between 0.38 - 0.50. In terms of the ΣFPVR, 50.00% of the combinations were additive, 21.43% were non-interactive, and 14.29% were undefined. No synergy or antagonism were observed. Overall, it can be seen that while the interactive profiles of (–)-β-Pinene in combination with the selected compounds could not be determined and compared to (+)-β-Pinene, from the MQSIC values it can be seen that (+)-β-Pinene often had reduced MQSIC values in combination, when compared to (–)-β-Pinene.

Snoussi *et al.* (2018) evaluated the anti-QS activity of *Carun capticum* L. essential oil, of which β-Pinene and *p*-Cymene were identified as being amongst the major constituents. The study reported an LC_{50} of 0.23 mg/mL for the essential oil. This means that at a concentration of 0.23 mg/mL, 50.00% or more of violacein was inhibited in *C. violaceum* and hence is the equivalent of an MQSIC value. The current investigation found that (+)-β-Pinene and (–)-β-Pinene, in combination with *p*-Cymene, had MQSIC values of 0.25 and 0.50 mg/mL, respectively. This suggests that the anti-QS activity of the *C. capticum* essential oil reported by Snoussi *et al.* (2018), is likely as a result of one of the combination of its major constituents.

Table 3.13: The mean MQSIC, interactive efficacy (ΣFQSIC and ΣFPVR) and interaction classification of the enantiomers of β-Pinene in combination

Selected compound	Mean MQSIC (mg/mL)		ΣFQSIC		ΣFPR at MQSIC	
	(+)-β-Pinene	(–)-β-Pinene	(+)-β-Pinene	(–)-β-Pinene	(+)-β-Pinene	(–)-β-Pinene
Camphene	> 0.50	> 0.50	nd; nd	nd; nd	nd; nd	nd; nd
β-Caryophyllene	**> 0.50**	**pr**	nd; nd	nd; nd	nd; nd	nd; nd
p-Cymene	0.25 ± 0.00	0.50 ± 0.00	2.25; Ind	nd; nd	1.42; Ind	nd; nd
Estragole	0.09 ± 0.05	0.13 ± 0.00	0.47; Syn	nd; nd	0.84; Add	nd; nd
Eucalyptol	0.50 ± 0.00	> 0.50	1.17; Ind	nd; nd	0.79; Add	nd; nd
Eugenol	0.13 ± 0.00	0.13 ± 0.00	1.13; Ind	nd; nd	0.98; Add	nd; nd
Geraniol	0.13 ± 0.00	0.13 ± 0.00	0.38; Syn	nd; nd	1.37; Ind	nd; nd
Isoeugenol	**0.06 ± 0.00**	**0.25 ± 0.00**	0.56; Add	nd; nd	1.44; Ind	nd; nd
Linalyl acetate	0.50 ± 0.00	0.50 ± 0.00	1.00; Add	nd; nd	0.69; Add	nd; nd
Menthol	0.13 ± 0.00	0.19 ± 0.09	nd; Ind	nd; nd	0.94; Add	nd; nd
Ocimene	> 0.50	> 0.50	nd; nd	nd; nd	nd; nd	nd; nd
Sabinene hydrate	0.25 ± 0.00	0.50 ± 0.00	1.25; Ind	nd; nd	0.62; Add	nd; nd
γ-Terpinene	**> 0.50**	**pr**	nd; nd	nd; nd	nd; nd	nd; nd
α-Terpineol	**0.06 ± 0.00**	**0.25 ± 0.00**	nd; Syn	nd; nd	0.58; Add	nd; nd

n = 2 replicates, with third consecutive replicate to confirm variations between enantiomers; Syn = synergy, Add = additive, Ind = non-interactive, 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).

ALrashidi *et al.* (2022) evaluated the anti-QS activity of *Pimenta dioica* L. essential oil and its major constituent, Eugenol. β-Pinene was also identified as one of the major constituents, however, it was not evaluated for anti-QS activity. The study reported that at a concentration of 0.05 mg/mL, the essential oil had inhibited violacein production by 71.30%, whereas Eugenol had only inhibited violacein by 48.29%, suggesting that the activity of the essential oil was likely as a result of the synergistic interactions of Eugenol with the other constituents. The current investigation evaluated the combined anti-QS activity of the enantiomers of the major constituents reported, β-Pinene in and Eugenol, and reported combined MQSIC values

as low as 0.13 mg/mL, similar to that of the *P. dioica* essential oil, reported by ALrashidi *et al.* (2022). In addition, the interactive efficacy studies revealed that while the combination of (+)-β-Pinene with Eugenol was non-interactive in terms of the effect on the effective concentration to inhibit violacein production (ΣFQSIC), the combination was additive in terms of the extent to which violacein production was inhibited (ΣFPVR), when compared to (+)-β-Pinene with Eugenol studied independently. The combined effects of (–)-β-Pinene in combination was undefined, however, it is therefore likely that the enantiomeric distribution of (+)-β-Pinene was greater than that of (–)-β-Pinene in *P. dioica* essential oil. The results of the anti-QS activity of the *P. dioica* essential oil correlate with the findings of this investigation, in which the major constituents of the oil were evaluated in combination. However, one needs to consider the influence of other compounds within neat essential oils and the ratios at which these compounds occur.

3.3.4 Summary of the interactive efficacy studies

3.3.4.1 Summary of the fractional quorum sensing inhibitory concentration (ΣFQSIC) interactive efficacy studies

The ΣFQSIC studies revealed that majority of the combinations were non-interactive, followed by additive and synergy (Figure 3.3). (+)-Limonene in combination with Estragole demonstrated the lowest (most synergistic) ΣFQSIC value (0.31) in combination. Only one combination was found to be antagonistic. This was demonstrated by (–)-Limonene in combination with *p*-Cymene (ΣFQSIC of 4.67). The (+)-enantiomers were involved in two-fold more combinations resulting in synergy, when compared to the (–)-enantiomers. It was noted that the enantiomers in combination with Geraniol mostly interacted synergistically, with the exception of the enantiomers of Borneol, which interacted additively, and (–)-α-Pinene and (–)-β-Pinene, which were undefined.

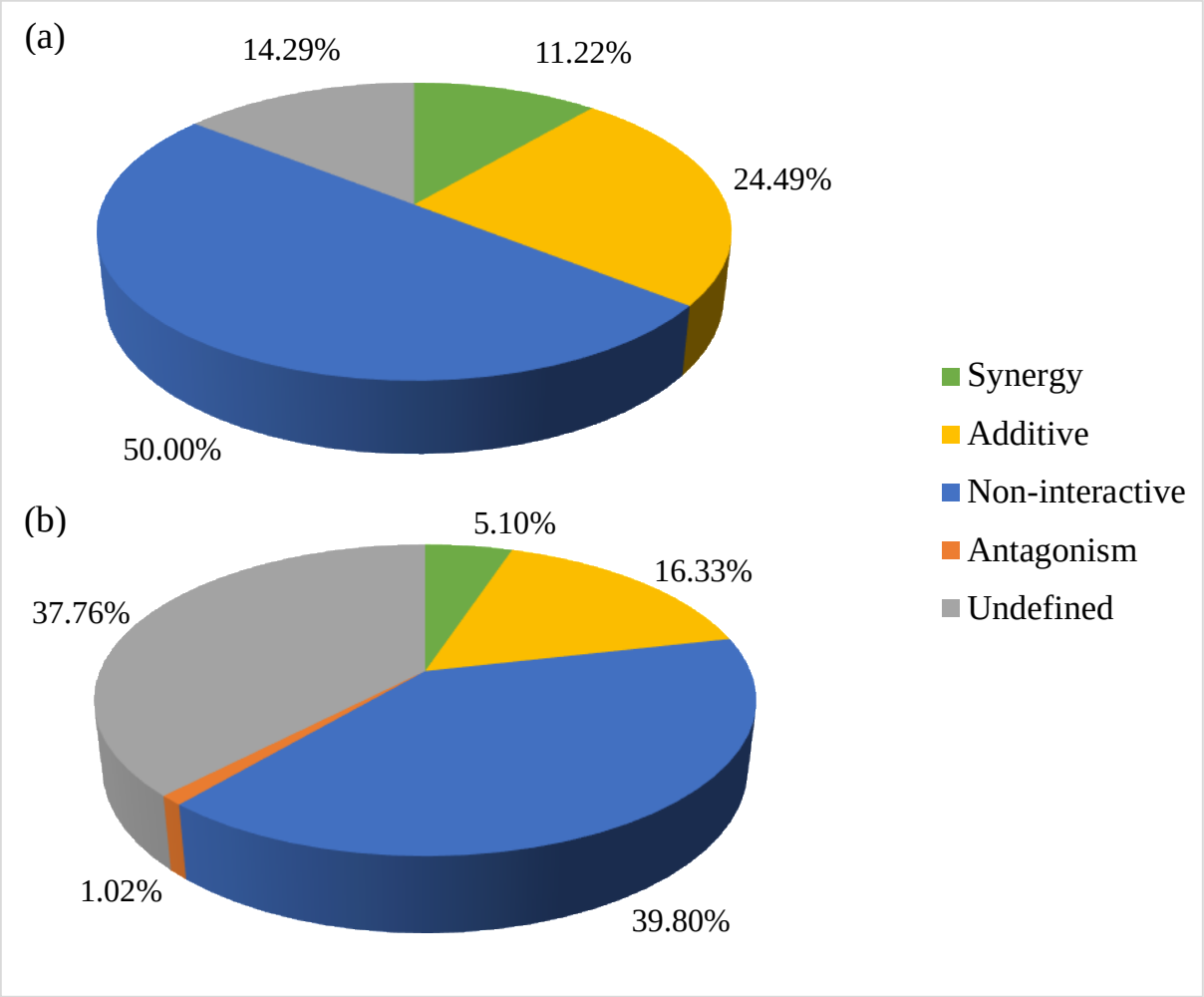


Figure 3.3: Summary of the ΣFQSIC observed with the (a) (+)-enantiomers (b) and (–)-enantiomers, in combination.

A total of 8.16% of the combinations displayed variations in terms of the ΣFQSIC, the majority of which were 'additive versus non-interactive'. It was revealed that the enantiomers of Limonene in combination varied the most in terms of ΣFQSIC, where (+)-Limonene interacted more favourably in those combinations, as compared to (–)-Limonene (Figure 3.4). This means that where variations were observed, (+)-Limonene interacted in a way that reduced the effective concentration required to disrupt bacterial communication by 50.00% or more, whereas (–)-Limonene often did not do so to the same extent.

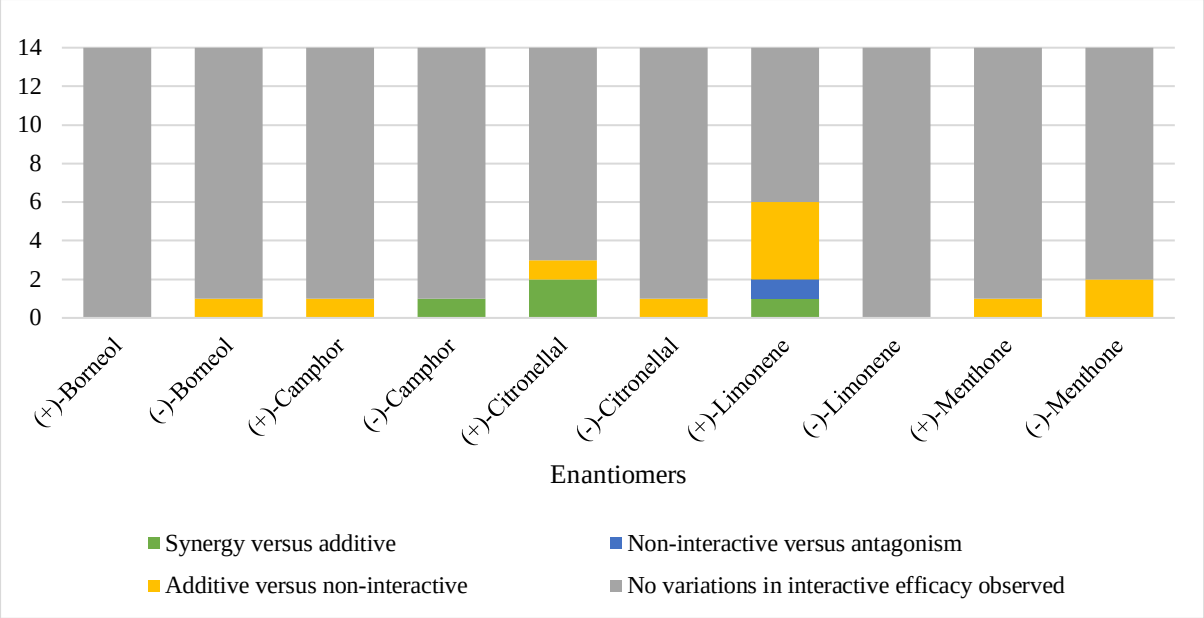


Figure 3.4: Summary of the types of variations seen between the enantiomers in combination, in terms of the interactive efficacy (ΣFQSIC).

The combination of the enantiomers of Limonene with *p*-Cymene displayed the biggest variation in terms of the ΣFQSIC interactive efficacy, where (+)-Limonene in combination was non-interactive (ΣFQSIC = 1.69), while (–)-Limonene in combination was antagonistic (ΣFQSIC = 4.67). This is illustrated in Figure 3.5.

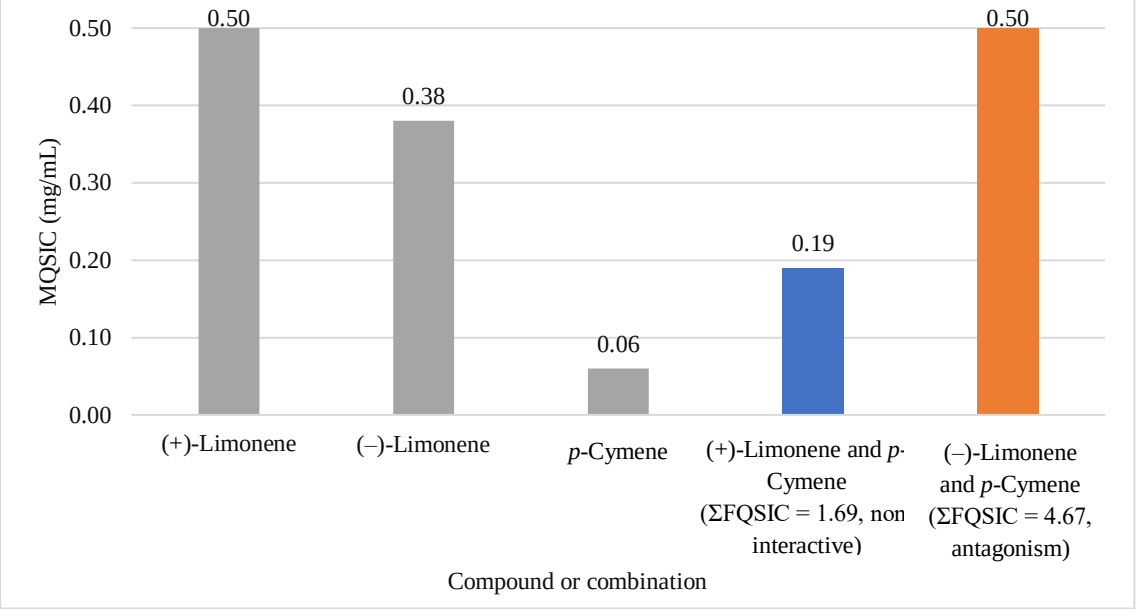


Figure 3.5: The mean MQSIC values and interactive efficacy (ΣFQSIC) of the enantiomers of Limonene and of *p*-Cymene, independently and in combination.

3.3.4.2 Summary of the fractional percentage violacein reduction (ΣFPVR) interactive efficacy studies

The ΣFPVR studies revealed that the majority of the combinations were non-interactive, followed by additive and less frequently, synergistic. (+)-Menthone in combination with Eucalyptol demonstrated the lowest ΣFPVR in combination of 0.26 (synergy). No antagonism was observed (Figure 3.6). The overall interactive profiles between the (+)- and (–)-enantiomers in combinations were mostly similar. The combinations that were undefined were mostly observed by (–)-α-Pinene and (–)-β-Pinene, resulting in the considerable variation observed.

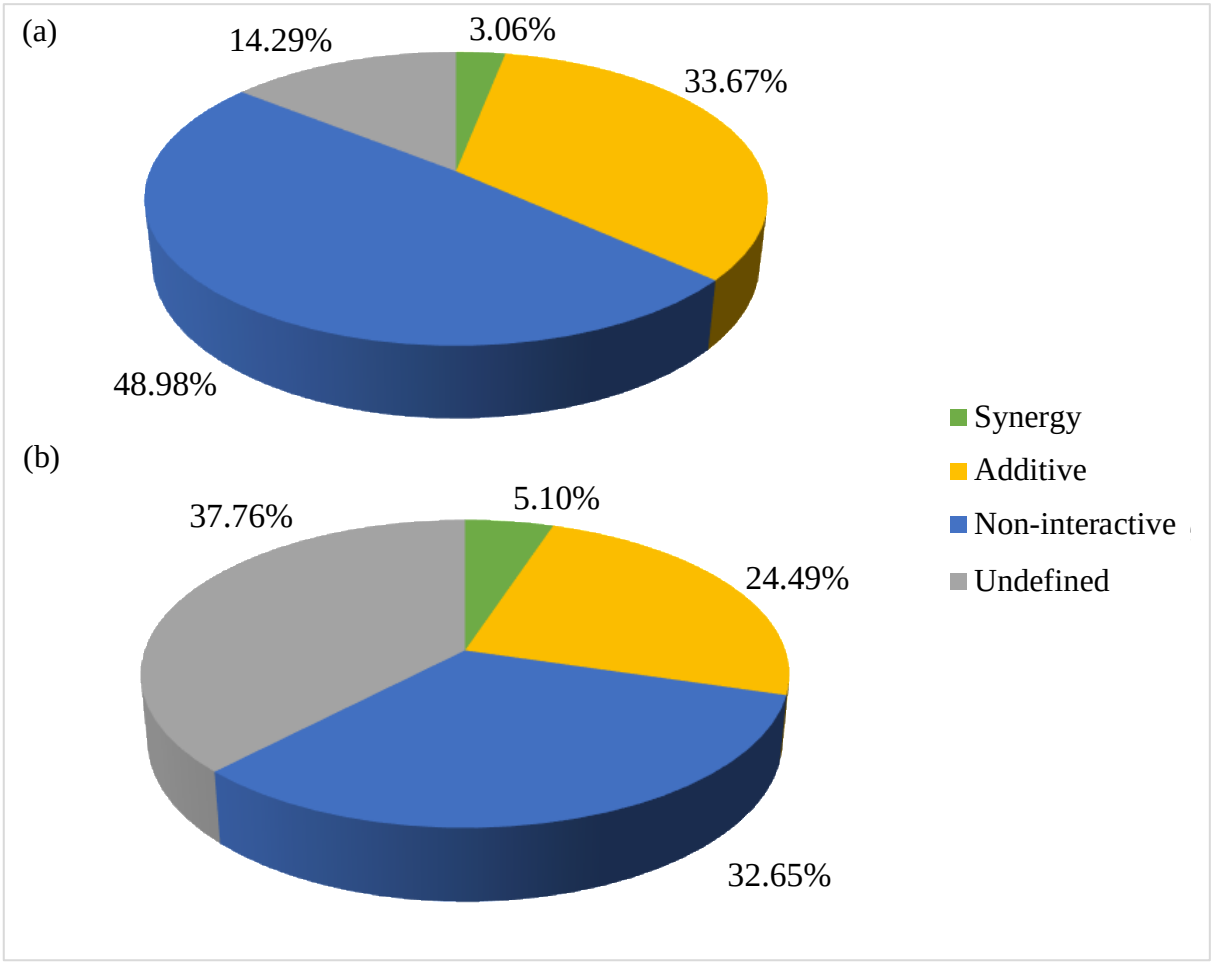


Figure 3.6: Summary of the ΣFPVR observed with the (a) (+)-enantiomers (b) and (–)-enantiomers, in combination.

A total of 15.82% of the combinations displayed variations in terms of the ΣFPVR. Although few, the most pronounced variation observed in terms interactive efficacy was ‘synergy versus

non-interactive'. This was evident with the enantiomers of Camphor in combination, where (–)-Camphor often displayed synergy in combination, whereas (+)-Camphor was non-interactive. In fact, where variations were observed in terms of the ΣFPVR interactive efficacy, (–)-Camphor interacted more favourably in those combinations (Figure 3.7). This means that (–)-Camphor inhibited the extent of bacterial communication to a greater extent in combination, when compared to (+)-Camphor.

