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The antimicrobial and toxicity properties of essential oil compounds combined with carrier oils

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THE ANTIMICROBIAL AND TOXICITY PROPERTIES OF ESSENTIAL OIL COMPOUNDS COMBINED WITH CARRIER OILS



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WITWATERSRAND,
JOHANNESBURG

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

Johannesburg, 2023

Declaration: Student's contribution to article and agreement of co-authors

I, Salehah Moola, student number 1645450, declare that this Dissertation is my own work and that I contributed in full towards research findings published in the article stated below which are included in my Dissertation.

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Agreement by co-authors: By signing this declaration, the co-authors listed below agree to the use of the article by the student as part of her Dissertation.

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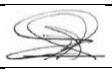
Author 2	Ané Orchard		15 December 2022
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Dedication

To my parents, Mohammed and Mumtaz, and my siblings, Mahdiyyah, Ahmed, and Ranaa. Thank you for your endless support throughout this journey, and for always standing by my side through both difficult and momentous times. You all have all been my rock, strength, and motivation and I am so grateful to have been blessed with the most loving family. May you all be rewarded for the happiness, laughter, and comfort you bring to my life.

In the name of Allah, The Most Gracious, The Most Merciful

List of presentations and accepted publication arising from this study

Presentations

Appendix A1 (Abstract)

Moola, S., Orchard, A., and van Vuuren, S.F., The antimicrobial and toxicity properties of essential oil compounds combined with carrier oils. 43rd Annual APSSA Conference on Pharmaceutical Sciences, 21-23 August, 2022, Rhodes University, Makhanda, (Podium presentation).

Appendix A2 (Poster)

Moola, S., Orchard, A., and van Vuuren, S.F., The antimicrobial and toxicity properties of essential oil compounds combined with carrier oils. Wits Faculty of Health Sciences Biennial Research Day, 15 September, 2022, University of the Witwatersrand, Johannesburg, (Poster presentation).

Publication (Chapter 3)

Moola, S., Orchard, A., and van Vuuren, S.F., The antimicrobial and toxicity influence of six carrier oils on essential oil compounds. *Molecules*., 2022. **28**(1): p.30.

Abstract

Essential oils contain a number of biologically active compounds that have been identified as alternative antimicrobials, however, their use is often limited due to their toxic nature. Carrier oils can reduce the toxicity of essential oils, which raises the question as to whether such activity would extend to the essential oil compounds if used in combination. Thus, this study aimed to investigate the toxicity, and the antimicrobial activity of 21 essential oil compounds in combination with six carrier oils against the ESKAPE pathogen group.

The antimicrobial properties of the essential oil compounds, alone and in combination with carrier oils, were determined using the broth microdilution assay to determine the minimum inhibitory concentration (MIC) against *Enterococcus faecium* (ATCC 27270), *Staphylococcus aureus* (ATCC 25923), *Klebsiella pneumoniae* (ATCC 13883), *Acinetobacter baumannii* (ATCC 17606), *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 8739) reference strains. A yeast reference strain, *Candida albicans* (ATCC 10231), was also included. The toxicity was determined using the brine shrimp lethality assay. The interactive profiles of the combinations of the compounds and carrier oils was determined by calculating the fractional inhibitory concentration index (ΣFIC) (MIC studies) and the fractional percentage mortality index (ΣFPM) (toxicity studies). The selectivity index (SI) of the combinations showing synergy in the broth microdilution assay was investigated. The time-kill effects of the essential oil compound: carrier oil combination that was synergistic in the broth microdilution assay and that demonstrated reduced toxicity, was further evaluated.