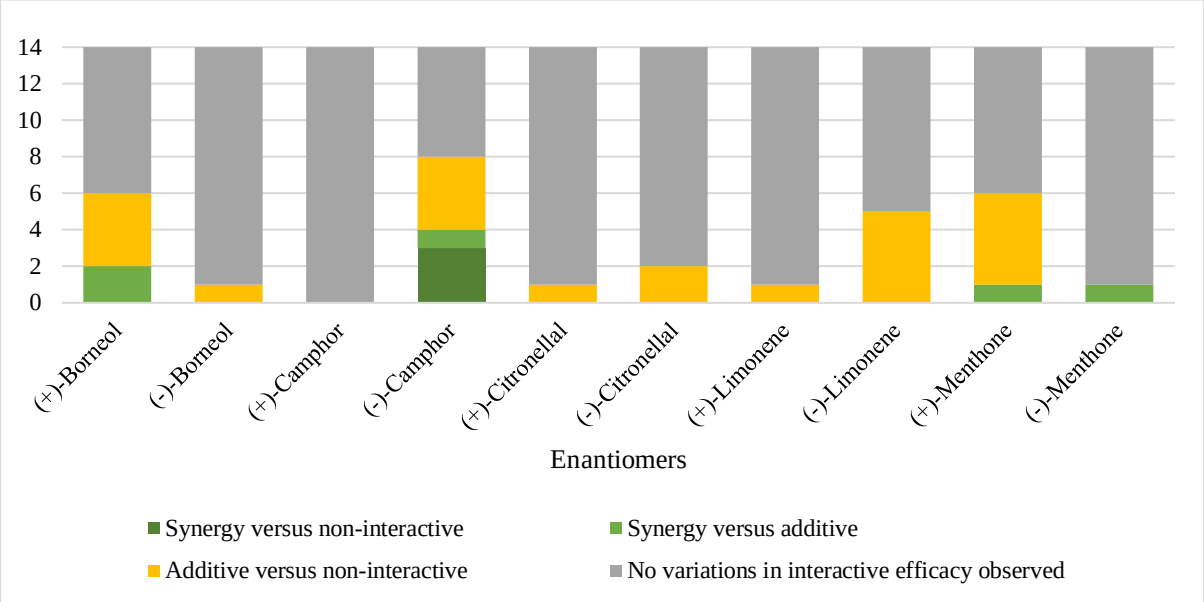


Figure 3.7: Summary of the types of variations seen between the enantiomers in combination, in terms of the interactive efficacy (ΣFPVR).

The combination of the enantiomers of Camphor with α-Terpineol displayed the biggest variation in terms of the ΣFPVR interactive efficacy, where (+)-Camphor in combination was non-interactive (ΣFPVR = 1.99), whereas (–)-Camphor in combination was synergistic (ΣFPVR = 0.44). This is further elaborated on in Figure 3.8.

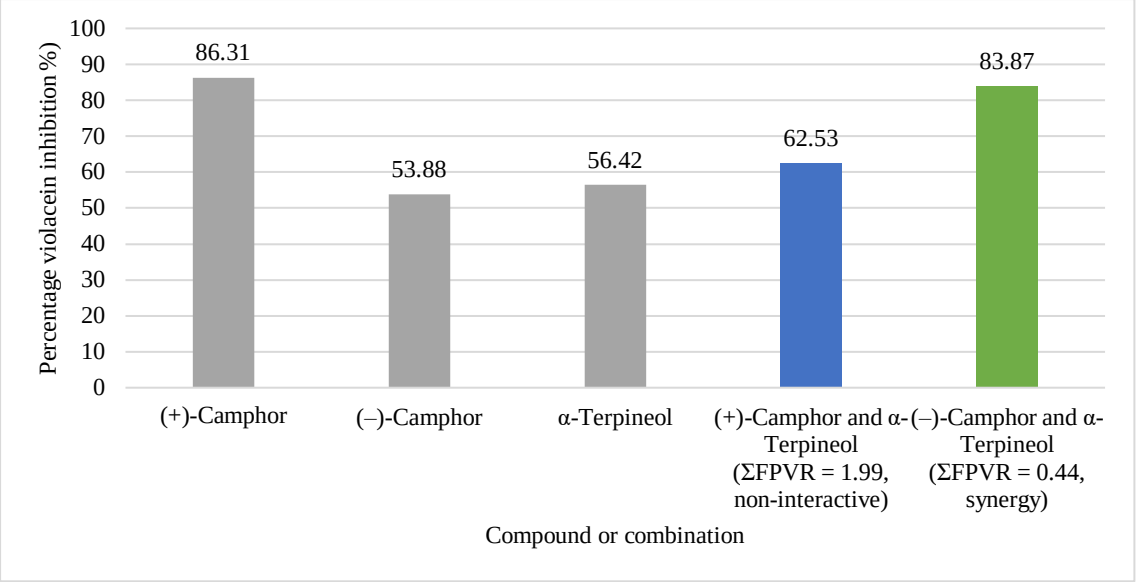


Figure 3.8: The mean percentage violacein inhibition and interactive efficacy (ΣFPVR) of the enantiomers of Camphor and of α-Terpineol, independently and in combination.

3.3.4.3 Comparisons between the fractional quorum sensing inhibitory concentration (ΣFQSIC) and fractional percentage violacein reduction (ΣFPVR)

It was often observed that although there were no variations in the effect of the combined MQSIC values (as determined through ΣFQSIC), the extent of violacein inhibition differed between the enantiomeric pairs in combination with certain selected compounds (as determined through ΣFPVR). This was evident with the enantiomers Borneol, Camphor and Menthone. An example of this was shown with the enantiomers of Camphor in combination with α-Terpineol (Figure 3.9). Both enantiomers were similarly non-interactive in terms of the effective concentration required to disrupt bacterial communication, when combined with α-Terpineol. However, where (+)-Camphor was non-interactive in terms of the extent of bacterial communication that was inhibited, (–)-Camphor interacted synergistically with α-Terpineol, thereby inhibiting communication to a greater extent. In some cases, one enantiomeric form interacted with the selected compounds more favourably in terms of the ΣFQSIC, while the other interacted more favourably in terms of the ΣFPVR. This was particularly evident with the enantiomers of Limonene, but was also evident with the enantiomers of Borneol, Citronellal and Menthone. An example of this is shown with the enantiomers of Citronellal in combination with Sabinene hydrate (Figure 3.10). (–)-Citronellal was additive in terms of reducing the effective concentration required disrupting bacterial communication when combined with Sabinene hydrate, whereas (+)-Citronellal was non-interactive (ΣFQSIC). However, (+)-

Citronellal was additive in terms of the extent to which bacterial communication was inhibited when combined with Sabinene hydrate, whereas (–)-Citronellal was non-interactive. It can therefore be seen that the enantiomeric configuration of an essential oil compound does have an effect on its anti-QS properties, especially in combination with other essential oil compounds. This is an important factor to consider when evaluating essential oils for potential anti-QS activity.

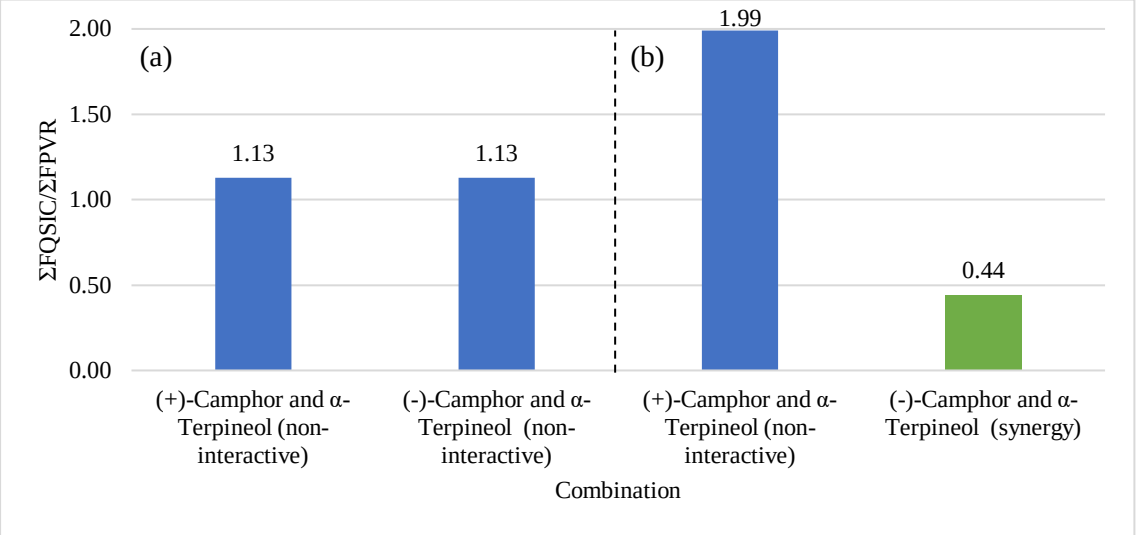


Figure 3.9: The enantiomers of Camphor in combination with α-Terpineol in terms of the (a) ΣFQSIC and (b) ΣFPVR interactive profiles.

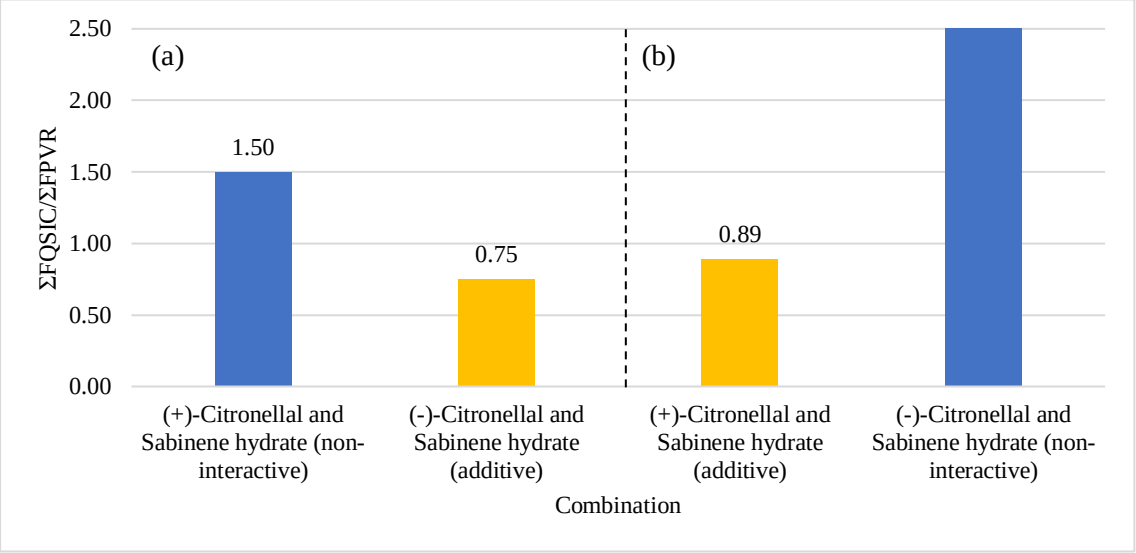


Figure 3.10: (+)-Citronellal in combination with Sabinene hydrate in terms of the (a) ΣFQSIC and (b) ΣFPVR interactive profiles.

3.4 Summary

- The anti-QS results revealed that all the enantiomers had strong anti-QS activity with MQSIC values ranging between 0.06 - 0.50 mg/mL and percentage violacein inhibition values ranging between 3.84 - 90.68% at the MQSIC.
- The only variation observed between enantiomers was observed with α-Pinene and β-Pinene, where the (+)-enantiomers had MQSIC values of 0.38 mg/mL and 0.50 mg/mL, respectively, whereas the MQSIC of the (–)-enantiomers were > 0.50 mg/mL.
- The selected compounds had MQSIC values ranging between 0.06 - 0.50 mg/mL, with the exception of Camphene (> 0.50 mg/mL). The percentage violacein inhibition values of the selected compounds ranged between 26.75 - 76.92% at the MQSIC.
- Eugenol, Isoeugenol, Menthol, Ocimene, *p*-Cymene, α-Terpineol and γ-Terpinene had the lowest MQSIC values of 0.06 mg/mL, of the selected compounds tested.
- The ΣFQSIC studies revealed that the majority of the combinations were non-interactive (44.90%), followed by additive (20.41%), synergistic (8.16%), and antagonistic (0.51%) interactions.
- Where variations were observed, (+)-Limonene and (+)-Citronellal often interacted more favourably in terms of the ΣFQSIC interactive profiles.
- In terms of ΣFQSIC, (+)-Limonene in combination with Estragole demonstrated the lowest (most synergistic) ΣFQSIC value of 0.31.
- The greatest variation, in terms of ΣFQSIC, was demonstrated by (–)-Limonene in combination with *p*-Cymene, which was antagonistic (ΣFQSIC = 4.67), whereas (+)-Limonene with *p*-Cymene was non-interactive (ΣFQSIC = 1.69).
- The ΣFPVR studies revealed that the majority of the combinations were non-interactive (40.82%), followed by additive (29.08%), and synergy (4.08%). Where variations were observed (–)-Camphor, (+)-Borneol and (+)-Menthone often interacted more favourably in terms of the ΣFPVR interactive profiles.
- In terms of the ΣFPVR, (+)-Menthone in combination with Eucalyptol demonstrated the lowest (most synergistic) ΣFPVR value of 0.26.
- The combination of the enantiomers of Camphor with α-Terpineol displayed the biggest variation in terms of the ΣFPVR interactive efficacy, where (+)-Camphor was non-interactive (ΣFPVR = 1.99), whereas (–)-Camphor in combination was synergistic (ΣFPVR = 0.44).

- In some cases, enantiomeric pairs displayed had similar combined ΣFQSIC, but varied in terms of ΣFPVR. In these cases, (+)-Borneol, (–)-Camphor and (+)-Menthone interacted more favourably in terms of the ΣFPVR.
- In other cases, one enantiomeric form interacted more favourably in terms of the ΣFQSIC, while the other enantiomeric form interacted more favourably in terms of ΣFPVR. This was seen with (+)-Limonene, which interacted more favourably in terms of ΣFQSIC, however, (–)-Limonene interacted more favourably than their enantiomeric counterparts, in terms of ΣFPVR.

Chapter 4 - Toxicity screening of enantiomers and combinations with the selected compounds

4.1 Introduction

The focus of Chapters 2 and 3 was on the antimicrobial activity of the enantiomers in terms of their stereoselective inhibitory and anti-quorum sensing (QS) activities, respectively. In order to evaluate their overall therapeutic potential, knowledge of the toxicological profiles of the enantiomers is important. Thus, the aim of the current chapter was to examine the impact enantiomeric configuration on the percentage mortality (PM) of brine-shrimp, after 24 and 48 hrs of exposure. In addition, the influence of the enantiomeric form of chiral compounds in combination with the selected compounds was also investigated. The selected compounds were additionally investigated independently in order to determine a baseline when comparing the combined toxicity of the enantiomers.

4.2 Materials and methods

4.2.1 Preparation of compounds and controls

The enantiomers and selected compounds were each diluted in a 2.00% aqueous solution of dimethyl sulfoxide (DMSO) to achieve a stock concentration of 1.00 mg/mL. Camphene and the enantiomers of Borneol and Camphor were not soluble in this solution, even when dissolved in a 50.00% aqueous solution of DMSO. Hence, these compounds were dissolved in a 50.00% solution of acetone, where no toxicity was observed for the solvent. Potassium dichromate (Sigma) was prepared at a concentration of 1.60 mg/mL and used as a positive control, as it is highly toxic to brine-shrimp. Artificial seawater, which mimics the brine-shrimp's natural environment, was prepared at 32.00 g/L using sodium chloride and distilled water. This, in addition to the 2.00% DMSO and 50.00% acetone aqueous solutions, were prepared as negative controls. This was to ensure that the solvents were not responsible for toxicity.

4.2.2 The Brine-shrimp lethality assay (BSLA)

The BSLA, adapted by Bussmann *et al*. (2011), was performed. 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^{TM}) (0.50 g) were then added to the saltwater. The eggs were left for 48 hrs 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 therefore studied at a final 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 and a mean value was obtained.

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 PM calculation. Dead brine-shrimp were counted at 24 and 48 hrs 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, using Equation 4.1.

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

Equation 4.1

A PM of 50.00% or greater is considered to be biologically toxic (Bussmann *et al*., 2011). A representation of the final microtiter plate is shown in Figure 4.1.

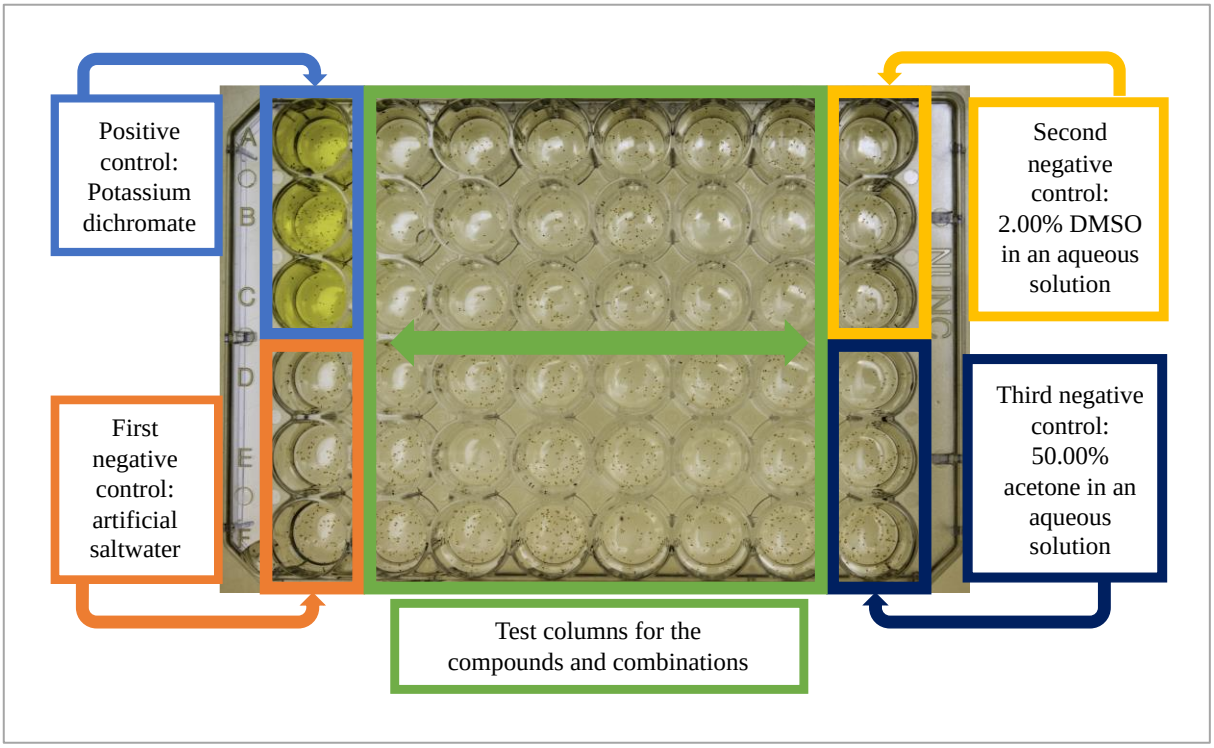


Figure 4.1: Layout of the BSLA on a 48-well microtiter plate.

4.2.3 The interactive efficacy studies

For the combinations, 200.00 µL of the enantiomers and 200.00 µL of the selected compounds at the stock concentration of 1.00 mg/mL were mixed, resulting in a 1.00 mg/mL combined sample. Thereafter, 400.00 µL of the combined sample was added to each well containing 400.00 µL of saltwater and the BLSA was performed as described in Section 4.2.2, with the combined samples being investigated at a final concentration of 0.50 mg/mL. The interactions between the compounds were then analyzed using the fractional percentage mortality (ΣFPM) index, as adapted from van Vuuren and Viljoen (2011) and Ramulondi (2017), and calculated as per Equation 4.2:

$$FPM^{(i)} = \frac{\text{PM (a*) in combination with (b)}}{\text{PM (a) independently}} \qquad FPM^{(ii)} = \frac{\text{PM (b) in combination with (a)}}{\text{PM (b) independently}}$$

Where (a) is the PM of the one compound in the combination and (b) is the PM of the other compound used in the combination.

Equation 4.2

The sum of the FPM, known as the FPM index, is calculated using Equation 4.3:

$$\Sigma FPM = FPM^{(i)} + FPM^{(ii)}$$

Equation 4.3

The ΣFPM was interpreted as either synergistic, additive, non-interactive or antagonistic (van Vuuren and Viljoen, 2011; Ramulondi, 2017). This is described in detail in Table 4.1.

Table 4.1: The interaction classification based on the ΣFPM values

ΣFPM	**Interaction classification**	**Description**
≤ 0.50	Synergy	The toxicity of the two compounds was markedly lower when combined, than when tested for each of the two compounds independently.
> 0.50 - 1.00	Additive	There was a reduction in the toxicity of the two compounds in combination, compared to the two compounds tested independently. However, not to the extent seen with the synergistic combinations.
> 1.00 ≤ 4.00	Non-interactive	The two compounds had no effect on the toxicity in combination, compared to the two compounds tested independently.
> 4.00	Antagonism	The toxicity of the two compounds when combined, was markedly higher than when the compounds were tested independently.

Once the interactive efficacy of the enantiomers in combination with the selected compounds were determined, the variations between the enantiomeric pairs in combination with the same selected compound were classified as described in Table 2.2 (Chapter 2).

4.3 Results and discussion

4.3.1 The mean percentage mortality (PM) of the enantiomers

The results of the BSLA conducted on the enantiomers when tested independently, are given in Table 4.2. The enantiomers of Citronellal and Menthone had toxic PM values between 97.44 - 100.00% from 24 hrs. However, the majority the enantiomers showed non-toxic PM values, ranging between 1.85 - 41.96% at 48 hrs. This was observed with the enantiomers of Borneol, Camphor, Limonene and α-Pinene The enantiomers of Borneol and Limonene were the least toxic at 48 hrs, with PM values of 2.45% and 4.67% for (+)-Borneol and (–)-Borneol, respectively; and 5.77% and 1.85% for (+)-Limonene and (–)-Limonene, respectively. The PM between the enantiomeric pairs were mostly equivalent, with one exception. The enantiomers of β-Pinene showed non-toxic PM values at 24 hrs (PM of 10.29 - 18.59%). However, at 48 hrs (–)-β-Pinene had a toxic PM of 93.82%, while (+)-β-Pinene was still non-toxic with a PM of 30.75%. This was the only variation in terms of toxic versus non-toxic, which was seen between the stereochemical configurations of each enantiomeric pair. In addition, (–)-β-Pinene was the only enantiomer that demonstrated toxicity after 24 hrs.

Table 4.2: Mean PM (%) of enantiomers against brine-shrimp

Enantiomer	Mean PM at 24 hr (%)	Mean PM at 48 hr (%)
(+)-Borneol	**0.46**	**2.45**
(–)-Borneol	**2.48**	**4.67**
(+)-Camphor	**1.93**	**25.62**
(–)-Camphor	**1.09**	**12.79**
(+)-Citronellal	100.00	100.00
(–)-Citronellal	100.00	100.00
(+)-Limonene	**4.82**	**5.77**
(–)-Limonene	**0.48**	**1.85**
(+)-Menthone	97.44	98.08
(–)-Menthone	100.00	100.00
(+)-α-Pinene	**23.45**	**41.96**
(–)-α-Pinene	**5.50**	**26.43**
(+)-β-Pinene	**10.29**	***30.75***

Enantiomer	Mean PM at 24 hr (%)	Mean PM at 48 hr (%)
(–)-β-Pinene	**18.59**	*93.82*

n = 2 replicates, with third consecutive replicate to confirm variations between enantiomers; **bold** - PM values < 50.00%; *italics* = variation in toxicity between enantiomeric pairs, where one enantiomeric form has a PM ≥ 50.00% and the other has a PM < 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%.

Studies on the comparative toxicity of enantiomers of essential oil compounds were often conducted through larvicidal or insecticidal assays. Nunes *et al.* (2018) investigated both the larvicidal activity (against mosquito larvae) and the brine-shrimp lethality activity of (–)-Borneol derivatives and found that the results of the two assays did not correlate, suggesting that the compounds may act differently on various test organisms. As a result, findings on toxicity that utilized assays other than the BSLA were not always comparable to the current findings. However, some correlations in terms of the stereochemistry of the enantiomers were observed and are discussed accordingly. To the best of my knowledge, few studies evaluated variations in the toxicity of the enantiomers. A further in-depth analysis of each set of enantiomers are given as follows;

4.3.1.1 Borneol

Yi *et al.* (2016) reported that Borneol displayed lower cytotoxicity or irritation in comparison to the well-established and standard permeation-enhancer, Azone. The oral lethal dose of Borneol is reported to be 300.00 - 5800.00 mg/kg in rodents and 3200.00 mg/kg in rats. Nunes *et al.* (2018) investigated the toxicological profiles of (–)-Borneol and its derivatives using the BSLA and found (–)-Borneol to be non-toxic up to a concentration of 1.00 mg/mL, which is in line with the findings of this study as low toxicity was observed at a concentration of 0.50 mg/mL.

4.3.1.2 Camphor

The toxicity profile of Camphor has been documented previously, and it was reported that 2.00 g of Camphor is toxic to adults, leading to congestion of the gastrointestinal tract, kidney and brain, and 3.50 g of Camphor may be lethal (Chen *et al.*, 2013; Sharma, 2021). Only one study could be found that investigated the toxicity of (–)-Camphor. Tak *et al.* (2006) investigated the

acaricidal activities of essential oil compounds against *Tyrophagus putrescentiae*, using direct contact and vapour phase toxicity bioassays, and found (1S)-(–)-Camphor to be toxic. The study reported the lethal concentration or dose, which kills 50% of the test subject LD_{50}, to be 10.45 mg/disc. However, (–)-Camphor was found to be non-toxic in the current investigation. This may be due to the use of different test organisms and method of testing.

4.3.1.3 Citronellal

According to the National Center for Biotechnology Information, animal studies conducted on Citronellal revealed that when injected into white leghorn embryos, dose-dependent morphological malformation occurred in the craniofacial area. In addition, Citronellal was reported to be a strong skin sensitizer in guinea pigs (NCIB, 2022). Tak *et al.* (2006), reported (±)-Citronellal to be toxic against *T. putrescentiae* (LD_{50} = 31.45 mg/disc), using direct contact and vapour phase toxicity bio-assays, which correlates with the findings of the current investigation for both enantiomers of Citronellal. Fouad *et al.* (2021) investigated the toxicity of the enantiomers of Citronellal against *Sitophilus oryzae* through contact, fumigant and repellency assays and the overall finding was that across the three assays, (–)-Citronellal displayed greater toxicity than (+)-Citronellal in terms of LC_{50} and LC_{90} values. However, the current investigation found both enantiomers of Citronellal to be highly toxic, with PM values of 100.00% at a concentration of 0.50 mg/mL. The discrepancy in results is likely due to the use of different test organisms and test methods, and the current investigation evaluated a higher concentration than those reported in Fouad *et al*. (2021).

4.3.1.4 Limonene

Kim *et al.* (2013) and Ravichandran *et al.* (2018) provide comprehensive reviews on the toxicological data, safety and risk evaluation of R-(+)-Limonene and categorize this compound as having low toxicity, which is in line with the results of the current study. The focus on R-(+)-Limonene over S-(–)-Limonene is due to the (+)-enantiomer being readily available as it is naturally produced by *Citrus* plants, as compared to the (–)-enantiomer (Bonaccorsi *et al*., 2011). Kim *et al.* (2013) reports that the dose of R-(+)-Limonene, up to which no adverse effects are observed, is 250.00 mg/kg/day. Both reviews report that R-(+)-Limonene appears to exert no serious risk for human exposure, except for the potential risk of skin irritation and sensitivity, which is due to the auto-oxidation nature of Limonene.

Lodhi *et al*. (2016) reported on the non-toxic effects of Limonene 1,2-epoxide (synthetically prepared) using the BSLA (LC_{50} = 7.95 mg/mL). Sutil *et al.* (2006) reported the LD_{50} to be 5.25 mg/mL for Limonene against brine-shrimp. These studies are consistent with the findings of the current investigation, which reports both enantiomers of Limonene to be non-toxic at a concentration of 0.50 mg/mL.,

Other studies evaluated the toxicity of Limonene enantiomers on organisms other than brine-shrimp. Tak *et al.* (2006) reported that S-(–)-Limonene was non-toxic against *T. putrescentiae* (LD_{50} = > 200 mg/disc), using direct contact and vapour phase toxicity bio-assays, which correlates with the findings of the current study. In terms of comparative toxicity evaluations between the enantiomers of Limonene, Giatropoulos *et al.* (2012) reported that R-(+)-Limonene and S-(–)-Limonene were toxic against *Aedes albopictus* (tiger mosquito). The study reported LC_{50} values of 35.99 mg/L (0.04 mg/mL) and 34.89 mg/L (0.03 mg/mL) for R-(+)-Limonene and S-(–)-Limonene, respectively. However, the current study reports that the enantiomers of Limonene were non-toxic against brine-shrimp at a dose of 0.50 mg/mL. This variation in results may be due to the use of different test organisms. Fouad and da Camara (2017) evaluated the insecticidal activity of R-(+)-Limonene and S-(–)-Limonene against *Sitophylus zeamais* (maize weevil) at concentrations ranging between 40.00 - 60.00 µL/mL, to evaluate their toxicity through contact, fumigant, and ingestion assays. A non-significant difference was reported between the two enantiomers in the fumigant and contact assays, which correlates with the findings of the current investigation. However, the ingestion assay revealed that of R-(+)-Limonene was marginally more toxic than S-(–)-Limonene. This may be due to compounds acting differently on the various test organisms. Batista *et al.* (2019) investigated the antileishmanial activity of the enantiomers of Limonene and reported LC_{50} values of 1.72 mM (0.23 mg/mL) for (+)-Limonene and 0.45 mM (0.06 mg/mL) for (–)-Limonene. These concentrations are not consistent with those of the current investigation, which may be due to the use of different test organisms and completely different assays investigated. Fouad *et al.* (2021) investigated the toxicity of the enantiomers of Limonene against *S. oryzae*, and the overall finding was that both enantiomers of Limonene displayed equivalent toxicity in terms of LC_{50} and LC_{90} values when investigated in the contact and fumigant assays. This correlates with the findings of the current investigation.

4.3.1.5 Menthone

Rossi *et al*. (2012) reported Menthone to be highly toxic against the fly *Musca domestica* (housefly), using the fumigant assay (LC_{50} value of 1.90 mg/dm^3), which was also observed in the current investigation. Sutil *et al.* (2006) reported an LD_{50} value of 1.12 mg/mL for Menthone against *A. salina*, while the current study reports that at a lower concentration of 0.50 mg/mL, the PM values of brine-shrimp ranged between 97.44 - 100.00%. Rajkumar *et al.* (2019) reported that Menthone (racemate) had considerable insecticidal activity in terms of fumigant toxicity, with LC_{50} values of 46.66 µL/L and 51.95 µL/L, making Menthone toxic at low concentrations, which correlates with the findings of the current investigation. Pang *et al.* (2020) reported that *l*-Menthone displayed significant ($P > 0.05$) insecticidal activity against *T. castaneum* (red flour beetle), *Lasioderma serricorne* (cigarette beetle), and *Liposcelis bostrychophila* (booklouse). The study conducted contact toxicity studies (LD_{50} ranging between 1.80 and 79.60 µg/cm^2) and fumigant toxicity studies (LC_{50} ranging between 0.20-14.80 mg/L of air). Therefore, *l*-Menthone was reported to be toxic at low concentrations, which correlates with the findings of the current investigation.

In terms of the comparative toxicity between the stereochemical enantiomers of Menthone, Giatropoulos *et al.* (2018) reported LC_{50} values of 53.90 mg/L (0.05 mg/mL) and 59.00 mg/L (0.06 mg/mL) for (+)-Menthone and (–)-Menthone, respectively, against the *A. albopictus* (tiger mosquito). This is in line with the findings of the current study which found (+)-Menthone and (–)-Menthone to be highly toxic at a higher concentration (0.50 mg/mL) against brine-shrimp. Fouad *et al.* (2021) investigated the toxicity of the enantiomers of Menthone against *S. oryzae*, and the overall finding was that (+)-Menthone was slightly less toxic, but not to an appreciable extent, in terms of LC_{50} and LC_{90} values, when evaluated through the contact and fumigant assays. This correlates with the findings of the current investigation.

4.3.1.6 α-Pinene and β-Pinene

A review conducted by Allenspach and Steuer (2021) on the biological activity of α-Pinene also considered studies on enantioselective activity. What was evident is the lack of research on the toxic potential of the enantiomeric forms of α-Pinene. Tak *et al.* (2006) reported that α-Pinene and β-Pinene (racemates) were non-toxic against the *T. putrescentiae*, using direct contact and vapour phase toxicity bioassays, which is in line with the findings of the current

study. The LD_{50} values reported by Tak *et al.* (2006) were 105.46 mg/disc and 150.15 mg/disc for α-Pinene and of β-Pinene, respectively. In terms of the comparative toxicity between the stereochemical enantiomers of α-Pinene and of β-Pinene, Michaelakis *et al.* (2009) evaluated the insecticidal activity of the enantiomers of α-Pinene and β-Pinene on *Culex pipiens* and reported LC_{50} values of 0.06 mg/mL for both enantiomers of α-Pinene and 0.07 mg/mL and 0.04 mg/mL for (+)-β-Pinene and (–)-β-Pinene respectively. Traboulsi *et al.* (2002) reported LC_{50} values of 0.06 mg/mL for the two enantiomers of α-Pinene against *C. pipiens*. In the current study, toxicity was not observed at a higher dose (0.50 mg/mL) than those reported in Michaelakis *et al.* (2009) and Traboulsi *et al.* (2002). This may be due to different screening methods and test organisms utilized. However, the overall findings are in line with the findings of the current study: the enantiomers of α-Pinene displayed negligible variations in toxicity, whereas the enantiomers of β-Pinene displayed enantioselective toxicity where (–)-β-Pinene was the more toxic compound. Fouad *et al*. (2021) investigated the toxicity of the enantiomers of α-Pinene against *S. oryzae*, and the overall finding was that (+)-α-Pinene was less toxic that (–)-α-Pinene in terms of LC_{50} and LC_{90} values, when evaluated through the contact, fumigant, and repellency assays. However, the current study found that both enantiomers of α-Pinene were non-toxic against brine-shrimp, and (–)-α-Pinene had slightly lower PM values, when compared to (+)-α-Pinene. However, the variation was not appreciable. Vourlioti-Arapi *et al.* (2012) also investigated the toxicity of the enantiomers of α-Pinene and β-Pinene against *C. pipiens* and reported LC_{50} values of 0.08 mg/mL and 0.07 mg/mL for (+)-β-Pinene and (–)-β-Pinene, respectively, and 0.08 mg/mL and 0.09 mg/mL for (+)-α-Pinene and (–)-α-Pinene, respectively. The study reported that amongst the essential oils investigated for their larvicidal activity, when the contained amount of (–)-α-Pinene was more than 50.00%, the LC_{50} values of the essential oils ranged from 65.69 - 96.69 mg/L, while for amounts between 19.00 - 50.00% the respective LC_{50} values ranged from 55.84 - 65.55 mg/L. Therefore, the higher the concentration of (–)-α-Pinene present, the lower the observed toxicity of the essential oil. This correlates to the findings of the current investigation, where (–)-α-Pinene had lower PM values than that of (+)-α-Pinene, despite both enantiomers being non-toxic. Giatropoulos *et al.* (2012) investigated the larvicidal activity of the enantiomers of α-Pinene and β-Pinene against *A. albopictus* (tiger mosquito) and reported LC_{50} values of 68.68 mg/L and 72.30 mg/L for (+)- and (–)-α-Pinene, respectively, and 47.33 mg/L and 42.39 mg/L for (+)-β-Pinene and (–)-β-Pinene, respectively (at 24 hrs). This is in line with the findings of the current study at 24 hrs of exposure, in terms of there being no variations between the enantiomeric pairs of α-Pinene and of β-Pinene.

4.3.2 The mean percentage mortality (PM) of the selected compounds

As the focus of this investigation was to examine the variability of the chiral compounds in combination, the first step was to determine the PM of the selected compounds which were to be combined with the enantiomers. The results of the PM of the selected compounds are given in Table 4.3. Camphene, β-Caryophyllene, Eucalyptol, Linalyl acetate, *p*-Cymene, Sabinene hydrate and γ-Terpinene displayed non-toxic percentage mortalities ranging between 0.85 - 6.87% at 24 hrs and 1.11 - 23.79% at 48 hrs. Eugenol, Geraniol, Isoeugenol and Menthol displayed toxic PM values of 100.00% from 24 hrs. Estragole was also toxic, with a PM of 81.00% at 24 hrs and 82.45% at 48 hrs. Ocimene had a PM of 42.16% at 24 hrs and 55.04% at 48 hrs. This trend of demonstrating some toxicity only at 24 hrs was also seen with α-Terpineol, which had a PM of 13.99% at 24 hrs and 63.17% at 48 hrs.

Table 4.3: Mean PM (%) of the selected compounds against brine-shrimp

Selected compound	Mean PM at 24 hr (%)	Mean PM at 48 hr (%)
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; **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%.

The toxic nature of Geraniol has been observed previously against nymphal lone star ticks and adult female yellow fever mosquitoes (Weldon *et al.*, 2011). The study also reported on the

repulsive activity of α-Terpineol, and Pavela (2014) reported on the larvicidal activity of α-Terpineol against *Spodoptera littoralis* (African cotton leaf worm) at a concentration of 0.30 mg/larva, which confirms the toxicity observed in the current study against brine-shrimp. Pattanasiri *et al*. (2017) reported Eugenol to cause 100.00% mortality of Siamese fighting fish at a concentration of 0.04 mg/mL, which confirms the findings of the current investigation, which reports 100.00% mortality of brine-shrimp at a dose of 0.50 mg/mL. Widiyarti *et al.* (2019) reported Eugenol to be toxic, with an LC_{50} value of 1.39 µg/mL, which confirms the findings of the current investigation where Eugenol was toxic at a higher concentration of 0.50 mg/mL. The toxic nature of Eugenol has been reported before against the mosquito *C. pipiens* and against *S. littoralis* (Kimbaris *et al*., 2012; Pavela, 2014). Pavela (2014) also reported Isoeugenol to be toxic, as was demonstrated in the current investigation. The non-toxic nature of *p*-Cymene and γ-Terpinene has been reported previously against *T. putrescentiae* (Tak *et al*., 2006). In addition, Pitarokili *et al.* (2011) and Weldon *et al.* (2011) have reported on the low toxicity of γ- Terpinene against *C. pipiens*, nymphal lone star ticks and adult female yellow fever mosquitoes. The low toxicity of β-Caryophyllene observed in the current investigation was confirmed by Kimbaris *et al.* (2012). Koliopoulos *et al.* (2010) investigated the larvicidal properties of 1,8-Cineole (Eucalyptol) against *C. pipiens* and reported that the compound had no toxicity, which is in line with the findings of the current study, where Eucalyptol had a PM against brine-shrimp as low as 1.11% at 48 hrs. This is also confirmed by Pitarokili *et al.* (2011), Kimbaris *et al.* (2012) and Giatropoulos *et al.* (2018).

4.3.3 The toxicity of the equal ratio (1:1) combinations

4.3.3.1 The mean percentage mortality (PM) of the equal ratio (1:1) combinations

The results of the BSLA conducted on the 1:1 combinations of the enantiomers with the selected compounds are given in Table 4.4. After 24 and 48 hrs of exposure, a total of 55.61% and 44.90% of the combinations, respectively, were non-toxic at a dose of 0.50 mg/mL, with PM values less than 50.00%. The majority of the non-toxic combinations were seen with both enantiomers of Citronellal, Limonene, Menthone, α-Pinene and β-pinene. Toxicity was mostly seen with the enantiomers of Camphor and Borneol in combination with the selected compounds. (+)-Menthone and (–)-Menthone were highly toxic when investigated independently, however, the results of the combination studies revealed that (+)-Menthone in

Table 4.4: The mean PM (%), ΣFPM and interaction classification (in parentheses) for the 1:1 combinations at 24 and 48 hrs

Selected compound	Enantiomers													
	(+)-Borneol		(–)-Borneol		(+)-Camphor		(–)-Camphor		(+)-Citronellal		(–)-Citronellal		(+)-Limonene	
	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr
Camphene	100.00 (135.25; Ant)	100.00 (33.66; Ant)	100.00 (47.40; Ant)	100.00 (23.99; Ant)	**47.64** (25.31; Ant)	70.39* (10.72; Ant)	**37.65** (27.47; Ant)	58.22* (10.01; Ant)	**0.78** (0.22; Syn)	**6.87** **(0.95; Add)**	**0.76** (0.21; Syn)	**9.14** **(1.26; Ind)**	**2.45** (0.92; Add)	**5.15** (1.13; Ind)
β-Caryophyllene	100.00 (108.62; Ant)	100.00 (20.98; Ant)	100.00 (20.77; Ant)	100.00 (11.32; Ant)	73.61 (27.38; Ant)	97.22 (6.71; Ant)	65.61 (37.42; Ant)	85.63 (7.58; Ant)	**2.54** **(0.30; Syn)**	**3.40** **(0.19; Syn)**	**9.16** **(1.08; Ind)**	**35.70** **(1.95; Ind)**	**1.04** (0.23; Syn)	**16.82** **(2.29; Ind)**
p-Cymene	92.93 (154.73; Ant)	100.00 (65.38; Ant)	100.00 (78.65; Ant)	100.00 (55.71; Ant)	60.54 (20.07; Ant)	68.79 (4.59; Ant)	100.00 (53.01; Ant)	100.00 (8.63; Ant)	**8.95** **(0.70; Add)**	**16.57** **(0.86; Add)**	**2.16** **(0.17; Syn)**	**8.07** **(0.42; Syn)**	***29.63*** (5.23; Ant)	***33.33*** (4.46; Ant)
Estragole	100.00 (108.50; Ant)	100.00 (20.88; Ant)	100.00 (20.65; Ant)	100.00 (11.21; Ant)	56.37 (14.93; Ant)	59.53 **(1.52; Ind)**	92.85 (43.03; Ant)	100.00 **(4.51; Ant)**	71.30 (0.80; Add)	78.15 **(0.86; Add)**	80.16 (0.90; Add)	96.04 **(1.06; Ind)**	**14.48** (1.59; Ind)	**35.63** **(3.30; Ind)**
Eucalyptol	100.00 (108.50; Ant)	100.00 (20.88; Ant)	100.00 (20.65; Ant)	100.00 (11.21; Ant)	*88.49* (74.67; Ant)	93.42 (43.86; Ant)	***40.71*** (42.43; Ant)	94.46* (46.20; Ant)	**0.00** (nd; nd)	**0.00** (nd; nd)	**0.00** (nd; nd)	**1.39** (0.63; Add)	**0.00** (nd; nd)	**6.01** (3.23; Ind)
Eugenol	100.00 (108.50; Ant)	100.00 (20.88; Ant)	100.00 (20.65; Ant)	100.00 (11.21; Ant)	100.00 (26.38; Ant)	100.00 **(2.45; Ind)**	100.00 (46.23; Ant)	100.00 **(4.41; Ant)**	100.00 (1.00; Add)	100.00 (1.00; Add)	100.00 (1.00; Add)	100.00 (1.00; Add)	100.00 (10.88; Ant)	100.00 (9.17; Ant)
Geraniol	93.89 (116.15; Ant)	100.00 (22.48; Ant)	100.00 (35.87; Ant)	100.00 (12.81; Ant)	100.00 (26.38; Ant)	100.00 **(2.45; Ind)**	100.00 (46.23; Ant)	100.00 **(4.41; Ant)**	**42.86** (0.43; Syn)	*52.38** **(0.52; Add)**	**27.81** (0.28; Syn)	***40.88*** **(0.41; Syn)**	100.00 (10.88; Ant)	100.00 (9.17; Ant)
Isoeugenol	100.00 (108.50; Ant)	100.00 (20.88; Ant)	100.00 (20.65; Ant)	100.00 (11.21; Ant)	96.56 (25.47; Ant)	100.00 **(2.45; Ind)**	65.53 (30.29; Ant)	100.00 **(4.41; Ant)**	**8.10** (0.08; Syn)	**27.46** (0.27; Syn)	**6.37** (0.06; Syn)	**30.10** (0.30; Syn)	100.00 (10.88; Ant)	100.00 (9.17; Ant)
Linalyl acetate	100.00 (109.19; Ant)	100.00 (21.29; Ant)	100.00 (21.34; Ant)	100.00 (11.62; Ant)	94.24 (39.19; Ant)	100.00 (4.05; Ant)	60.70 (37.30; Ant)	84.02 (5.05; Ant)	**0.00** (nd; nd)	**0.78** (0.02; Syn)	**2.33** (0.38; Syn)	**6.25** (0.16; Syn)	**0.00** (nd; nd)	**3.26** **(0.35; Syn)**
Menthol	100.00 (115.28; Ant)	100.00 (25.10; Ant)	100.00 (27.44; Ant)	100.00 (15.43; Ant)	**31.97** **(8.43; Ant)**	**43.65** **(1.07; Ind)**	**0.90** **(0.42; Syn)**	**3.40** **(0.15; Syn)**	**38.45** (0.38; Syn)	**39.70** (0.40; Syn)	**15.44** (0.15; Syn)	**26.95** (0.27; Syn)	***41.43*** (4.51; Ant)	89.66* (8.22; Ant)
Ocimene	100.00 (141.96; Ant)	100.00 (25.17; Ant)	100.00 (54.11; Ant)	100.00 (15.51; Ant)	66.24 (17.92; Ant)	90.64 **(2.59; Ind)**	68.25 (32.02; Ant)	98.32 **(4.74; Ant)**	**0.00** (nd; nd)	**1.65** (0.02; Syn)	**2.28** (0.04; Syn)	**3.01** (0.04; Syn)	**8.55** **(0.99; Add)**	**16.82** **(1.61; Ind)**
Sabinene hydrate	100.00 (111.57; Ant)	100.00 (21.17; Ant)	100.00 (23.73; Ant)	100.00 (11.50; Ant)	***46.78*** (27.99; Ant)	57.61* **(3.89; Ind)**	*69.92* (55.72; Ant)	80.69 **(7.02; Ant)**	**20.04** (6.91; Ant)	**25.23** (1.34; Ind)	**46.72** (16.10; Ant)	74.81* (3.96; Ind)	**0.00** (nd; nd)	**5.57** **(0.75; Add)**
γ-Terpinene	100.00 (119.31; Ant)	100.00 (25.33; Ant)	100.00 (31.47; Ant)	100.00 (15.66; Ant)	***37.67*** (14.27; Ant)	52.02* (4.76; Ant)	*72.61* (41.92; Ant)	94.50 (10.50; Ant)	**3.13** (0.39; Syn)	**22.31** **(1.72; Ind)**	**1.11** (0.14; Syn)	**2.78** **(0.21; Syn)**	***0.98*** **(0.22; Syn)**	***3.84*** **(0.61; Add)**
α-Terpineol	100.00 (120.00; Ant)	100.00 (27.58; Ant)	100.00 (32.15; Ant)	100.00 (17.91; Ant)	*100.00* **(29.45; Ant)**	*100.00* **(2.74; Ind)**	***0.81*** **(0.40; Syn)**	***3.87*** **(0.18; Syn)**	**4.38** (0.18; Syn)	**6.52** (0.08; Syn)	**1.71** (0.07; Syn)	**4.27** (0.06; Syn)	***1.44*** **(0.20; Syn)**	***2.41*** **(0.23; Syn)**