Of the combinations tested in the broth microdilution assay, 3% resulted in synergy (ΣFIC ≤ 0.50), with the compound thymoquinone and the carrier oil *Prunus armeniaca* demonstrating broad-spectrum synergistic activity. The carrier oils reduced the toxicity of the compounds, where at 24 and 48 hrs, the combinations showed 8% and 6% synergy, respectively. *Calendula officinalis* and *P. armeniaca* carrier oils were responsible for most of the reduced toxicity observed. The compound thymoquinone was present most often in combinations which showed SI values > 4. The combination of *Aloe vera* with α-terpinene demonstrated synergy in the broth microdilution assay (ΣFIC value of 0.41), as well as reduced toxicity (ΣFIC value of 0.49) and was thus evaluated in the time-kill assay. The combination provided bacteriostatic activity over 6 hrs.

This study provides evidence of the essential oil compound: carrier oil interactions where favourable several combinations such as *A. vera* with α-terpinene, *P. armeniaca* with thymoquinone, *P. americana* with thymoquinone and *H. perforatum* with *p*-cymene, could be identified as ideal candidates for further research into developing novel combination therapy against the ESKAPE pathogens. Furthermore, the interactions demonstrate the added value of carrier oils in combination with several essential oil compounds.

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Equation 1

The ΣFIC values of the combinations were calculated using the following equations;

$$\text{FIC (i)} = \frac{\text{Value of (a} *\text{) combined with value of (b} *\text{)}}{\text{Value of (a) independently}}$$

$$\text{FIC (ii)} = \frac{\text{Value of (b) combined with value of (a)}}{\text{Value of (b) independently}}$$

The (a*) represents the essential oil compound in combination and (b*) represents the carrier oil.

The FIC index could then be calculated: ΣFIC = FIC (i) + FIC (ii) or ΣFPM = FPM (i) + FPM (ii)

Equation 2

The percentage mortality was calculated using the following equation;

$$\%\ \text{Mortality} = \frac{\text{dead shrimp at 24 hours (or 48 hours) (before acetic acid)} - \text{dead shrimp (time 0)}}{\text{dead shrimp (after acetic acid)}} \times 100$$

Equation 3

The selectivity index (SI) indicates the ratio of toxicity to antimicrobial activity of a sample and was calculated using the following equation;

$$\text{SI} = \frac{\text{LC}_{50}}{\text{MIC}}$$

Equation 4

The logarithm of CFU (colony forming units) /ml present in the original well, in the time-kill studies, was calculated using the following equation;

$$\text{Log (CFU/ml)} = \text{Log}_{10}\,(\text{CFU x } 0.05 \text{ x } 10^{*})$$

*1 if counting colonies of well B; 2, if counting colonies of well C; 3, if counting colonies of well D; 4, if counting colonies of well E; 5, if counting colonies of well F.

List of abbreviations

α	Alpha
β	Beta
γ	Gamma
µg	Microgram
µl	Microlitre
µl/l	Microlitre per litre
µl/ml	Microlitre per millilitre
µM	Micromolar
°C	Degrees Celsius
%	Percentage
<	Less than
>	Greater than
≤	Less than or equal to
≥	Greater than or equal to
AHRI	Animal Health Research Institute
ATCC	American type culture collection
CC_{50}	Cytotoxic concentration for 50% culture
CFU	Colony forming units
CIP	Institut Pasteur Collection
DCS	DuPont collection of strains
DMSO	Dimethyl sulfoxide
FIC	Fractional inhibitory concentration
ΣFIC	Sum of the fractional inhibitory concentration
FPM	Fractional percentage mortality
ΣFPM	Sum of the fractional percentage mortality
g	Gram
hrs	Hours
IC_{50}	Half-maximal inhibitory concentration
INT	*p*-iodonitrotetrazolium violet