Selected compound	Enantiomers															
	(–)-Limonene		(+)-Menthone		(–)-Menthone		(+)-α-Pinene		(–)-α-Pinene		(+)-β-Pinene		(–)-β-Pinene	
	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr
Camphene	**0.00** (nd; nd)	**7.69** (3.10; Ind)	**0.72** (0.20; Syn)	**2.85** (0.39; Syn)	**0.93** (0.26; Syn)	**6.39** (0.88; Add)	**0.88** (0.26; Syn)	**10.53** **(1.52; Ind)**	**1.08** (0.39; Syn)	**3.64** **(0.55; Add)**	**0.00** (nd; nd)	**0.00** (nd; nd)	**0.00** (nd; nd)	**2.33** (0.32; Syn)
β-Caryophyllene	**0.00** (nd; nd)	**2.04** **(0.65; Add)**	**1.21** **(0.14; Syn)**	***11.98*** **(0.65; Add)**	**23.75** **(2.81; Ind)**	*67.95** **(3.70; Ind)**	**0.00** (nd; nd)	**1.85** **(0.11; Syn)**	**3.74** (0.76; Add)	**10.15** **(0.69; Add)**	*100.00* **(16.17; Ant)**	*100.00* **(6.58; Ant)**	***7.76*** **(1.09; Ind)**	***25.00*** **(1.37; Ind)**
p-Cymene	*100.00* (110.78; Ant)	*100.00* (31.72; Ant)	***44.40*** **(3.46; Ind)**	***48.01*** **(2.51; Ind)**	*100.00* **(7.78; Ant)**	*100.00* **(5.22; Ant)**	**3.20** (0.30; Syn)	**10.10** **(0.60; Add)**	**0.00** (nd; nd)	**1.72** **(0.11; Syn)**	**15.30** **(1.86; Ind)**	**20.28** **(1.29; Ind)**	**1.96** **(0.20; Syn)**	**10.13** **(0.53; Add)**
Estragole	**1.55** (1.62; Ind)	**46.56** **(12.85; Ant)**	***11.34*** **(0.13; Syn)**	***18.49*** **(0.21; Syn)**	*92.11* **(1.03; Ind)**	*94.89* **(1.05; Ind)**	**7.76** **(0.21; Syn)**	**45.56** **(0.82; Add)**	**9.29** **(0.90; Add)**	**44.71** **(1.12; Ind)**	***4.55*** **(0.25; Syn)**	65.30* **(1.46; Ind)**	*50.69* **(1.68; Ind)**	81.71 **(0.93; Add)**
Eucalyptol	**0.00** (nd; nd)	**2.53** (1.82; Ind)	***4.29*** **(2.53; Ind)**	***14.82*** (6.75; Ant)	*76.92* **(45.38; Ant)**	*78.35* (35.65; Ant)	**3.08** **(1.87; Ind)**	**8.51** **(3.93; Ind)**	**7.49** **(5.06; Ant)**	**11.59** **(5.43; Ant)**	**2.46** (1.56; Ind)	**4.09** (1.91; Ind)	**2.51** (1.54; Ind)	**7.53** (3.43; Ind)
Eugenol	100.00 (104.00; Ant)	100.00 (27.50; Ant)	100.00 **(1.01; Ind)**	100.00 **(1.01; Ind)**	100.00 **(1.00; Add)**	100.00 **(1.00; Add)**	100.00 **(2.63; Ind)**	100.00 (1.69; Ind)	100.00 **(9.59; Ant)**	100.00 (2.39; Ind)	*100.00* **(5.36; Ant)**	*100.00* **(2.13; Ind)**	***2.26*** **(0.07; Syn)**	***5.19*** **(0.05; Syn)**
Geraniol	100.00 (104.00; Ant)	100.00 (27.50; Ant)	98.89 (1.00; Add)	100.00 **(1.01; Ind)**	100.00 (1.00; Add)	100.00 **(1.00; Add)**	100.00 **(2.63; Ind)**	100.00 (1.69; Ind)	100.00 **(9.59; Ant)**	100.00 (2.39; Ind)	100.00 **(5.36; Ant)**	100.00 (2.13; Ind)	100.00 **(3.19; Ind)**	100.00 (1.03; Ind)
Isoeugenol	100.00 (104.00; Ant)	100.00 (27.50; Ant)	70.97 (0.72; Add)	90.32 (0.91; Add)	98.99 (0.99; Add)	100.00 (1.00; Add)	100.00 **(2.63; Ind)**	100.00 (1.69; Ind)	84.96 **(8.15; Ant)**	100.00 (2.39; Ind)	65.85 (3.53; Ind)	99.19 (2.11; Ind)	93.43 (2.98; Ind)	100.00 (1.03; Ind)
Linalyl acetate	**0.51** (0.61; Add)	**2.01** **(0.58; Add)**	**0.63** **(0.10; Syn)**	***1.71*** **(0.04; Syn)**	**16.22** **(2.63; Ind)**	*55.80** **(1.45; Ind)**	**0.00** (nd; nd)	**2.50** **(0.08; Syn)**	**13.86** (3.44; Ind)	**33.52** **(1.34; Ind)**	**8.12** **(1.67; Ind)**	***9.63*** **(0.36; Syn)**	**48.89** **(9.00; Ant)**	*92.53** **(2.44; Ind)**
Menthol	*78.46* (81.60; Ant)	97.78 (26.89; Ant)	100.00 **(1.01; Ind)**	100.00 **(1.01; Ind)**	73.75 **(0.74; Add)**	95.29 **(0.95; Add)**	**36.02** **(0.95; Add)**	83.49* (1.41; Ind)	**41.38** **(3.97; Ind)**	79.61* (1.90; Ind)	**25.45** **(1.36; Ind)**	81.82* **(1.74; Ind)**	**25.67** **(0.82; Add)**	78.89* **(0.81; Add)**
Ocimene	**4.85** **(5.07; Ant)**	95.31* **(26.60; Ant)**	**16.54** (0.28; Syn)	**22.25** (0.32; Syn)	**11.42** (0.19; Syn)	**18.68** (0.26; Syn)	**34.44** **(1.14; Ind)**	**43.75** **(0.92; Add)**	**4.21** **(0.43; Syn)**	**8.42** **(0.24; Syn)**	**0.00** (nd; nd)	**18.79** (0.48; Syn)	**0.00** (nd; nd)	**9.87** (0.14; Syn)
Sabinene hydrate	**2.27** (3.13; Ind)	**37.14** **(11.81; Ant)**	**2.89** **(1.00; Add)**	**12.43** **(0.66; Add)**	**6.34** **(2.19; Ind)**	**29.32** **(1.55; Ind)**	**1.26** **(0.45; Syn)**	**26.12** (1.56; Ind)	**3.88** **(1.67; Ind)**	**35.41** (2.37; Ind)	**7.73** (3.00; Ind)	*60.63** (3.89; Ind)	**4.12** (1.51; Ind)	***26.24*** (1.40; Ind)
γ-Terpinene	*100.00* **(115.50; Ant)**	*100.00* **(34.20; Ant)**	**3.15** **(0.39; Syn)**	***8.48*** **(0.65; Add)**	**30.70** **(3.84; Ind)**	*68.34** **(5.26; Ant)**	**3.21** (0.45; Syn)	**20.63** **(1.73; Ind)**	**2.34** (0.49; Syn)	**7.26** **(0.66; Add)**	***0.61*** **(0.10; Syn)**	***43.17*** **(3.81; Ind)**	*100.00* **(14.69; Ant)**	*100.00* **(7.73; Ant)**
α-Terpineol	*51.93* **(55.60; Ant)**	*80.01* **(22.24; Ant)**	**5.84** (0.24; Syn)	*51.70** **(0.67; Add)**	**0.51** (0.02; Syn)	***35.79*** **(0.46; Syn)**	**0.79** (0.05; Syn)	**7.41** (0.15; Syn)	**0.48** (0.06; Syn)	**4.37** (0.12; Syn)	**15.31** **(1.29; Ind)**	*70.09** **(1.69; Ind)**	**1.63** **(0.10; Syn)**	***12.10*** **(0.16; Syn)**

n = 2 replicates, with third consecutive replicate to confirm variations between enantiomers; **bold** = PM < 50%; *italics* = variation in toxicity between enantiomeric pairs, where one enantiomeric form has a PM ≥ 50.00% and the other has a PM < 50.00%; *combinations that were non-toxic at 24 hrs, but demonstrated toxicity at 48 hrs; **red bold** = variations in interactive efficacy between enantiomers in combination at 24 hrs, and at 48 hrs; Syn = synergy, Add = additive, Ind = non-interactive, Ant = antagonism, 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%.

combination with 10 of the 14 selected compounds resulted in non-toxic PM's as low as 0.63% (in combination with Linalyl acetate). The same was seen with (–)-Menthone in combination with seven of the 14 selected compounds with a PM as low as 0.51% (in combination with α-Terpineol). Geraniol, Eugenol and Isoeugenol were the selected compounds involved in the majority of the toxic combinations, which correlates with the toxicity observed independently. Menthol displayed a PM = 100.00% when investigated independently, however, in combination with nine of the 14 enantiomers the combined PM was non-toxic with a PM as low as 0.90% (in combination with (–)-Camphor).

A total of 7.65% and 10.20% of the enantiomers in combination displayed variations in terms of toxicity, at 24 hrs and 48 hrs, respectively. These have been highlighted in Table. 4.4. Of particular interest were the combinations that varied where one enantiomeric form was non-toxic in combination at 24 and 48 hrs, whereas the other demonstrated toxicity from 24 hrs. This was evident with the enantiomers of Camphor, Limonene, Menthone and β-Pinene in combination. The most pronounced variation variation seen in terms of PM was the enantiomers of Limonene in combination with γ-Terpinene. (+)-Limonene in combination with γ-Terpinene had a PM value of 0.98% and 3.84%, at 24 hrs and 48 hrs, respectively. However, (–)-Limonene in combination with γ-Terpinene was toxic with a PM value of 100.00% from 24 hrs (Figure 4.2).

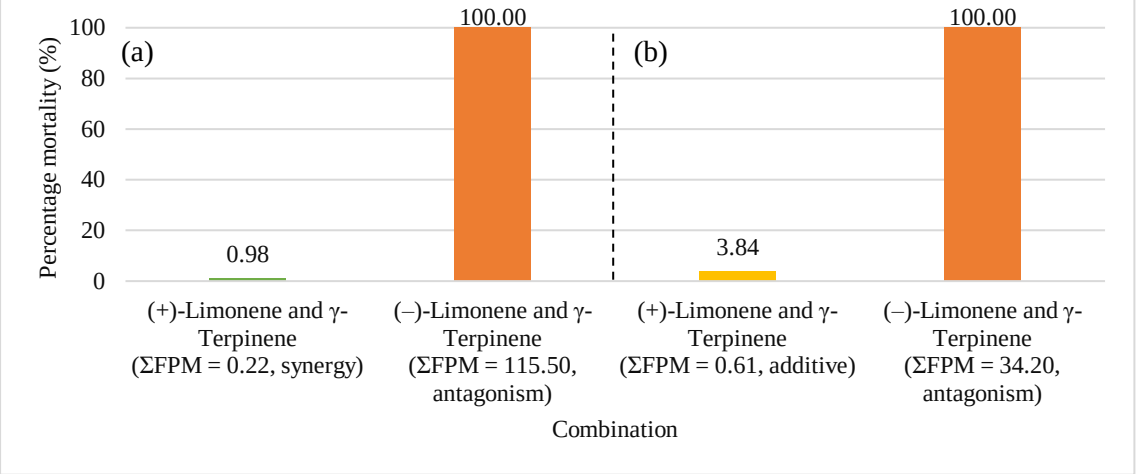


Figure 4.2: The mean PM and interactive efficacy of the enantiomers of Limonene in combination with γ-Terpinene, at (a) 24 hrs and (b) 48 hrs.

A total of 10.71% of the combinations only showed toxicity at 48 hrs, and are highlighted in Table 4.4. It was interesting to note that the enantiomers of the three monoterpenes, (+)-

Limonene, and the enantiomers of α-Pinene and of β-Pinene, displayed the same trend of demonstrating toxicity only at 48 hrs, when combined with Geraniol, highlighting a possible structure-activity relationship between simple monoterpene hydrocarbons and the terpene alcohol, Geraniol. Some variations were observed, where both enantiomers were non-toxic in combination at 24 hrs, however at 48 hrs, one enantiomeric form in combination demonstrated toxicity while the other was still non-toxic. This was evident with all of the enantiomers, besides Borneol. For example, (–)-Menthone was non-toxic at 48 hrs (PM = 35.79%) when combined with α-Terpineol, whereas (+)-Menthone exhibited toxicity (PM = 51.70%) after 48 hrs. When in combination with either β-Caryophyllene, Linalyl acetate or γ-Terpinene, (+)-Menthone was non-toxic at 48 hrs, with PM values ranging between 1.71 - 11.98%, whereas (–)-Menthone was toxic, with PM values ranging between 55.80 - 68.34%. An example of this has been demonstrated in Figure 4.3.

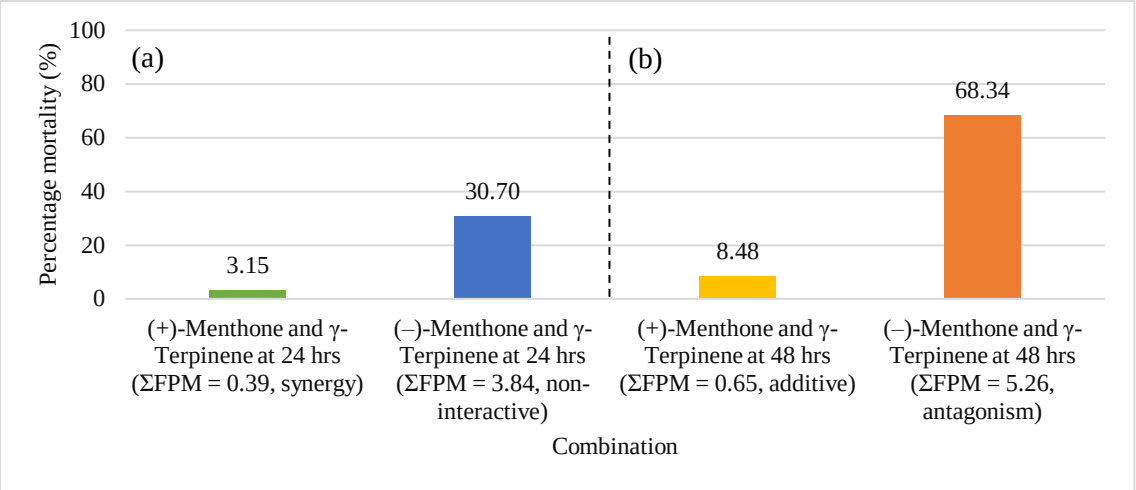


Figure 4.3: The mean PM and interactive efficacy of the enantiomers of Menthone in combination with γ-Terpinene, at (a) 24 hrs and (b) 48 hrs.

4.3.3.2 The interactive efficacy of the 1:1 combinations at 24 hrs of exposure

At 24 hrs, it was observed that the majority of the combinations were antagonistic (40.82%), followed by synergistic (23.47%), non-interactive (17.35%) and additive (9.69%). The interactive efficacy of some (8.67%) of the combinations were undefined, as either the PM value of the enantiomer, the selected compound or the combination was 0.00%, which meant that an absolute ΣFPM could not be calculated. The enantiomers of Citronellal demonstrated the most synergy in combination (ΣFPM ranging between 0.04 - 0.43), followed by the

enantiomers of Menthone (ΣFPM ranging between 0.02 - 0.39) and α-Pinene (ΣFPM ranging between 0.05 - 0.49). The most synergistic combination was exhibited by (–)-Menthone with α-Terpineol, which had a ΣFPM value of 0.02. In fact, the lowest ΣFPM values were observed with (–)-Menthone, (+)-α-Pinene, (–)-α-Pinene and (–)-Citronellal (ΣFPM values ranging between 0.02 - 0.07) in combination with α-Terpineol. All of the combinations involving Borneol and the majority of the combinations involving Camphor were antagonistic.

A total of 19.39% of the combinations displayed variations in terms of the interactive efficacy. Those observed were 'synergy versus antagonism' (3.06%), 'synergy versus non-interactive' (6.12%), 'synergy versus additive' (2.55%), 'additive versus antagonism' (0.51%), 'additive versus non-interactive' (2.55%), and 'non-interactive versus antagonism' (4.59%). The greatest variation was 'synergy versus antagonism' and 'additive versus antagonism'.

4.3.3.3 The interactive efficacy of the 1:1 combinations at 48 hrs of exposure

At 48 hrs, it was observed that the majority of the combinations were antagonistic (34.69%), followed by non-interactive (30.61%), synergistic (18.37%), and additive (15.31%). The interactive efficacy of some (1.02%) of the combinations were undefined. The enantiomers of Citronellal demonstrated the most synergy in combination (ΣFPM ranging between 0.02 - 0.42), followed by the enantiomers of Menthone (ΣFPM ranging between 0.04 - 0.46), α-Pinene (ΣFPM ranging between 0.08 - 0.24), and β-pinene (ΣFPM ranging between 0.05 - 0.48). The most synergistic combination was exhibited by (–)-Citronellal with Linalyl acetate or Ocimene, which had a ΣFPM values of 0.02. The synergy demonstrated by (–)-Menthone, (+)-α-Pinene, (–)-α-Pinene and (–)-Citronellal, in combination with α-Terpineol was still evident at 48 hrs (ΣFPM between 0.06 - 0.46). All of the combinations involving the enantiomers of Borneol, and the majority of the combinations involving the enantiomers of Camphor were still antagonistic at 48 hrs. However, (+)-Camphor was non-interactive with either Geraniol, Eugenol, Isoeugenol, Ocimene, Estragole, Sabinene hydrate, Menthol or α-Terpineol (ΣFPM between 1.07 - 3.89), whereas these combinations were antagonistic at 24 hrs (ΣFPM between 8.43 - 29.45).