l	Litre
LC_{25}	Lethal concentration to cause 25% mortality
LC_{50}	Lethal concentration to cause 50% mortality
LD_{25}	Lethal dose to cause 25% mortality
LD_{50}	Lethal dose to cause 50% mortality
mg	Milligram
mg/l	Milligram per litre
mg/ml	Milligram per millilitre
MIC	Minimum inhibitory concentration
MIC_{50}	Minimum inhibitory concentration required to inhibit 50% of micro-organism growth
MIC_{90}	Minimum inhibitory concentration required to inhibit 90% of micro-organism growth
min	Minutes
ml	Millilitre
mm	Millimetre
mM	Millimolar
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltretrazolium bromide
NCTC	National Collection of Type Cultures
RSKK	Refik Saydam Central Hygiene Institute-Culture Collection
sec	Seconds
SD	Standard deviation
TSA	Tryptone Soya agar
TSB	Tryptone Soya broth
UNSW	University of New South Wales
v/v	Volume per volume
w/v	Weight per volume

Dissertation structure

A dissertation by publication was undertaken and comprises of the following;

- Chapter one introduces the study and explains the design.
- Chapter two provides a literature review.
- Chapter three provides the publication entitled, 'The antimicrobial and toxicity influence of six carrier oils on essential oil compounds' published by *Molecules* journal containing the results of the *in vitro* findings of carrier oils combined with essential oil compounds.
- Chapter four shows further time-kill studies undertaken.
- Chapter five concludes the study.

Chapter 1 - Introduction and study flow

1.1 Background

Globally, antimicrobial resistance is a grave and growing health concern which must be prioritised at international and local levels. The World Health Organization lists priority micro-organisms requiring urgent antimicrobial development, due to their growing multidrug resistance, termed the ESKAPE pathogens: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Escherichia coli* [1]. Prescribers, policy-makers, and researchers have the responsibility of the difficult task of mitigating antibiotic resistance in an era where there are limited new treatments for bacterial infections [2]. Essential oils and essential oil compounds have been identified as alternative treatment options due to their antimicrobial properties which have been observed [3-5].

1.2 Essential oils

An essential oil is a volatile concentration of hydrophobic liquid, comprising of a complex mixture of volatile components of plant metabolites from aromatic plants [6, 7]. These oils are produced naturally by plants for alternative uses such as such as attraction of pollinators, pollination, and protection against enemies [8]. For example, coniferous plants secrete a complex mixture of monoterpenes and sesquiterpenes when attacked by predators or insects [9].

Essential oils are collected either by steam distillation or by mechanical processes known as ‘dry distillation’ [10, 11]. Essential oils have a number of uses such as in cosmetics and aromatherapy, as well as an ecological role when used as insect repellents, or as inhibitors of seed germination [12]. For hundreds of years, essential oils and their constituents have been widely used for cosmetic and medicinal applications due to the various biological activities such as bactericidal, antiparasitic, insecticidal, and cosmetic applications [13]. Essential oils were also used in both World War I and II and were believed to prevent gangrene, treat burns, and wounds of injured soldiers [14]. They are known to possess antimicrobial activity against a wide range of pathogens [3, 5, 15-19]. These antimicrobial properties of essential oils may be a solution to combat antimicrobial resistance [5].

1.3 Essential oil compounds

Essential oils comprise of a number of compounds that can range anywhere between 10 to more than 300 compounds with molecular weights of less than 300 units [12, 20]. The compounds belong to many different chemical classes such as alcohols, oxides or ethers, aldehydes, esters, ketones, amides, amines, heterocycles, phenols, and terpenes [12]. The resultant biological activity of essential oils may be due to the major occurring compounds in the specific essential oil. As a result, an interest continues to grow around the study of these compounds for their potential use in drug utilization [21, 22]. These compounds possess antimicrobial activity, with different compounds portraying variable degrees of activity [12].

The compounds can be referred to as bio-active markers/bio-active compounds, defined as secondary plant metabolites which exhibit pharmacological or toxicological activity in humans, animals, or pests [12, 23]. These compounds have also been used in the pharmaceutical industry. For example, limonene has been used as a wetting agent, dispersing agent, and as a solvent or resin [24]. Essential oil compounds have been shown to display biological activities such as anti-inflammatory activity [25, 26], antileukemic [27], anticancer properties [28] and antimicrobial activity [3, 5, 10, 29, 30]. Despite the extensive use of these compounds and the plethora of studies reporting the antimicrobial potential, their use in humans is limited by their toxicity which has been displayed against human lymphocytes, hepatocytes, and skin [31, 32].

1.4 Carrier oils

Carrier oils, also known as fixed oils, are made of a number of lipids such as waxes or fatty acids (Omega 3 and 6) as well as vitamins (Vitamins E and A) and minerals [33]. Carrier oils are produced by methods of centrifugation, maceration, cold press or extraction from the fatty component of a plant [33]. Essential oils are rarely used without being diluted with a carrier oil [34]. Carrier oils have recently been shown to reduce the toxicity of essential oils and affect their antimicrobial activity [35]. The constituents in carrier oils responsible for the reduction of toxicity is not known, however, the carrier oils often contain vitamin E, and studies reporting the decrease in toxicity of toxic medicines by vitamin E are available [35-37].

A previous study [35], found that six carrier oils, chosen for their frequent dermatological use and antimicrobial properties, displayed poor antimicrobial activity, overall, against 11 pathogens associated with dermatological conditions. Although noteworthy (≤ 1.00 mg/ml) antimicrobial activity is not expected for carrier oils [33], since carrier oils are predominantly made up of free fatty acids and vitamins which are substances associated with poor antimicrobial activity [33], it was found that selected carrier oils demonstrated some antimicrobial activity against the pathogens *P. aeruginosa* and *Brevibacterium epidermidis* at an MIC value of 1.00 mg/ml [35]. The carrier oils, *Calendula officinalis* (calendula) and *Persea americana* (avocado) have also demonstrated antimicrobial activity [35] and this activity may be due to the free fatty acids within the carrier oils which at high concentrations have been reported to display antimicrobial activity against *S. aureus* [38]. A previous study [35] showed that when carrier oils were combined with essential oils, the combinations resulted in additive or synergistic antimicrobial reactions. Synergy was mostly exhibited by the carrier oils, *Aloe vera* (aloe vera) and *P. americana* [35].

1.5 Problem statement

While there has been a study which has shown that the carrier oils demonstrated no level of toxicity [35], research on the effect of carrier oils on the toxicity and antimicrobial activity of compounds is lacking. Furthermore, combinational studies incorporating carrier oils (which are almost always used in combination in aromatherapy) are deficient. This study therefore sought to investigate the antimicrobial and toxicity effects of carrier oils when combined with essential oil compounds, as well as the time-kill effects of certain synergistic combinations.

1.6 Compounds selected

The essential oil compounds were selected based on previous reports on the antimicrobial activity or identification from previous studies, for example from chemometric analysis identifying compounds responsible for antimicrobial activity [5], against the ESKAPE pathogens (**Table 1.1**).

Table 1.1: Compounds reported and identified as having antimicrobial activity against the ESKAPE pathogens

Compound	**Pathogen**	**Reference**
Carvacrol	*S. aureus* *A. baumannii* *P. aeruginosa* *E. coli*	[39-41]
β-Caryophyllene	*E. faecium* *S. aureus* *K. pneumoniae* *A. baumannii* *P. aeruginosa* *E. coli*	[42]
Cinnamaldehyde	*S. aureus* *A. baumannii* *P. aeruginosa* *E. coli*	[43-45]
Citral	*S. aureus* (Methicillin resistant) *K. pneumoniae* *A. baumannii* *P. aeruginosa* *E. coli*	[45-48]
p-Cymene	*S. aureus* *K. pneumoniae* *A. baumannii* *P. aeruginosa* *E. coli*	[39, 49-51]
Eugenol	*S. aureus* *K. pneumoniae* *P. aeruginosa* *E. coli*	[5, 39, 40]