A total of 26.02% of the combinations displayed variations in terms of the interactive efficacy. Those observed were 'synergy versus antagonism' (0.51%), 'synergy versus non-interactive' (5.10%), 'synergy versus additive' (4.59%), 'additive versus antagonism' (1.53%), 'additive

versus non-interactive' (7.14%), and 'non-interactive versus antagonism' (6.12%). The greatest variations was 'synergy versus antagonism' and 'additive versus antagonism'. 'Synergy versus antagonism' was observed only once at 48 hrs.

At 24 hrs, (–)-Camphor was synergistic in combination with Menthol or α-Terpineol (ΣFPM between 0.40 - 0.42), whereas (+)-Camphor was antagonistic (ΣFPM between 8.43 - 29.45). (+)-β-pinene demonstrated synergy in combination with γ-Terpinene (ΣFPM = 0.10), whereas (–)-β-pinene was antagonistic (ΣFPM = 14.69). However, in combination with Eugenol, (–)-β-pinene was synergistic (ΣFPM = 0.07), whereas (+)-β-pinene was antagonistic (ΣFPM = 5.36).

The variation 'additive versus antagonism' was observed once, when (+)-Limonene was combined with Ocimene the interaction was additive (ΣFPM = 0.99), whereas (–)-Limonene interacted antagonistically (ΣFPM = 5.07). Another variation with (+)-Limonene demonstrating the greatest variability is that with γ-Terpinene (Fig 4.4) or α-Terpineol. (+)-Limonene in combination with γ-Terpinene or α-Terpineol was synergistic (ΣFPM between 0.20 - 0.22), whereas (–)-Limonene was antagonistic in combination (ΣFPM between 55.60 - 115.50). An example of this is demonstrated in Figure 4.4.

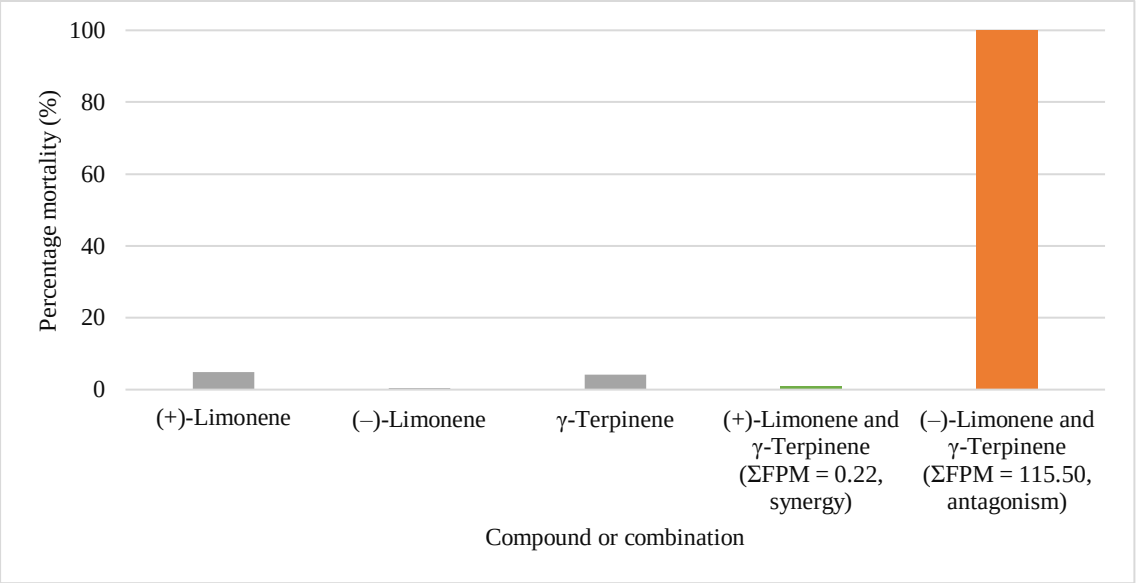


Figure 4.4: The mean PM and interactive efficacy of the enantiomers of Limonene and of γ-Terpinene, independently and in combination, at 24 hrs.

At 48 hrs, (+)-Limonene in combination with Sabinene hydrate was additive (ΣFPM = 0.75), whereas (–)-Limonene was antagonistic (ΣFPM = 11.81). In addition, (+)-Limonene in

combination with γ-Terpinene was additive (ΣFPM = 0.61), whereas (–)-Limonene was antagonistic (ΣFPM = 34.20). This is similar to what was observed at 24 hrs, which was 'synergy versus antagonism'. (+)-Menthone was also additive in combination with γ-Terpinene (ΣFPM = 0.65), whereas (–)-Menthone was non-interactive (ΣFPM = 5.26). At 24 hrs, (+)-Menthone was synergistic in combination with γ-Terpinene (ΣFPM = 0.39), whereas (–)-Menthone was non-interactive (ΣFPM = 3.84). The biggest variation was observed with (+)-Limonene in combination with α-Terpineol was synergistic (ΣFPM = 0.23), whereas (–)-Limonene was antagonistic in combination (ΣFPM = 22.24) (Figure 4.5). This was observed at 24 hrs as well.

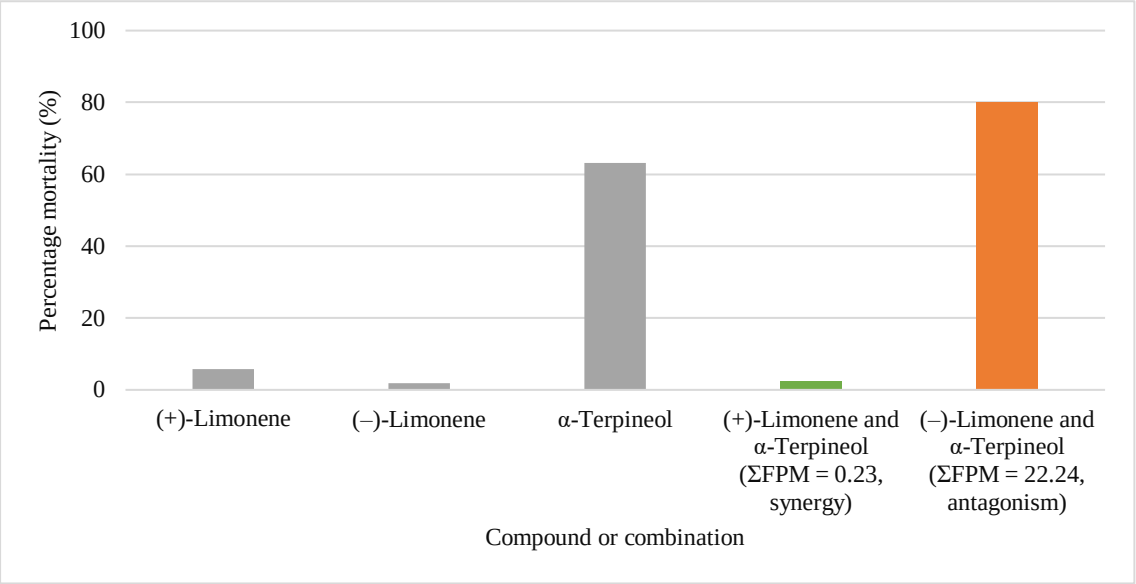


Figure 4.5: The mean PM and interactive efficacy of the enantiomers of Limonene and of α-Terpineol, independently and in combination, at 48 hrs.

The combined interactive efficacy of essential oil compounds in terms of toxicity were previously investigated (Ntalli *et al.*, 2011; Pavela, 2014; Wu *et al.*, 2017; Gaire, *et al.*, 2019; Radwan and Gad, 2021). However, few studies considered the enantiomeric configuration of the essential oil compounds. Acute toxicity through insecticidal and larvicidal activity have been evaluated, however, to the best of my knowledge, no combination studies have been conducted on toxicity screening of essential oil compounds against brine-shrimp. It is important to note that studies that were evaluated reported synergy to be potentiated, or enhanced toxicity, 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 has therapeutic relevance.

Tak and Isman, (2015) reported the combination of Camphor with 1,8-Cineole (Eucalyptol) to have enhanced toxic effects against *Trichoplusia ni* (cabbage looper moth) through fumigation and contact assays, which is in line with the findings of the current study for both enantiomers of Camphor in combination with Eucalyptol, which exhibited antagonism at 24 and 48 hrs. Pavela (2014) investigated the acute toxicity of 30 aromatic compounds against the larvae of *S. littoralis* at a dose of 0.30 mg/larva at 24 hrs of exposure. The enantiomers included were: R-(+)-Limonene, (–)-β-Pinene, (+)-Camphor and (–)-Borneol, however, their enantiomeric counterparts were not evaluated. The racemates of Citronellal, Menthone and α-Pinene were also investigated, however, their enantiomeric distribution was not specified. Other compounds that were included were *p*-Cymene, α-Terpineol, γ-Terpinene, Camphene, Eugenol, Isoeugenol and 1,8-Cineole (Eucalyptol). Of the 49 combinations in common with this investigation, the interactive profiles of 31 of the combinations (63.27%) were in line with the findings of the current study. Most interestingly, it was reported that (–)-Borneol and Camphor had a low PM value when investigated alone, however, enhanced toxicity was observed in combination with other compounds, which was observed in the current study as antagonism was exhibited by both enantiomers of Borneol and Camphor in majority of the combinations. Pavela (2014) also reported that the combination of α-Pinene (racemate) with Eucalyptol had enhanced toxic effects, which correlates to the findings of the current study, which reports (–)-α-Pinene in combination with Eucalyptol to be antagonistic while (+)-α-Pinene in combination with Eucalyptol was non-interactive. This suggests that (–)-α-Pinene likely had a greater enantiomeric distribution than (+)-α-Pinene in the racemate evaluated by Pavela (2014). However, one needs to consider the influence of other compounds within the neat essential oils.

Lahlou and Berrada (2001) reported on the toxicity of *Citrus aurantium* var. amara (Rutaceae), of which Limonene and *p*-Cymene are the major constituents. The study found that it was highly toxic against *Bulinus truncates* (freshwater snail) (LC_{50} of 0.001 mg/mL). The current investigation evaluated the combined toxicity of the enantiomers of Limonene and *p*-Cymene against brine-shrimp, and found that (+)-Limonene was non-toxic in combination, with a PM of 29.63 - 33.33 at 24 and 48 hrs. However, (–)-Limonene was found to be highly toxic in combination with *p*-Cymene at 24 hrs, with a PM of 100.00%. Therefore it is likely that the sample of *Citrus aurantium* likely had a greater enantiomeric distribution of (–)-Limonene than (+)-Limonene.

Sousa *et al.* (2017) evaluated the toxicity of the essential oil *Cuminum cyminum* L. against *Radix peregra* (wandering pond snail) and reported it to be highly toxic with an LC_{50} of 0.04 mg/mL. The main constituents of *C. cyminum* essential oil were β-Pinene and γ-Terpinene. The current investigation evaluated the toxicity of the enantiomers of β-Pinene and γ-Terpinene, and reported the combination of (–)-β-Pinene to be highly toxic, with a PM of 100.00%. In addition, the combination was antagonistic. However, (+)-β-Pinene was non-toxic at 24 and 48 hrs, with PM values of 0.61 - 43.97%. In addition, the combination was synergistic, resulting in reduced toxicity in combination, when compared to individual toxicity. Orchard *et al.* (2019) evaluated the toxicity of *Helichrysum italicum* (Roth) G.Don essential oil against brine-shrimp. Amongst the major constituents of *H. italicum*, were β-Pinene (7.6%) and β-Caryophyllene (12.4%). The study found the oil to have low toxicity, with PM values of 20.41% at 24 hrs and 40.09% at 48 hrs. The current investigation evaluated the toxicity of the enantiomers of β-Pinene in combination with β-Caryophyllene against brine-shrimp and found that (+)-β-Pinene was highly toxic in combination (PM = 100.00% at 24 hrs), whereas (–)-β-Pinene had low toxicity in combination, with a PM of 2.26% and 5.19%, at 24 and 48 hrs, respectively. Therefore this suggests that there was a greater enantiomeric distribution of (–)-β-pinene > in *C. cyminum* and *H. italicum* essential oils, reported by Sousa *et al.* (2017) and Orchard *et al.* (2019), respectively, however, one needs to consider the influence of other compounds within neat essential oils. The importance of considering the stereochemical configuration of the compound in combination, as the toxicity profiles may not always coincide, has been clearly demonstrated in the current investigation.

4.3.3.4 Summary of the interactive efficacy studies

4.3.3.4.1 Summary of the interactive efficacy at 24 hrs of exposure

The overall interactive profiles between the (+)- and (–)-enantiomers in combinations were mostly similar (Figure 4.6). A total of 19.39% of the combinations displayed variations in terms of the ΣFPM at 24 hrs, majority of which were ‘synergy versus non-interactive’, followed by ‘non-interactive versus antagonism’.

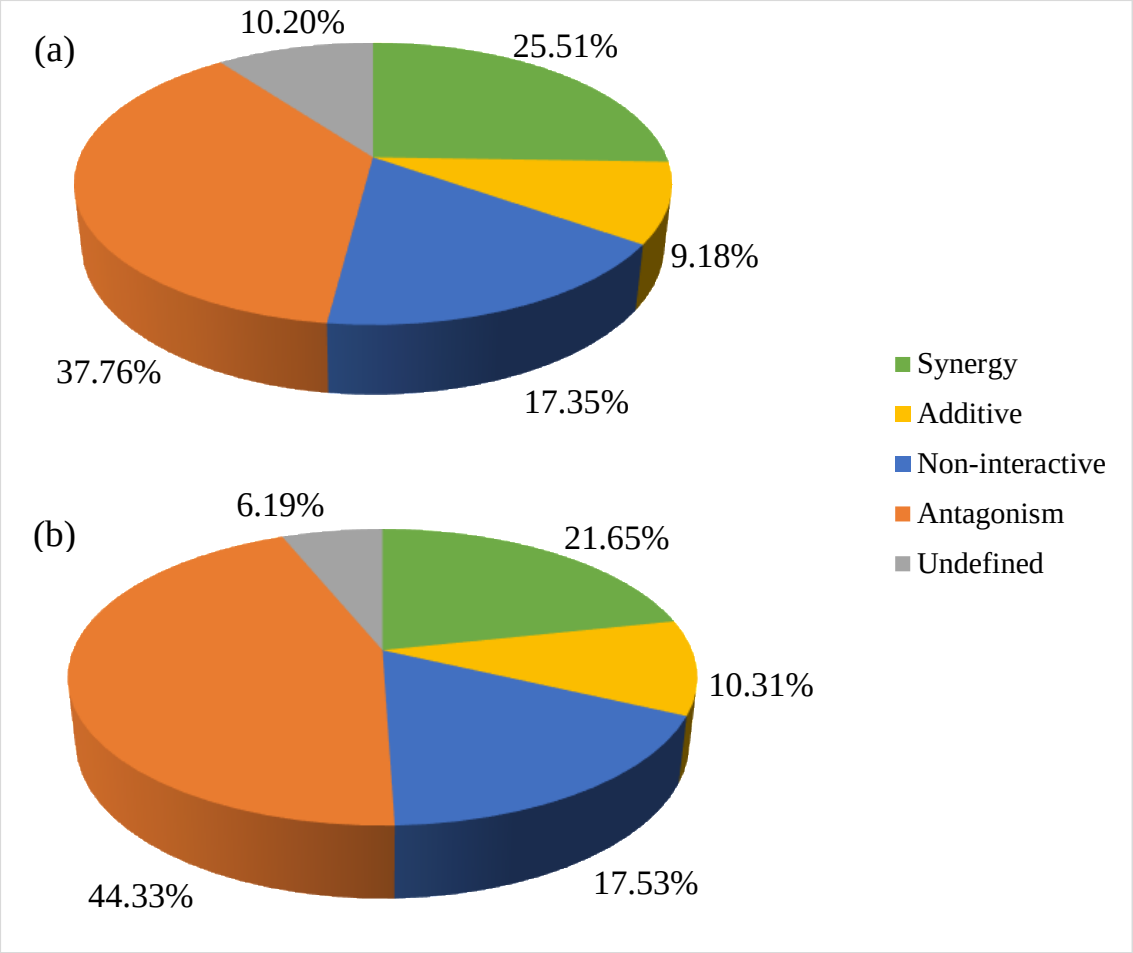


Figure 4.6: Summary of the interactive efficacy studies observed with the (a) (+)-enantiomers (b) and (–)-enantiomers, in combination, at 24 hrs.

The greatest variation in interactive efficacy that was observed was 'synergy versus antagonism', which was observed with (+)-Camphor, (+)-Limonene and both enantiomers of β-pinene. Where variations were observed in terms of the interactive efficacy at 24 hrs, (+)-Limonene, (+)-Menthone and (+)-α-Pinene often interacted more favourably in combination (Figure 4.7). This means that the toxicity of (+)-Limonene, (+)-Menthone and (+)-α-Pinene was often lower when combined, when compared to the (–)-enantiomers. It was also observed that (–)-β-Pinene often interacted more favourably than (+)-β-Pinene in combination. The enantiomers of Borneol did not vary in terms of the combined interactive profiles.

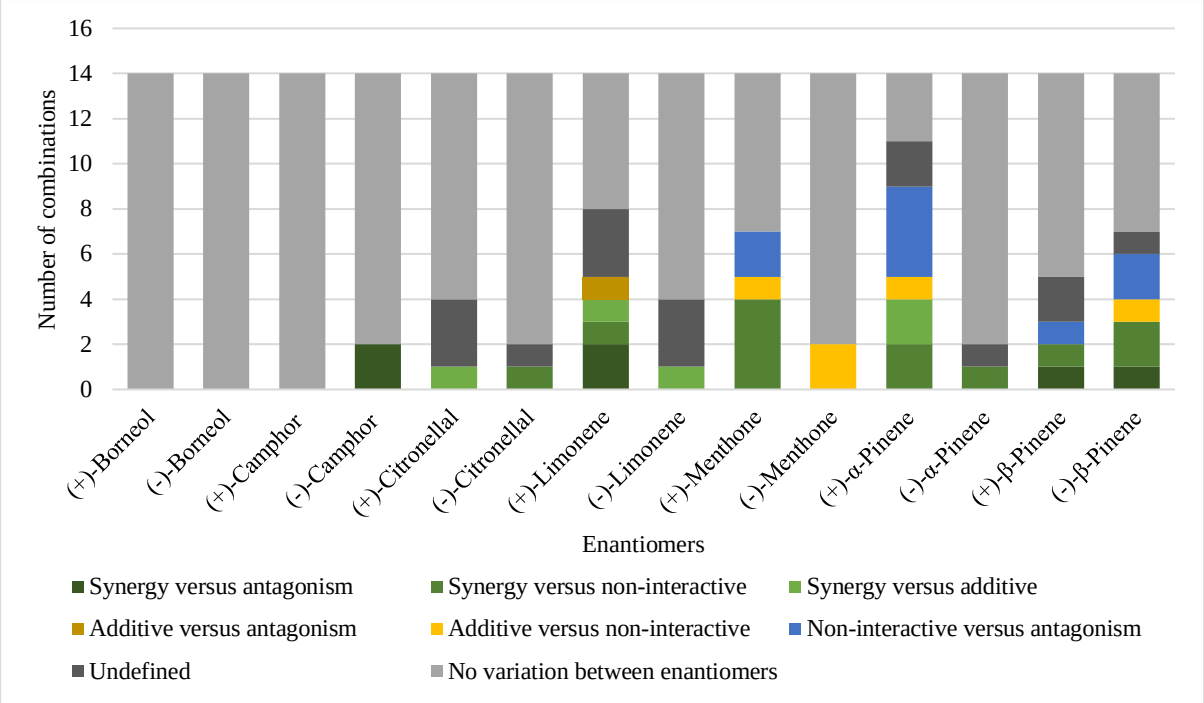


Figure 4.7: Summary of the types of variations seen between the enantiomers in combination, in terms of the interactive efficacy, at 24 hrs

4.3.3.4.2 Summary of the interactive efficacy at 48 hrs of exposure

The overall interactive profiles between the (+)- and (–)-enantiomers in combinations were mostly similar, however, the (–)-enantiomers were involved in almost two-fold more combinations resulting in antagonism, as compared to the (+)-enantiomers (Figure 4.8). A total of 26.02% of the combinations displayed variations in terms of the ΣFPM at 48 hrs, majority of which were 'additive versus non-interactive', followed by 'non-interactive versus antagonism'. The greatest variation in interactive efficacy that was observed was 'synergy versus antagonism', which was observed with only one combination at 48 hrs. This was seen with the enantiomers of Limonene in combination with α-Terpineol, where (+)-Limonene interacted synergistically (ΣFPM = 0.23), whereas (–)-Limonene interacted antagonistically (ΣFPM = 22.24).

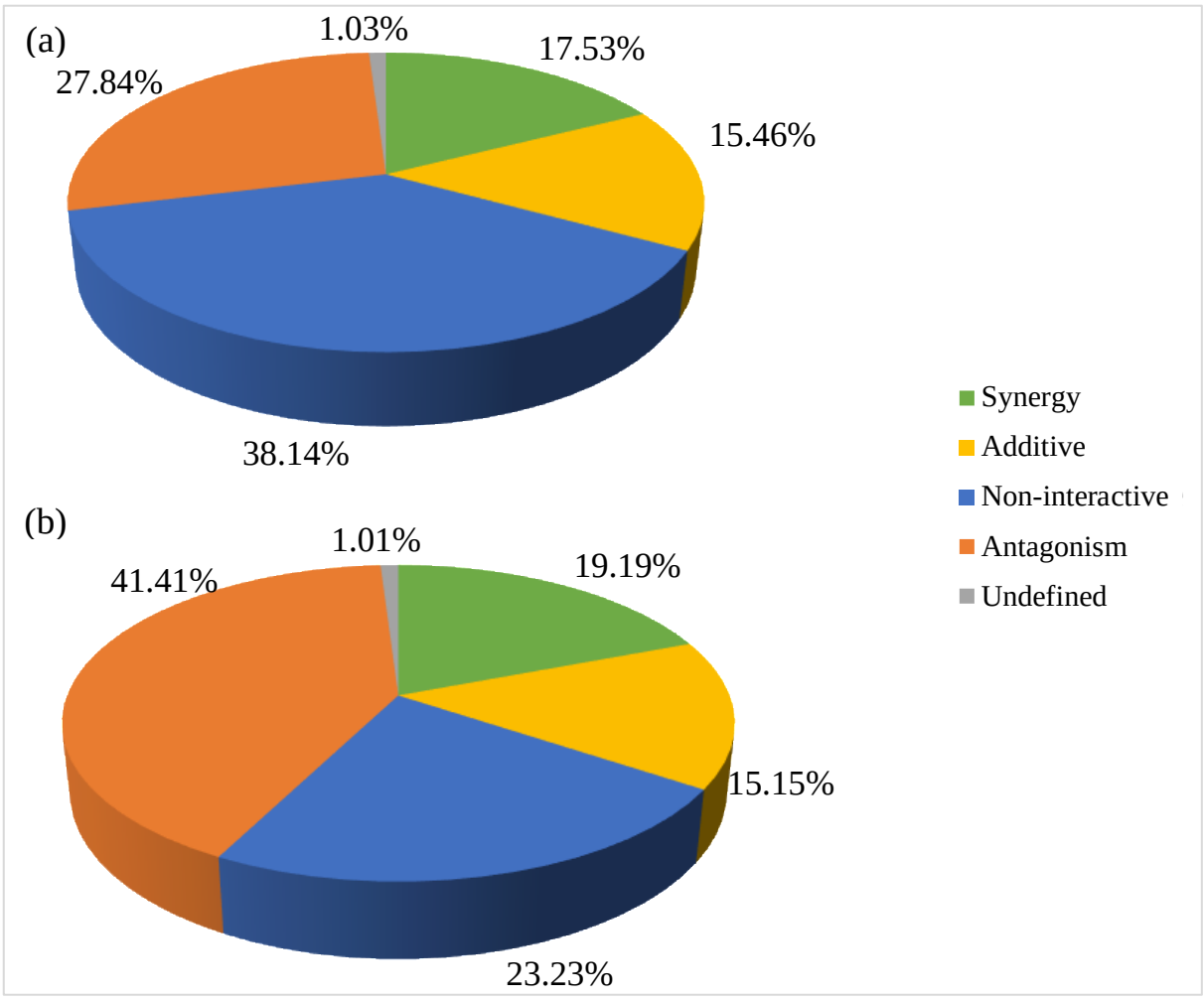


Figure 4.8: Summary of the interactive efficacy studies observed with the (a) (+)-enantiomers (b) and (–)-enantiomers, in combination, at 48 hrs.

Where variations were observed in terms of the interactive efficacy at 48 hrs, (+)-Limonene, (+)-Menthone and (–)-β-Pinene often interacted more favourably in those combinations, as was observed at 24 hrs (Figure 4.9). This means that the toxicity of (+)-Limonene, (+)-Menthone and (–)-β-Pinene was often lower when combined, when compared to their enantiomeric counterparts. It was also observed that at 48 hrs, (+)-Camphor often interacted more favourable, when compared to (–)-Camphor, whereas at 24 hrs the variations were minimal, and no trend was observed. In terms of (+)-α-Pinene often interacting more favourably than (–)-α-Pinene at 24 hrs, this was not observed at 48 hrs. The enantiomers of Borneol did not vary in terms of the combined interactive profiles, as was observed at 24 hrs.

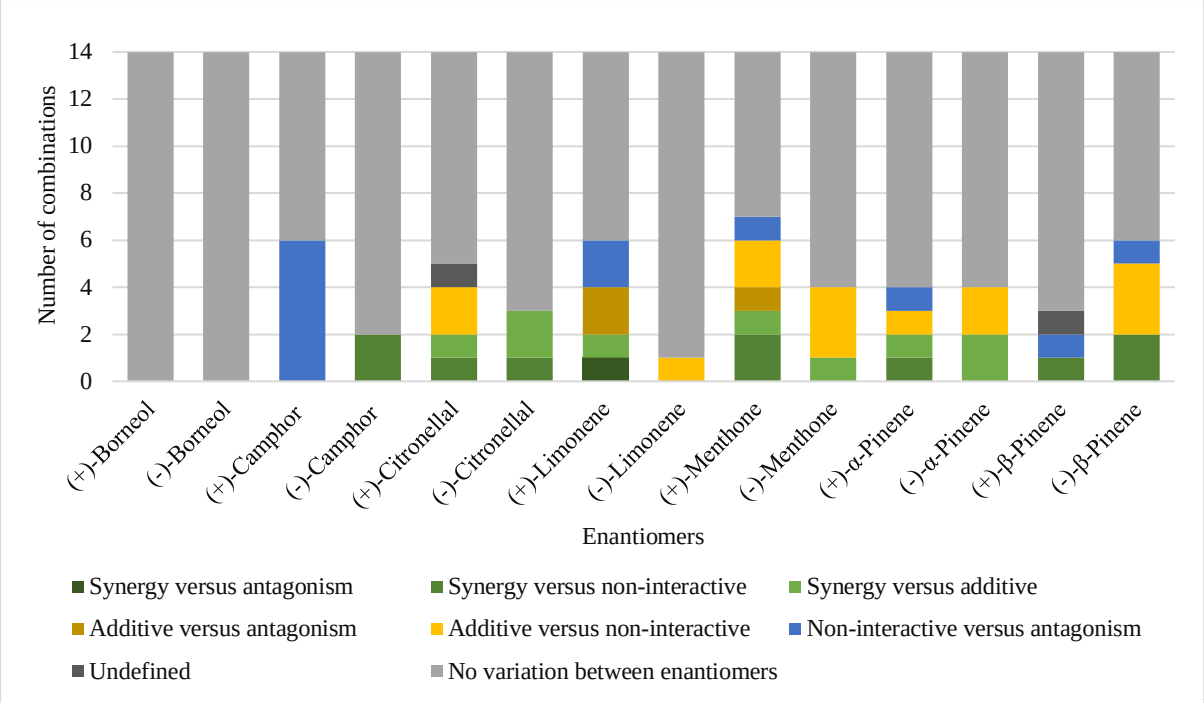


Figure 4.9: Summary of the types of variations seen between the enantiomers in combination, in terms of the interactive efficacy, at 48 hrs.

Overall, the most interesting combination observed with the most synergistic combination was exhibited by (–)-Citronellal and Linalyl acetate and Ocimene, which had a ΣFPM values of 0.02 at 48 hrs. The enantiomers of Limonene in combination with α-Terpineol displayed one of the biggest variations between the enantiomeric pairs in combination. After 24 and 48 hrs of exposure, (+)-Limonene was synergistic in combination with α-Terpineol, whilst (–)-Limonene was antagonistic (Figure 4.10). Overall, it has been clearly demonstrated that the enantiomeric configuration of an essential oil compound does have considerable effects on its toxicity, especially in combination with other essential oil compounds, and is an important factor to consider in the evaluation of essential oil compounds, as well as essential oils.

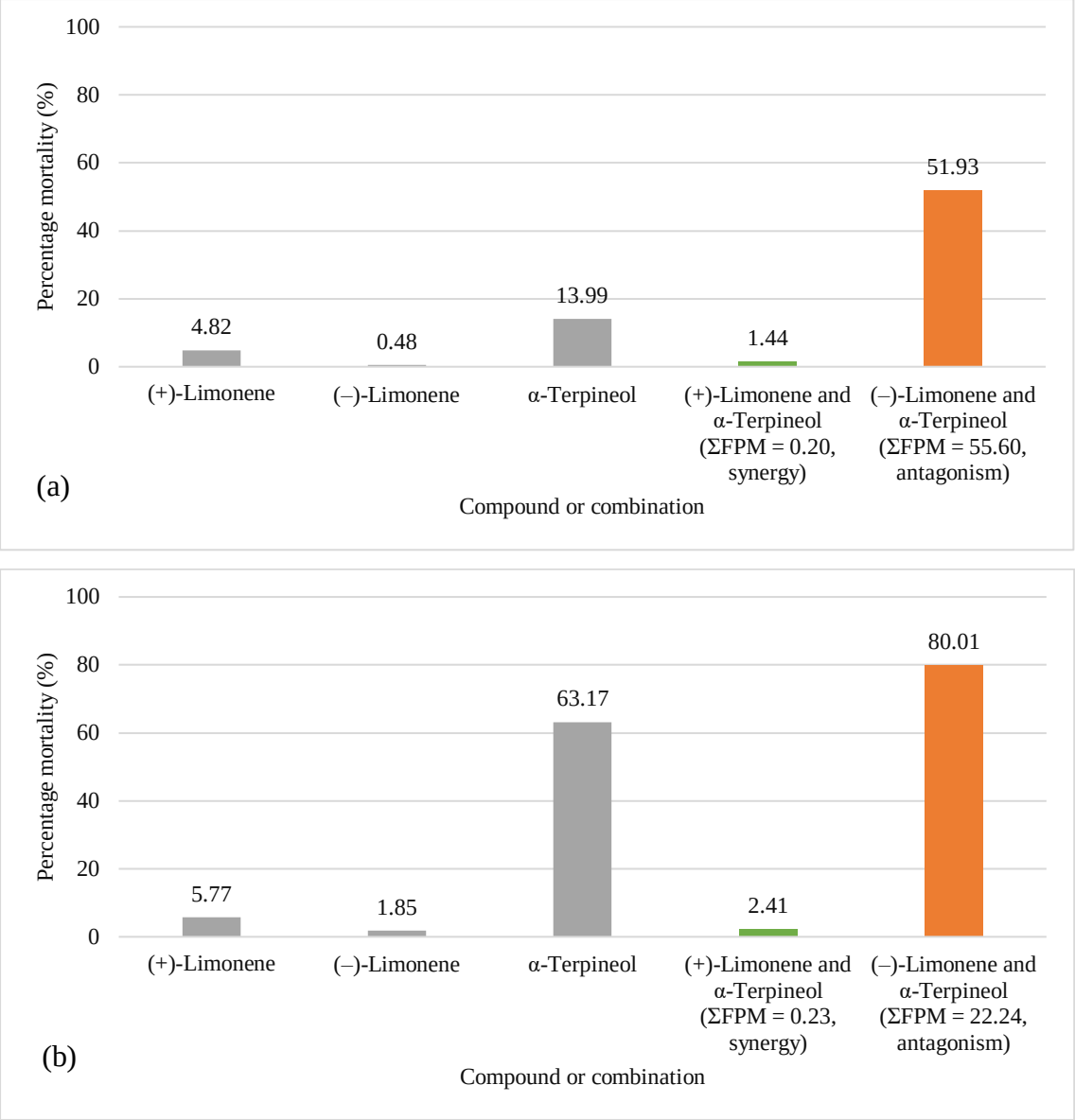


Figure 4.10: The mean PM of the enantiomers of Limonene and of α-Terpineol, independently and in combination, at (a) 24 hrs and (b) 48 hrs.

4.4 Summary

- The enantiomers of Citronellal and Menthone had toxic PM values between 97.44 - 100.00% from 24 hrs.
- The enantiomers of Borneol, Camphor, Limonene and α-Pinene showed non-toxic PM values, ranging between 1.85 - 41.96% at 48 hrs, with the enantiomers of Borneol and Limonene being the least toxic.
- The variations in toxicity displayed by the enantiomers investigated independently were mostly equivalent. The only variation observed in the singular studies was

between the enantiomers of β-Pinene after 48 hrs, where (+)-β-Pinene had a PM of 30.75% and (–)-β-Pinene had a PM of 93.82%.

- Camphene, β-Caryophyllene, Eucalyptol, Linalyl acetate, *p*-Cymene, Sabinene hydrate and γ-Terpinene displayed non-toxic percentage mortalities ranging between 0.85 - 6.87% at 24 hrs and 1.11 - 23.79% at 48 hrs.
- Eugenol, Geraniol, Isoeugenol, Estragole and Menthol displayed toxic PM values of 81.00% - 100.00% at 24 hrs and 82.45 - 100.00% at 48 hrs.
- Ocimene and α-Terpineol were non-toxic at 24 hrs (13.99 - 42.16%), but showed toxicity at 48 hrs (55.04 - 63.17%).
- The results of the combination ΣFPM studies revealed that at 24 and 48 hrs, the majority of the combinations were antagonistic (34.69 - 40.82%), followed by non-interactive (17.35 - 30.61%), synergistic (18.37 - 23.47) and additive (9.69 - 15.51%).
- Both the enantiomers of Citronellal were responsible for most of the synergy observed. Overall, the most synergistic combination was exhibited by (–)-Citronellal and Linalyl acetate and Ocimene, which had a ΣFPM values of 0.02 at 48 hrs.
- Where variations in terms of ΣFPM were observed, (+)-Menthone, (+)-Limonene and (–)-β-Pinene (at 24 and 48 hrs), (–)-Camphor (at 48 hrs) often interacted more favourably, when compared to their enantiomeric counterparts.
- Overall, the enantiomers of Limonene in combination with α-Terpineol displayed the biggest variation between enantiomeric pairs in combination that was observed. After 24 and 48 hrs of exposure, (+)-Limonene was synergistic in combination with α-Terpineol (ΣFPM = 0.20 - 0.23), whilst (–)-Limonene was antagonistic (ΣFPM = 22.24 - 55.60).

Chapter 5 - Overview and conclusion

The current investigation has demonstrated that stereochemistry is an important consideration in the evaluation of the biological activity of essential oil compounds. This study investigated the antimicrobial activity of seven chiral essential oil compounds in their enantiomeric forms, namely: Borneol, Camphor, Citronellal, Limonene, Menthone, α-Pinene, and β-Pinene. The antimicrobial inhibitory activity was investigated. Furthermore, combinations with a selection of compounds, on planktonic bacteria and yeasts were studied. In addition, their effect on quorum sensing (QS) and toxicity were examined. Variations in the antimicrobial, anti-quorum sensing (QS) and toxicity profiles between enantiomeric pairs of seven essential oil compounds were identified. An overview of the objectives and outcomes are as follows;

Objective one: To determine the antimicrobial activity of a selection of enantiomers and compare variability of efficacy using the MIC assay (Chapter 2).

For the investigation against the yeast pathogens, the MIC values of the enantiomers ranged between 0.50 - 2.00 mg/mL (*Candida albicans* ATCC 10231) and 0.13 - 1.00 mg/mL (*Cryptococcus neoformans* ATCC 14116). When investigated against the Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 27853 and *Klebsiella pneumoniae* ATCC 13887), the MIC values ranged between 1.00 - 4.00 mg/mL. Against the two Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923 and *Enterococcus faecium* ATCC 27270), MIC values of the enantiomers ranged between 1.00 - 4.00 mg/mL. The most noteworthy inhibitory activity of the enantiomers was observed against *C. neoformans*. This was observed with (+)-Borneol, with an MIC of 0.13 mg/mL.

There was little variation in the antimicrobial activity between the enantiomeric pairs. A trend was observed in terms of stereoselectivity for certain pathogens, where one enantiomeric form was moderately more active than the other. (+)-α-Pinene showed moderately stronger inhibitory activity than (–)-α-Pinene against all the pathogens investigated. (–)-Limonene displayed moderately stronger inhibitory activity against the yeast pathogens and *P. aeruginosa*, when compared to (+)-Limonene. This was also observed with (+)-Camphor, when compared to (–)-Camphor. (–)-β-Pinene displayed moderately stronger inhibitory activity against *C. albicans* and *P. aeruginosa*, when compared to (+)-β-Pinene. (+)-Borneol displayed

moderately stronger inhibitory activity against the yeast pathogens, when compared to (–)-Borneol. Citronellal and Menthone displayed equivalent inhibitory activity between their enantiomers, against all the pathogens investigated.

Objective two: To determine the interactive antimicrobial activity of the enantiomers in combination with the selected compounds by calculating the fractional inhibitory concentration (ΣFIC) and compare the differences between enantiomers (Chapter 2).

The combinations were carried out to evaluate the combined activity of the enantiomers with the selected compounds against the six pathogens. The results demonstrated that the most prevalent interaction observed was additivity (56.46%), followed by non-interactive (37.93%) interactions. A total of 66 (5.61%) the combinations were found to be synergistic, the majority of which were against the two yeast pathogens, *C. neoformans* and *C. albicans*, followed by *S. aureus*. The most synergistic combinations had ΣFIC values of between 0.38 - 0.50 against all the pathogens, except *E. faecium*, against which no synergy was observed. The majority of the additivity observed was against *K. pneumoniae* and *E. faecium*. No antagonism was observed in any of the combinations investigated. Overall, the combination of (–)-Borneol with Menthol was the most interesting, demonstrating synergy against *S. aureus*, *K. pneumoniae* and *C. neoformans*, with ΣFIC values ranging between 0.38 - 0.50. In addition, additivity was observed against the remaining pathogens, namely: *E. faecium*, *P. aeruginosa* and *C. albicans*, with ΣFIC values ranging between 0.58 - 1.00.

Of the 1176 combinations tested, a total of 17.18% of the combinations displayed variations in terms of the ΣFIC interactive efficacy, the majority of which were 'additive versus non-interactive' (12.93%), followed by 'synergy versus additive' (2.64%) and 'synergy versus non-interactive' (1.62%). The enantiomers of Borneol, Limonene, α-Pinene and β-Pinene varied the most in terms of interactive efficacy. This means that where variations were observed, the (–)-enantiomers of Borneol, Limonene and α-Pinene often interacted in a way that reduced the effective concentration required to inhibit the pathogen, when compared to the (+)-enantiomers. However, in the case of β-Pinene, (+)-β-Pinene often interacted more favourably in combination than (–)-β-Pinene (Figure 5.1).

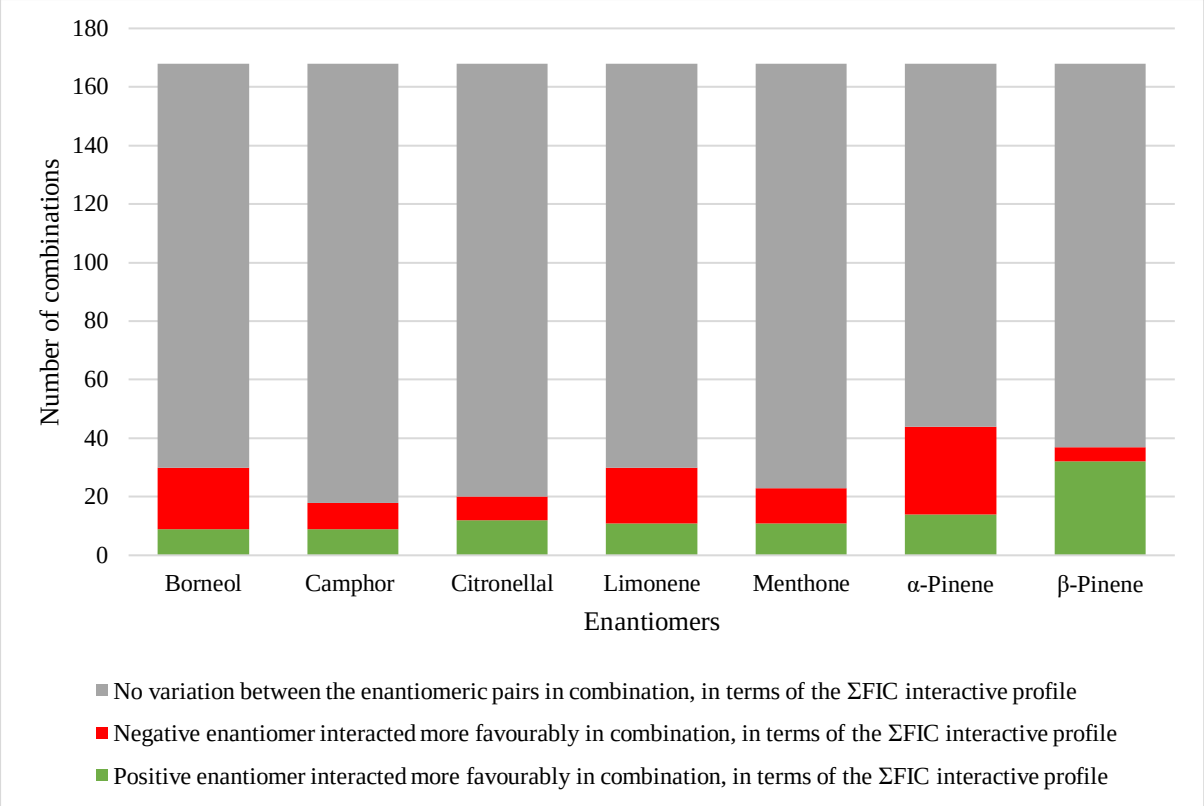


Figure 5.1: Summary of the enantiomeric configurations of the compounds which displayed favourable combined activity, when compared to its enantiomeric counterpart, in terms of the ΣFIC interactive profiles.

The overall greatest variations in terms of ΣFIC that were observed with the enantiomers in combination with the selected compounds were observed with; (+)-β-Pinene in combination with β-Caryophyllene, and (–)-α-Pinene in combination with Estragole, which interacted either additively or synergistically against the majority of the micro-organisms tested, whereas their enantiomeric counterparts were often non-interactive. While the same trend was not observed for the enantiomers of Limonene in combination with γ-Terpinene, where there were variations (against *P. aeruginosa* and *S. aureus*), they were amongst the greatest variations observed. (+)-Limonene with γ-Terpinene interacted either additively or synergistically, with ΣFIC values ranging between 0.38 - 0.67, whereas (–)-Limonene with γ-Terpinene was non-interactive with ΣFIC values ranging between 1.50 - 1.67.