Compound	Pathogen	Reference
Geraniol	*S. aureus* *P. aeruginosa* *E. coli*	[39, 45]
Isoeugenol	*S. aureus* *P. aeruginosa* *E. coli*	[5, 52, 53]
R (+)-Limonene	*S. aureus* *K. pneumoniae* *A. baumannii* *P. aeruginosa* *E. coli*	[7, 54, 55]
Linalool	*E. faecium* *S. aureus* *K. pneumoniae* *A. baumannii* *P. aeruginosa* *E. coli*	[42]
Linalyl acetate	*S. aureus* *K. pneumoniae* *P. aeruginosa* *E. coli*	[5, 39, 56]
Menthol	*S. aureus* *K. pneumoniae* *P. aeruginosa* *E. coli*	[17, 48, 57, 58]
Nerol	*S. aureus* *K. pneumoniae* *A. baumannii* *P. aeruginosa* *E. coli*	[5, 48, 58, 59]

Compound	Pathogen	Reference
(+)-α-Pinene	*S. aureus* *K. pneumoniae* *A. baumannii* *P. aeruginosa* *E. coli*	[39, 50, 60]
Santalol	*S. aureus* *P. aeruginosa* *E. coli*	[61]
α-Terpinene	*S. aureus* *E. coli*	[62, 63]
γ-Terpinene	*S. aureus* *E. coli*	[39]
(+)-Terpinen-4-ol	*S. aureus* *A. baumannii* *P. aeruginosa* *E. coli*	[45, 55, 64]
α-Terpineol	*E. faecium* *S. aureus* *K. pneumoniae* *A. baumannii* *P. aeruginosa* *E. coli*	[42]
Thymol	*E. faecium* *S. aureus* *K. pneumoniae* *A. baumannii* *P. aeruginosa* *E. coli*	[41, 49, 65]
Thymoquinone	*E. faecium* *S. aureus* *P. aeruginosa* *E. coli*	[66, 67]

Essential oil compounds have various chemical classes which are briefly summarized in **Table 1.2**, such as terpenes (sesquiterpenes and monoterpenes), terpenoids, alcohols, aldehydes, *p*-benzoquinones, esters, ketones, oxides, phenols and phenylpropanoids [12, 68-71]. The majority of the occurring essential oil compounds belong to the terpene family [12, 21]. A previous study [72] says that terpenoids (heterogenous groups of terpenes) and phenylpropanoids are the most commonly occurring chemical classes; whereby most plants contain about 80% terpenoids. Several compounds form part of more than one chemical class [73-80].

Table 1.2: Summary of essential oil compounds and their chemical class

Chemical class	**Essential oil compound**	**Reference**
Terpenes: ***Monoterpenes***	Carvacrol, citral (monoterpene aldehyde), *p*-cymene, geraniol (monoterpene alcohol), limonene, linalool (monoterpene alcohol), menthol (monoterpene alcohol), (+)-α-pinene, α-terpinene, γ-terpinene, thymol	[73, 75, 77-79, 81-86]
Sesquiterpenes	*β*-Caryophyllene, santalol (sesquiterpene alcohol)	[80, 87, 88]
Terpenoids	Carvacrol, *β*-Caryophyllene, citral, *p*-cymene, eugenol, geraniol, isoeugenol, limonene, linalool (monoterpenoid), linalyl acetate, menthol, nerol, (+)-α-pinene, santalol, α-terpinene, γ-terpinene, (+)-terpinen-4-ol (monoterpenoid), α-terpineol, thymol	[72, 74, 89-99]
Alcohols	Geraniol (monoterpene alcohol), linalool (monoterpene alcohol), menthol (monoterpene alcohol), nerol, santalol (sesquiterpene alcohol), α-terpineol (terpene alcohol), (+)-terpinen-4-ol	[39, 75, 76, 78-80, 90]
Aldehydes	Citral (monoterpene aldehyde), cinnamaldehyde	[77, 100, 101]
***p*-Benzoquinones**	Thymoquinone	[71]
Esters	Linalyl acetate (tertiary alcohol ester)	[102]
Phenols	Carvacrol, eugenol, isoeugenol, thymol	[39, 73, 74, 76, 103, 104]
Phenylpropanoids	Cinnamaldehyde, eugenol, isoeugenol	[68-70]