Objective three: To determine the anti-QS activity of the enantiomers and compare the difference, using the MQSIC assay and quantification of the percentage violacein inhibition (Chapter 3).

The anti-QS results revealed that the enantiomers had anti-QS activity at the concentrations investigated (0.06 - 0.50 mg/mL), with percentage violacein inhibition values ranging between 3.84 - 90.68%. The only exceptions were (–)-α-Pinene and (–)-β-Pinene, which did not display violacein inhibition of 50.00% or greater at the highest concentration investigated, and therefore the MQSIC could not be determined. However, (+)-α-Pinene and (+)-β-Pinene had MQSIC values of 0.38 and 0.50 mg/mL, respectively. These were the only variations observed between the enantiomeric pairs, when investigated independently. The enantiomers of Borneol had the lowest MQSIC values (0.13 mg/mL) of the enantiomers investigated, with percentage violacein inhibition values of 56.75% and 69.61% for (+)-Borneol and (–)-Borneol, respectively. The lack of studies related to the anti-QS effects of Borneol is surprising as the current investigation demonstrates that the anti-QS effects of both enantiomers of Borneol are appreciable, as they required the lowest effective dose to achieve considerable violacein inhibition, when compared to the other enantiomers investigated.

Objective four: To determine the interactive antimicrobial activity of the enantiomers in combination with the selected compounds by calculating the fractional quorum sensing inhibitory concentration (ΣFQSIC) and the fractional percentage violacein reduction (ΣFPVR) and compare the differences between enantiomers (Chapter 3).

The interactive efficacy of the combinations was determined in two ways: 1) Through ΣFQSIC analysis of the enantiomers and selected compounds, and 2) Through ΣFPVR analysis of the enantiomers and the selected compounds. The ΣFQSIC studies evaluated the influence of the combinations in terms of their effect on the concentration required to inhibit violacein production by 50.00% or more, using the MQSIC values determined. The ΣFPVR studies evaluated the influence of the combinations in terms of their effect on the extent to which violacein was inhibited, using the percentage violacein reduction values.

The ΣFQSIC studies revealed that the majority of the combinations were non-interactive (44.90%), followed by additive (20.41%), then synergy (8.16%). (+)-Limonene in combination with Estragole demonstrated the lowest ΣFQSIC value 0.31 (synergy). Only one combination

was found to be antagonistic. This was evident for (–)-Limonene in combination with *p*-Cymene (ΣFQSIC = 4.67). A few (26.02%) of the combinations remained 'undefined', particularly as (–)-α-Pinene and (–)-β-Pinene had MQSIC values greater than the highest concentration investigated.

Of the 196 combinations investigated, a total of 8.16% of the combinations displayed variations in terms of the ΣFQSIC, the majority of which were 'additive versus non-interactive' (5.61%), followed by 'synergy versus additive' (2.04%), and 'non-interactive versus antagonism' (0.51%). It was revealed that the enantiomers of Limonene and Citronellal in combination varied the most in terms of ΣFQSIC, where the (+)-enantiomers often interacted more favourably in those combinations, when compared to the (–)-enantiomers (Figure 5.2).

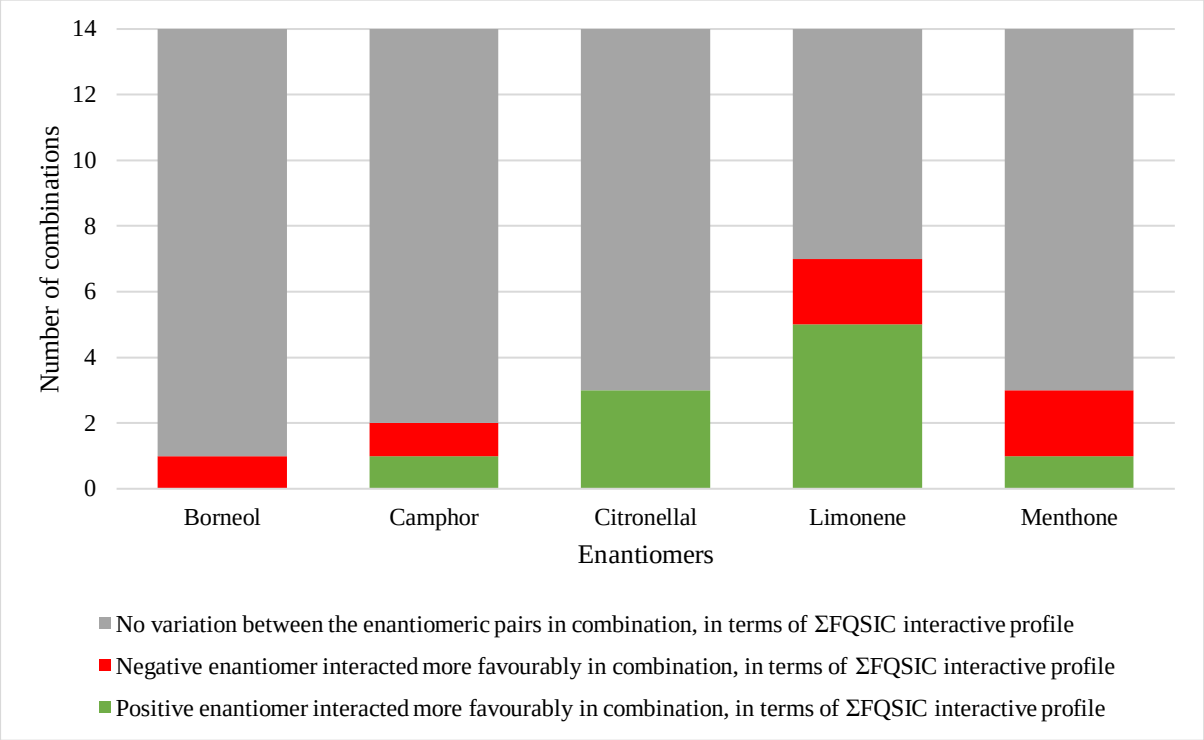


Figure 5.2: Summary of the enantiomeric configurations of the compounds which displayed favourable combined activity, when compared to its enantiomeric counterpart, in terms of the ΣFQSIC interactive profiles.

This means that where variations were observed, (+)-Limonene and (+)-Citronellal often interacted in a way that reduced the effective concentration required to disrupt bacterial communication by 50.00% or more, when compared to their (–)-counterparts. The combination of the enantiomers of Limonene with α-Terpineol displayed the biggest variation in terms of

the ΣFQSIC interactive efficacy, where (+)-Limonene in combination was additive (ΣFQSIC = 0.56), while (–)-Limonene in combination was non-interactive (ΣFQSIC = 2.33). Another considerable variation was demonstrated by (–)-Limonene in combination with *p*-Cymene, which was antagonistic (ΣFQSIC = 4.67), whereas (+)-Limonene was non-interactive (ΣFQSIC = 1.69).

The ΣFPVR studies revealed that the majority of the combinations were non-interactive (40.82%), followed by additive (29.08%), and synergy (4.08%). (+)-Menthone in combination with Eucalyptol demonstrated the lowest ΣFPVR in combination of 0.26 (synergy). No antagonism was observed. A few (26.02%) of the combinations remained 'undefined'. Of the 196 combinations investigated, 15.82% of the combinations displayed variations in terms of the ΣFPVR, the majority of which were 'additive versus non-interactive' (11.73%), followed by 'synergy versus additive' (2.55%). Although few, the greatest variation observed in terms interactive efficacy observed was 'synergy versus non-interactive' (1.53%). This was only evident with the enantiomers of Camphor in combination, where (–)-Camphor often displayed synergy in combination, whereas (+)-Camphor was non-interactive. Where variations were observed in terms of the ΣFPVR interactive efficacy, (+)-Borneol, (–)-Camphor, (–)-Limonene and (+)-Menthone often interacted more favourably in those combinations. This means that they inhibited the extent of bacterial communication to a greater extent in combination, when compared to their enantiomeric counterparts. This is visually demonstrated in Figure 5.3.

The combination of the enantiomers of Camphor with α-Terpineol displayed the biggest variation in terms of the ΣFPVR interactive efficacy, where (+)-Camphor in combination was non-interactive (ΣFPVR = 1.99), whereas (–)-Camphor in combination was synergistic (ΣFPVR = 0.44).

When the ΣFQSIC and ΣFPVR studies were compared, two observations were made: Firstly, enantiomers can interact similarly in terms of their combined effect on the effective concentration required to disrupt bacterial communication (ΣFQSIC), however, they may differ in terms of the extent to which bacterial communication is inhibited (ΣFPVR). This was observed with (+)-Borneol, (–)-Camphor and (+)-Menthone, which displayed similar combined ΣFQSIC values but often varied in terms of ΣFPVR, when compared to their enantiomeric counterparts.

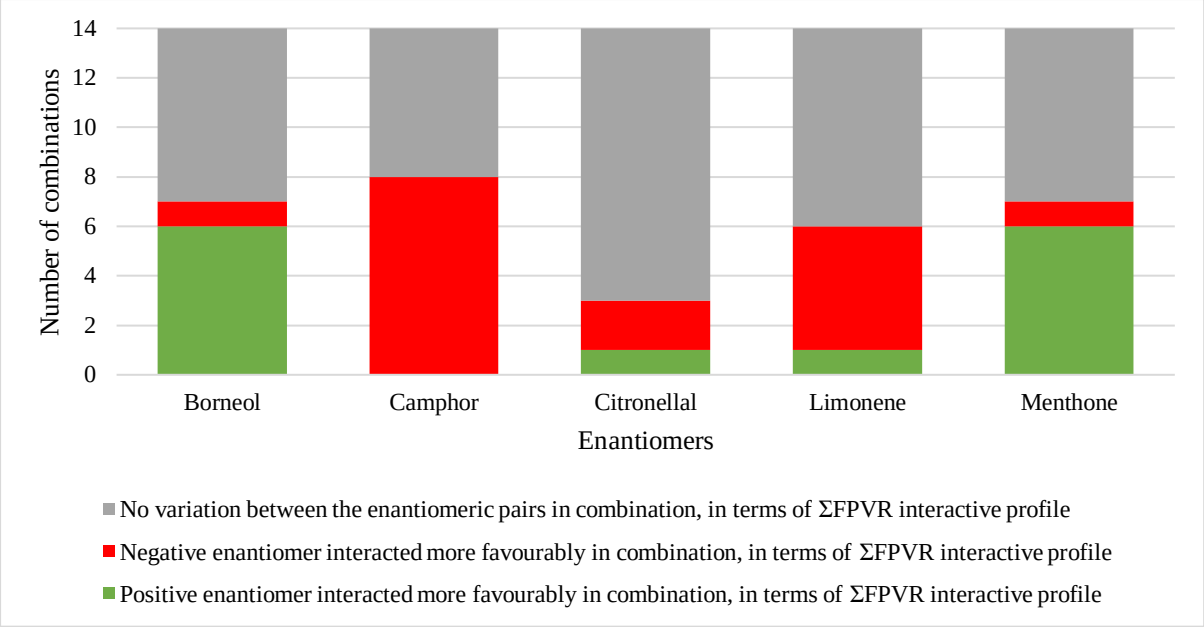


Figure 5.3: Summary of the enantiomeric configurations of the compounds which displayed favourable combined activity, when compared to its enantiomeric counterpart, in terms of the ΣFPVR interactive profiles.

This means that although they inhibited bacterial communication at a similar concentration, the extent to which bacterial communication was inhibited varied at that concentration. The second observation made was, one enantiomeric form may interact more favourably in terms of the effect on the combined MQSIC, while the other enantiomeric form may interact more favourably in terms of inhibiting violacein to a greater extent, when compared to the other (ΣFPVR). This was particularly evident with the enantiomers of Limonene, where (+)-Limonene often interacted more favourably in terms of requiring a lower effective concentration to disrupt bacterial communication (ΣFQSIC), whereas (–)-Limonene interacted more favourable in terms of the extent to which bacterial communication was disrupted (ΣFPVR).

Objective five: To screen the toxicity of the enantiomers and compare the difference, between enantiomers using the brine-shrimp lethality assay (Chapter 4).

The enantiomers of Borneol, Camphor, Limonene and α-Pinene were found to be non-toxic, with percentage mortality (PM) values ranging between 0.46 - 41.96%. (+)-Borneol and (–)-Limonene had the lowest PM values ranging between 0.46 - 0.48% at 24 hrs, and 1.85 - 2.45% at 48 hrs. The enantiomers of Citronellal and Menthone were found to be toxic with PM values

ranging between 97.44 - 100.00%. The toxicological profiles of the enantiomeric pairs were mostly equivalent. The only exception was the enantiomers of β-Pinene where both enantiomers were non-toxic at 24 hrs (PM values ranging between 10.29 - 18.59%), however, at 48 hrs (+)-β-Pinene had PM values of 30.75% and (–)-β-Pinene was three-fold more toxic, with a PM of 93.82%.

Objective six: To determine the interactive toxicity of the enantiomers in combination with the selected compounds by calculating the fractional percentage mortality (ΣFPM) and differences between enantiomers (Chapter 4).

The results of the combination ΣFPM studies revealed that at 24 and 48 hrs, the majority of the combinations were antagonistic (34.69 - 40.82%), followed by non-interactive (17.35 - 30.61%), synergistic (18.37 - 23.47) and additive (9.69 - 15.51%). A few combination (1.02 - 8.67%) remained undefined. The enantiomers of Citronellal demonstrated the most synergy in combination (ΣFPM ranging between 0.02 - 0.42), followed by the enantiomers of Menthone (ΣFPM ranging between 0.04 - 0.46), α-Pinene (ΣFPM ranging between 0.08 - 0.24), and β-pinene (ΣFPM ranging between 0.05 - 0.48). The most synergistic combination was exhibited by (–)-Citronellal and Linalyl acetate and Ocimene, which had a ΣFPM values of 0.02 at 48 hrs. The enantiomers of Borneol and Camphor were responsible for majority of the antagonism demonstrated and were highly toxic in combination, which is interesting given that, individually, they were considered non-toxic with PM values 0.46 - 2.48% at 24 hrs, and 2.45 - 25.62% at 48 hrs.

Variations in terms of the toxicity of the enantiomers were observed in 19.39% and 26.02% of the 196 combinations, at 24 hrs and 48 hrs, respectively. The most prevalent variation observed was ‘synergy versus non-interactive’ (6.12%) at 24 hrs, and ‘additive versus non-interactive’ (7.14%) at 48 hrs. The greatest variation observed was ‘synergy versus antagonism’. This was observed with (+)-Limonene in combination with α-Terpineol at both 24 hrs and 48 hrs, which was synergistic (ΣFPM = 0.22 - 0.23), whereas (–)-Limonene was antagonistic in combination (ΣFPM = 22.24 - 115.50). Where variations in terms of ΣFPM were observed, (+)-Menthone, (+)-Limonene and (–)-β-Pinene (at 24 and 48 hrs), (–)-Camphor (at 48 hrs) often showed reduced toxicity in combination, when compared to their enantiomeric counterparts (Figure 5.4).

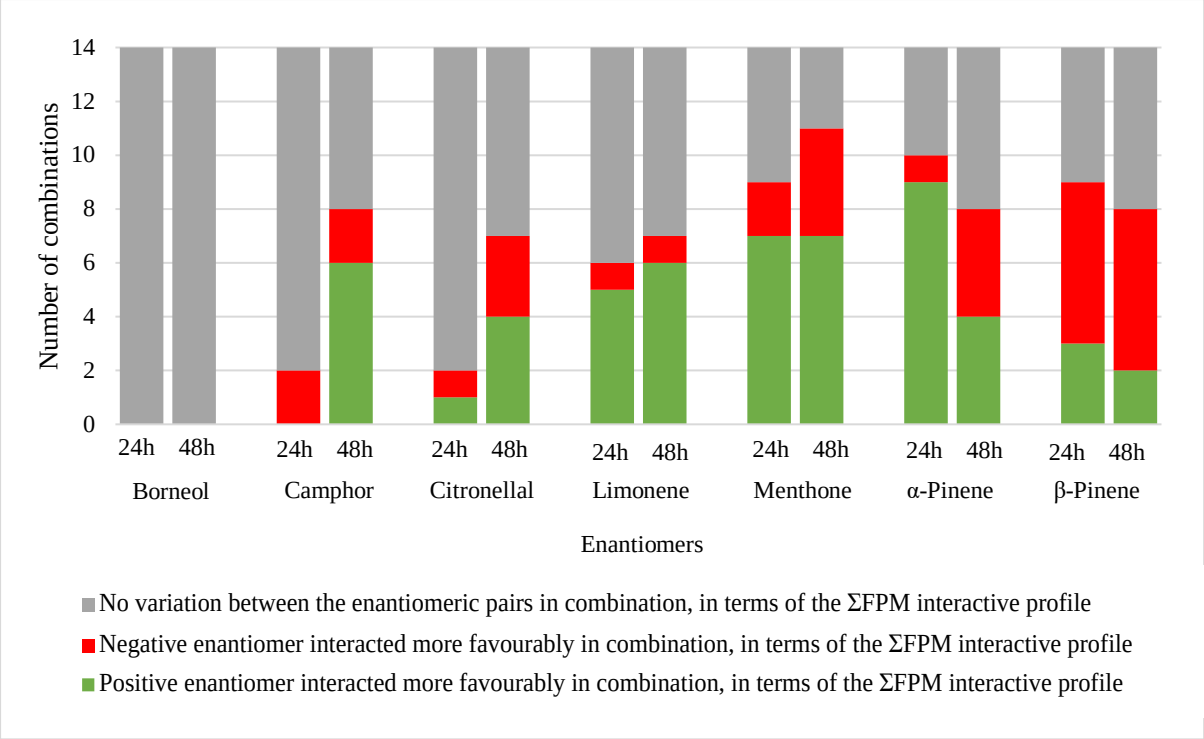


Figure 5.4: Summary of the enantiomeric configurations of the compounds which displayed favourable combined activity, when compared to its enantiomeric counterpart, in terms of the ΣFPM interactive profiles.

5.1 Observations and trends between the antimicrobial, anti-quorum sensing and brine-shrimp lethality assays.

Overall, the best activity across the three assays was observed for (–)-Limonene in combination with Estragole which was synergistic against *S. aureus* (ΣFIC = 0.50) and additive against the remaining pathogens, namely: *E. faecium*, *P. aeruginosa*, *K. pneumoniae*, *C. albicans* and *C. neoformans*, with ΣFIC values ranging between 0.75 - 1.00. In addition, the combination was additive in terms of both ΣFQSIC (0.67) and ΣFPVR (0.58). This means that the combined effective concentration was reduced, and the extent to which bacterial communication was disrupted was increased, compared to the activity of the compounds independently. In addition, the combination was observed to be biologically non-toxic, with a PM value of 1.55% at 24 hrs and 46.56% at 48 hrs.

This study has clearly demonstrated that while variations in the antimicrobial activity and the toxicological profiles were few, variations in the combined activity of enantiomeric pairs were often observed. Some of the highlights include: the enantiomers of α-Pinene in combination

with Estragole which both had similar, non-toxic profiles at 24 and 48 hrs. However, in terms of the antimicrobial activity of this combination, (–)-α-Pinene in combination with Estragole was considerably more active against the pathogens investigated, when compared to (–)-α-Pinene and Estragole. Another interesting example is the combination of the enantiomers of Limonene with α-Terpineol. Both enantiomers had similar inhibitory activity against the pathogens in combination with Limonene with α-Terpineol, however, varied greatly in terms of the anti-QS activity and toxicity. (+)-Limonene was 50.49 - 77.60% less toxic and the MQSIC was four-fold less than that of (–)-Limonene in combination with α-Terpineol. Table 5.1 summarises the synergistic combinations (ΣFIC) observed against the six pathogens investigated, and the corresponding anti-QS activity and toxicity of the combinations. In the case of the enantiomers of Borneol and Camphor, the combined antimicrobial activity observed may have been as a result of their toxic interactions with the selected compounds. This could lead one to assume that the observed antimicrobial activity may be as a result of the toxic nature of essential oil compounds. However, this was not always the case, as highlighted in Table 5.1. This was especially evident with (+)-Citronellal, and the enantiomers of Limonene, Menthone, α-Pinene and β-pinene. The enantiomers displayed synergy against the pathogens mentioned, when combined with certain selected compounds, and often had PM values that were less than 50.00%, making them non-toxic. It is important to note that often when synergy was observed by only one enantiomer, the other was likely additive in combination. However, highlighted in Table 5.1 are the combinations that showed synergy, whereas their enantiomeric counterparts were non-interactive in combination with the selected compound (not included in Table 5.1), and therefore displayed the greatest variation in interactive efficacy of the combinations investigated.

Table 5.1: Summary of the synergistic combinations (ΣFIC) observed against the six pathogens investigated, and the corresponding anti-QS activity and toxicity

Synergistic combination (antimicrobial inhibitory)		**Pathogen**	**Combined MIC (mg/mL)**	**ΣFIC**	**MQSIC**	**ΣFQSIC**	**ΣFPVR**	**PM (%) after 24 hrs at 0.50 mg/mL**	**PM (%) after 48 hrs at 0.50 mg/mL**	**Toxic/non-toxic**
Enantiomer	**Selected compound**									
(+)-Borneol	β-Caryophyllene	*S. aureus*	1.00	0.38*	0.25	1.25	**0.81**	100.00	100.00	Toxic
	γ-Terpinene		1.00	0.50	0.13	1.50	**0.91**	100.00	100.00	Toxic
	Menthol		1.00	0.50	0.13	1.50	**0.96**	100.00	100.00	Toxic
	α-Terpineol		1.00	0.50	0.13	1.50	**0.98**	100.00	100.00	Toxic
	Estragole	*K. pneumoniae*	1.00	0.50	0.13	**1.00**	1.43	100.00	100.00	Toxic
	Sabinene hydrate		1.00	0.50	0.13	**1.00**	**0.92**	100.00	100.00	Toxic
(–)-Borneol	Menthol	*S. aureus*	1.00	0.50	0.13	1.50	1.28	100.00	100.00	Toxic
	Menthol	*K. pneumoniae*	0.50	0.38	0.13	1.50	1.28	100.00	100.00	Toxic
	Geraniol	*C. neoformans*	0.13	0.50*	0.13	**0.75**	1.30	100.00	100.00	Toxic
	β-Caryophyllene		0.13	0.50*	0.25	1.25	**0.56**	100.00	100.00	Toxic
	Isoeugenol		0.13	0.50	0.13	1.50	1.53	100.00	100.00	Toxic
	Estragole		0.13	0.38*	0.13	**1.00**	1.71	100.00	100.00	Toxic
	Menthol		0.13	0.38*	0.13	1.50	1.28	100.00	100.00	Toxic
(+)-Camphor	β-Caryophyllene	*S. aureus*	2.00	0.50*	> 0.50	nd	nd	73.61	97.22	Toxic
	Estragole		2.00	0.50*	0.13	**0.63**	1.27	56.37	59.53	Toxic
(–)-Camphor	Camphene	*C. albicans*	0.50	0.38	0.50	nd	nd	37.65	58.22	Toxic only after 48 hrs
(+)-Citronellal	Eugenol	*P. aeruginosa*	0.50	0.50	0.09	**0.94**	1.90	100.00	100.00	Toxic

Synergistic combination (antimicrobial inhibitory)		Pathogen	Combined MIC (mg/mL)	ΣFIC	MQSIC	ΣFQSIC	ΣFPVR	PM (%) after 24 hrs at 0.50 mg/mL	PM (%) after 48 hrs at 0.50 mg/mL	Toxic/non-toxic
Enantiomer	**Selected compound**									
	Isoeugenol		0.50	0.50	0.13	1.25	1.58	8.10	27.46	Non-toxic
	γ-Terpinene		0.50	0.42	0.25	2.50	**0.63**	3.13	22.31	Non-toxic
	Ocimene	*P. aeruginosa*	0.50	0.50	0.19	1.88	2.82	0.00	1.65	Non-toxic
	p-Cymene	*P. aeruginosa*	0.50	0.50	0.13	1.25	1.43	8.95	16.57	Non-toxic
	α-Terpineol	*C. neoformans*	0.25	0.38	0.13	1.25	1.65	4.38	6.52	Non-toxic
(+)-Limonene	γ-Terpinene	*S. aureus*	1.00	0.38*	0.13	1.13	**0.97**	0.98	3.84	Non-toxic
	Estragole	*C. neoformans*	0.19	0.38	0.06	**0.31**	**0.95**	14.48	35.63	Non-toxic
	Camphene		0.25	0.50	> 0.50	nd	nd	2.45	5.15	Non-toxic
(–)-Limonene	Estragole	*S. aureus*	2.00	0.50	0.13	**0.67**	**0.58**	1.55	46.56	Non-toxic
	β-Caryophyllene	*C. neoformans*	0.13	0.50*	> 0.50	nd	nd	0.00	2.04	Non-toxic
	Ocimene		0.13	0.50	0.25	2.33	1.16	4.85	95.31	Toxic only after 48 hrs
	p-Cymene		0.13	0.38	0.50	4.67	**0.69**	100.00	100.00	Toxic
(+)-Menthone	Linalyl acetate	*C. albicans*	1.00	0.50	0.25	**0.75**	**0.94**	0.63	1.71	Non-toxic
	Eucalyptol		1.00	0.50	0.50	1.67	**0.26**	4.29	14.82	Non-toxic
	γ-Terpinene		1.00	0.50	0.13	1.25	**0.80**	3.15	8.48	Non-toxic
	Ocimene		1.00	0.50	0.13	1.25	1.15	16.54	22.25	Non-toxic
(–)-Menthone	γ-Terpinene	*C. albicans*	1.00	0.50	0.25	2.50	**0.54**	30.70	68.34	Toxic only after 48 hrs
	Ocimene		1.00	0.50	0.06	**0.63**	1.60	11.42	18.68	Non-toxic

Synergistic combination (antimicrobial inhibitory)		Pathogen	Combined MIC (mg/mL)	ΣFIC	MQSIC	ΣFQSIC	ΣFPVR	PM (%) after 24 hrs at 0.50 mg/mL	PM (%) after 48 hrs at 0.50 mg/mL	Toxic/non-toxic
Enantiomer	**Selected compound**									
	Estragole		1.00	0.50	0.13	**0.75**	1.39	92.11	94.89	Toxic
	Camphene		1.00	0.50	0.25	nd	nd	0.93	6.39	Non-toxic
	Eucalyptol	*C. neoformans*	0.13	0.38*	0.50	1.67	**0.89**	76.92	78.35	Toxic
(+)-α-Pinene	Menthol	*C. albicans*	0.50	0.50	0.13	1.17	**0.67**	36.06	83.49	Toxic only after 48 hrs
	Eugenol	*C. neoformans*	0.13	0.50	0.06	**0.58**	1.16	100.00	100.00	Toxic
	Isoeugenol		0.13	0.50	0.13	1.17	1.30	100.00	100.00	Toxic
	γ-Terpinene	*C. neoformans*	0.13	0.50*	0.25	2.33	1.06	3.21	20.63	Non-toxic
	Ocimene	*C. neoformans*	0.13	0.50*	0.25	2.33	**0.87**	34.44	43.75	Non-toxic
(–)-α-Pinene	β-Caryophyllene	*S. aureus*	2.00	0.50*	0.50	nd	nd	3.74	10.15	Non-toxic
	Ocimene		2.00	0.50*	0.25	nd	nd	4.21	8.42	Non-toxic
	Estragole		2.00	0.50*	0.13	nd	nd	9.29	44.71	Non-toxic
	Eucalyptol	*P. aeruginosa*	1.00	0.50*	0.50	nd	nd	7.49	11.59	Non-toxic
	Camphene	*K. pneumoniae*	2.00	0.50	0.25	nd	nd	1.08	3.64	Non-toxic
	Menthol	*C. albicans*	0.50	0.42	0.09	nd	nd	41.38	79.61	Toxic only after 48 hrs
	Eugenol	*C. neoformans*	0.13	0.41	0.19	nd	nd	100	100.00	Toxic
	Isoeugenol		0.13	0.41	0.13	nd	nd	84.96	100.00	Toxic
(+)-β-Pinene	β-Caryophyllene	*C. albicans*	1.00	0.50*	> 0.50	nd	nd	100.00	100.00	Toxic
	Linalyl acetate		1.00	0.50	0.50	**1.00**	**0.69**	8.12	9.63	Non-toxic

Synergistic combination (antimicrobial inhibitory)		Pathogen	Combined MIC (mg/mL)	ΣFIC	MQSIC	ΣFQSIC	ΣFPVR	PM (%) after 24 hrs at 0.50 mg/mL	PM (%) after 48 hrs at 0.50 mg/mL	Toxic/non-toxic
Enantiomer	**Selected compound**									
	Eucalyptol		1.00	0.50	0.50	1.17	**0.79**	2.46	4.08	Non-toxic
	Ocimene		1.00	0.50	> 0.50	nd	nd	0.00	18.79	Non-toxic
	Estragole		1.00	0.50	0.09	**0.47**	**0.84**	4.55	65.30	Toxic only after 48 hrs
	Sabinene hydrate		0.50	0.38	0.25	1.25	**0.62**	7.73	60.63	Toxic only after 48 hrs
	Camphene		1.00	0.50	> 0.50	nd	nd	0.00	0.00	Non-toxic
	Menthol		0.50	0.38	0.13	1.13	**0.94**	25.45	81.82	Toxic only after 48 hrs
	Eugenol	*C. neoformans*	0.13	0.50	0.13	1.13	**0.98**	100.00	100.00	Toxic
	Estragole		1.13	0.38	0.09	**0.47**	**0.84**	4.55	65.30	Toxic only after 48 hrs
	α-Terpineol		0.13	0.38*	0.06	**0.50**	**0.58**	15.31	70.09	Toxic only after 48 hrs
(–)-β-Pinene	Eugenol	*C. neoformans*	0.13	0.50	0.13	nd	nd	2.26	5.19	Non-toxic
	Isoeugenol	*C. neoformans*	0.13	0.50*	0.25	nd	nd	93.43	100.00	Toxic
	p-Cymene		0.13	0.38	0.50	nd	nd	1.96	10.13	Non-toxic
	Estragole		0.13	0.38	0.13	nd	nd	50.69	81.71	Toxic

Red = combinations that demonstrated synergy in terms of ΣFIC and were simultaneously non-toxic; *combinations in which the enantiomeric counterpart was non-interactive in terms of ΣFIC (not included in Table 5.1); **bold** = ΣFQSIC and ΣFPVR values that are ≤ 1.00 (synergistic or additive).

5.2 Future recommendations

5.2.1 Varied ratio combinations

The combination studies in this investigation were carried out at a 1:1 ratio. However, the compounds that are naturally present in essential oils do not occur at an equal ratio and may be present in major or even minor concentrations. Investigating the combinations at varied ratios could provide more insight into the influence of the stereochemical configuration on the antimicrobial activity, or even the toxicity. Alternatively, investigating the combinations at ratios that replicate their occurrence in a specific essential oil may provide more insight into the influence of the stereochemical configuration on the antimicrobial activity, or the toxicity. Van Vuuren and Viljoen (2007) conducted varied ratio combinations and found that antagonistic profiles were observed when the ratio of (+)-Limonene was greater than 1,8-Cineole (Eucalyptol), whereas (–)-Limonene still displayed additivity when the ratio of (–)-Limonene was greater than 1,8-Cineole (Eucalyptol). Another consideration is that it may not just be two compounds in an essential oil that are responsible for observed antimicrobial activity. Kharsany *et al.* (2019) demonstrated that triple ratio (1:1:1) combinations of certain bioactive compounds resulted in a three-fold increase in synergistic interactions in terms of antimicrobial activity. As such, the recommended next step is varied and/or triple ratio combinations to evaluate variations in the interactive efficacy.

5.2.2 In-depth toxicity analysis

The BSLA which was undertaken in this study is one measure of *in vitro* toxicity, however, as it is a screening test it is recommended that further in-depth toxicity assays on the enantiomeric pairs of essential oil compounds be undertaken. Animal testing is often the standard in determining safety profiles, as responses in animal models can often predict human responses. However, the ethical and financial implications, special skill requirements and time consuming protocols make it a contentious option (Doke and Dhawale, 2015). The use of cell line cultures is a viable alternative to animal testing, and allows for accurate tissue response to investigated compounds (Allen *et al*., 2005). Cells from various tissues, such as from the brain, skin, liver, kidney etc., are grown and kept viable through sub-culturing in an appropriate growth medium. The cells are then exposed to the test agent, and their cytotoxic effects or effects on cell proliferation, are then measured through viability assays (Verma *et al*., 2020). This method of

toxicity testing is advantageous as it is less expensive and time-consuming, the protocols are relatively easy to follow and important pharmacokinetic information can be obtained (Doke and Dhawale, 2015; Jaroch *et al*., 2018). Therefore, an evaluation of the toxicological variations of enantiomers of essential oil compounds using cell lines, are the recommended next step, specifically in combination with other essential oil compounds.

5.2.3 Structure-activity relationships (SARs)

Structure-activity relationships are utilised to relate molecular descriptors to biological activity. Essential oil compounds are good candidates for the investigation of SARs, as the assessment of molecules from the same chemical family with small structural changes allows for a more comprehensive understanding of the role of certain functional groups in bioactivity. A recent review conducted by Fikri *et al*. (2020) demonstrated the limited research done on SARs of essential oil compounds and particularly, the influence of the stereochemical configuration of the compounds on SARs. The hydrophobicity of essential oil compounds is believed to be amongst the principle causes of their antimicrobial activity, as this results in the partitioning of the bacterial cell-membrane (Arfa *et al*., 2006). In some cases, the role of the hydroxyl moiety alongside a phenolic group in a molecule in bioactivity has been demonstrated (Andrade-Ochoa *et al*., 2015). As was demonstrated in the current investigation, enantiomeric pairs of an essential oil compound do not always have the same activity, especially in combination. Iraji *et al*. (2020) attributed the greater potency of (+)-α-Pinene over (–)-α-Pinene to stereoselective inhibition pathways against micro-organisms. The potency of (+)-α-Pinene over (–)-α-Pinene was observed in the current investigation against the six pathogens investigated, as reported in Chapter 2. Quantitative structure-activity relationships (QSAR) and molecular docking through *in silico* modelling allows one to develop mathematical or computer-generated models in order to accurately predict the biological activity and potency of compounds through structure-based virtual screening (Rudrapal and Chetia, 2020). This well-established method relates the bioactivity of a compound to its chemical descriptors, and has been largely applied in drug discovery (Muratov *et al*., 2020). As the principal variance between a pair of enantiomeric compounds is related to their chemistry, it is recommended to further investigate the influence the stereochemistry of these and other enantiomeric compounds, particularly in combination, have on the structure-activity relationships through QSAR modelling.

5.2.4 Anti-biofilm forming studies

This investigation evaluated the anti-QS activity of the enantiomeric pairs of a selection of essential oil compounds, and variations between certain enantiomeric pairs, particularly in combination, were observed. Biofilm formation is amongst the virulence factors controlled by QS and, in addition, is one of the major causes of infection (Sharma *et al*., 2019). A biofilm is a protective encasing that contains micro-organisms within an extracellular matrix, forming a complex three-dimensional structure that adheres to surfaces. Biofilms tend to form on any surface, whether it is a biological or non-biological environment, as long as micro-organisms are present with sufficient water and nutrients (Verderosa *et al.*, 2019). From a biological perspective, biofilms play a significant part in infection due to their recalcitrant nature. This is seen in infections such as cystic fibrosis pneumoniae, urinary tract infections and dental plaque formation, to name a few (Donlan and Costerton, 2002). When the biofilm has matured and it reaches a maximum cell density, micro-colonies are released allowing them to migrate and find new surfaces to attach themselves to and begin the process of biofilm formation on that new surface. This final phase in which micro-colonies migrate is the main cause of the spread and recurrence of infections in biological systems (Stoodley *et al*., 2002; Reichling, 2020). Several experimental studies on the anti-biofilm and virulence factor reducing activity of essential oils and essential oil compounds have been investigated. Reichling (2020) published a comprehensive review of the literature pertaining to this. It was demonstrated in the current investigation that the anti-QS activity can vary between enantiomeric pairs of a compound, particularly in combination. This was particularly evident with (+)-Limonene and (+)-Citronellal, which often interacted more favourably in terms of the effect of the MQSIC, and (–)-Camphor, (+)-Borneol and (+)-Menthone often interacted more favourably in terms of the effect on the extent to which bacterial communication was inhibited. As biofilm formation is a QS-regulated process, it stands to reason that enantiomers may affect this process differently, and it suggested that this is further evaluated. The two ways in which the effects of potential anti-biofilm agents can be investigated are: 1) Through evaluation of potential agents in preventing biofilm formation, and 2) Eradication of formed biofilms.

5.3 Limitations

The main limitation of this study was the implication of the COVID-19 pandemic and subsequent national lockdown. As a result, laboratory access was restricted and there were

delays in the procurement and delivery of the consumables and other materials required to conduct this investigation. In addition, opportunities to participate in research conferences were substantially limited. An additional limitation is the cost and procurement of the compounds investigated. In total, 21 samples were investigated. Many of the compounds had high costs for a small quantity of sample, for example, the cost of (+)-Citronellal was approximately R8607.00 for 5.00 g. Whilst the antimicrobial activities of these compounds (particularly when used in combination) were found to be promising and further, the preferred enantiomeric form of certain essential oil compounds could be ascertained, their therapeutic applications may be limited due to their economic implications. Before considering future formulations incorporating these compounds, thought should be given to cost and feasibility.

5.4 Final conclusion

This investigation compared the activity of a selection of enantiomeric pairs of essential oil compounds and concludes with a number of points: Firstly, enantiomeric pairs of a compound often interact differently when combined with certain other compounds. This is of particular importance when considering that the antimicrobial activity of essential oils is attributed to the combinations of the constituent compounds, rather than one singular compound. Secondly, the enhanced antimicrobial activity of certain combinations does not always correlate with their toxicity, suggesting structure activity-relationships where the stereochemical configuration plays a role. For example, if we consider the example of Limonene and the frequent co-occurrence with other compounds such as γ-Terpinene and α-Terpineol in *Citrus* essential oils (Singh *et al*., 2021), that are often evaluated for the antimicrobial properties. Therefore, considering the variations in the antimicrobial activity and toxicity demonstrated in the current investigation, it can be seen that an often-neglected evaluation of the enantiomeric distribution of the compounds of an essential oil has been demonstrated to be an important consideration. What is clearly evident in this study is that the biological activity of individual essential oil compounds cannot be extrapolated towards whole essential oils. Even the different chirality's when combined can alter the overall biological outcome and this aspect should be considered in all studies going forward.

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APPENDIX A: ABSTRACT FOR PRESENTATION

The role of stereochemistry on the biological activity of essential oil compounds

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The antimicrobial activity of essential oil compounds has been comprehensively investigated. However, the influence of the stereochemical configuration of these essential oil compounds has often been overlooked. This study aimed to explore the antimicrobial activity of the enantiomeric configuration of a selection of essential oil compounds, both alone and in 1:1 combination with a selection of essential oil compounds. The studies undertaken in this investigation included minimum inhibitory concentrations (MIC), as well as toxicity studies using the brine shrimp lethality assay (BSLA). Six enantiomeric pairs were combined with fourteen essential oil compounds identified as putative biomarkers against six pathogens, resulting in a total of 1008 combinations. While majority of the combinations showed no difference in activity for both assays, results of the MIC assay showed that 183 of those combinations resulted in each enantiomer showing different combined antimicrobial activity. This was particularly evident with β-Pinene, where the R-(+)-enantiomer showed better combined antimicrobial activity in more combinations than S-(–)-β-Pinene. An example of this was seen in the combination with β-Caryophyllene against C. albicans, where a three-fold difference in the MIC value was observed. R-(+)-β-Pinene combined with β-Caryophyllene had an MIC of 0.50 mg/ml (synergistic combination), while S-(–)-β-Pinene combined with β-Caryophyllene had an MIC of 1.50 mg/ml (indifferent combination). Results from the BSLA study showed that differences in toxicity between enantiomers can also be seen when they are combined with other essential oil compounds. Of the 168 1:1 combinations investigated, 37 combinations resulted in each enantiomer showing different combined toxicity. This was

particularly evident with the enantiomers of Limonene and Camphor. An example of this was seen in the combination of the enantiomers of Limonene with α-Terpineol, where R-(+)-Limonene combined with α-Terpineol had a percentage mortality of 2% (synergistic combination), while S-(–)-Limonene combined with α-Terpineol had a percentage mortality of 80% (antagonistic combination). Interestingly, there appeared to be an inverse correlation between the antimicrobial and toxicity studies, where the enantiomeric configurations that were responsible for favourable antimicrobial activity also displayed higher toxicity, and vice versa. The results of this study highlight the importance of considering the stereochemical configuration when investigating potential therapeutic use.

APPENDIX B: ABSTRACT FOR PUBLICATION (DRAFT)

Investigating the interactive efficacy of the enantiomers of Limonene

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Abstract

Limonene is often the major constituent in many essential oils and can naturally occur in either its (+)- and (–)- enantiomeric forms. Limonene has been previously evaluated for its antimicrobial activity and toxicity, however, what is often overlooked is the influence of enantiomerism on its biological activity, particularly in combination with other essential oil compounds. The aim of this study was to conduct an *in vitro* antimicrobial analysis, anti-quorum sensing (QS) analysis and toxicological screening of the enantiomers of Limonene, both independently and in combination with a selection of compounds, to identify variations in the independent and combined activity of the enantiomers. The enantiomers of Limonene and the selected compounds were investigated for interactive antimicrobial activity by determining the minimum inhibitory concentrations (MIC), anti-QS activity, and toxicity studies (brine shrimp lethality assay). The enantiomers of Limonene often displayed variations in their antimicrobial and anti-QS activity, and toxicity, specifically in combination with the selected compounds. This was particularly evident in combination with γ-Terpinene, α-Terpineol, Ocimene and *p*-Cymene. Overall, where variations were observed in combination, (–)-Limonene often interacted more favourably in terms of the MIC assay, (+)-Limonene often interacted more favourably in terms of the toxicity. (+)-Limonene often interacted more favorably in terms of ΣFQSIC, whereas (–)-Limonene interacted more favorably in terms of ΣFPVR. The results obtained in this study demonstrate that the enantiomers of Limonene often

displayed similar antimicrobial activity or toxicity to one another, however, variations in terms of their combined interactions with other essential oil compounds were observed.

APPENDIX C: ETHICS WAIVER

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Ref: W-CP-201028-2

28 October 2020

TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigator:	**Miss Nazihah Hoosen (Student no 1349444)**
Supervisor:	**Prof Sandy van Vuuren**
Faculty:	Health Sciences
School:	Therapeutic Sciences
Department:	Pharmacy and Pharmacology
Project title:	The role of stereochemistry on the biological activity of essential oil compounds
Reason:	In vitro laboratory for degree purposes using cell lines, bacterial cultures, materials with no humans, human data or human tissues.

Dr Clement Penny

Chair: Human Research Ethics Committee (Medical)

Copy – HREC (Medical) Secretariat: Zanele Ndlovu, Rhulani Mkansi, Mapula Ramaila and Iain Burns.

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