1.7 Carrier oils selected

The carrier oils selected were *Aloe vera* (Aloe vera); *Calendula officinalis* (Calendula); *Hypericum perforatum* (St John's wort); *Persea americana* (Avocado); *Prunus armeniaca* (Apricot kernel) and *Simmondsia chinensis* (Jojoba).

1.8 Aims and objectives

This study aimed to investigate the antimicrobial and toxicity effects of carrier oils in combination with essential oil compounds.

The objectives of this study were:

1. To determine the antimicrobial properties of essential oil compounds, singularly and in combination with carrier oils, using the broth microdilution assay.
2. To determine the toxicity of essential oil compounds and carrier oils, singularly and in combination, using the brine-shrimp lethality assay.
3. To determine the interactive profiles of the combinations of compounds and carrier oils in each method by calculating the fractional inhibitory concentration index (ΣFIC) (for use in MIC (minimum inhibitory concentration) studies), and the fractional percentage mortality index (ΣFPM) (for use in toxicity studies).
4. To calculate the selectivity index of the combinations which showed synergy in the broth microdilution assay.
5. To determine the bacteriostatic or bactericidal activity of a combination showing synergy in the broth microdilution assay and brine-shrimp lethality assay, using the time-kill assay.

Chapter 2 - Literature review on the antimicrobial activity, toxicity, and time-kill effects of selected essential oil compounds and carrier oils

2.1 Antimicrobial activity of essential oil compounds against ESKAPE pathogens

There are various groups of essential oil compounds that can be considered for use against the ESKAPE (*E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *E. coli*) pathogens with 21 having been selected for this study. A summary of the studies that have investigated the antimicrobial activity of the selected compounds against the ESKAPE pathogens are given in **Table 2.1**. It is evident that the essential oil compounds have notable antimicrobial activity and most importantly, against highly problematic pathogens such as *A. baumannii* (**Table 2.1**). Cinnamaldehyde and thymoquinone are observed as having displayed the most noteworthy activity against the ESKAPE pathogens, followed by the compounds isoeugenol, thymol, and santalol [41, 61, 105]. Previous studies investigating the antimicrobial activity of the selected compounds against the pathogens *E. faecium* and *A. baumannii* were less common thus highlighting the need for a study including these pathogens.

Phenolic compounds could be potential agents to fight against antimicrobial resistance as data has shown the ability of phenolics to display antimicrobial activity against resistant bacteria namely *E. coli*, *P. aeruginosa* and *Staphylococcus* and *Klebsiella* species [106-108]. The lipophilic nature of phenolic compounds (carvacrol, eugenol, isoeugenol and thymol) results in their high affinity for cell membranes and ultimately the disruption of the membrane's physico-chemical properties. This therefore results in a reduction in the integrity of the membrane through disturbance of lipids [109], enzymes, and proteins of the pathogen's membrane, causing loss of intracellular constituents through leakage, ultimately causing bacterial cell death [109, 110].

Cinnamaldehyde, an aldehyde, is made up of a benzene ring connected to an acrolein structure [111]. The hydrophobic nature of cinnamaldehyde allows it to enter and cause disturbance to the lipid bilayer of the cell membrane, resulting in increased proton permeability of the cell

[111]. Citral, a monoterpene aldehyde, may have its antimicrobial activity attributed to its ability to crosslink amino groups within the cytoplasm and cell walls of bacteria [111]. Geraniol is a monoterpene alcohol. It has a lipophilic character and this results in its ability to adhere to lipids in the cell membrane of the micro-organism. This makes the membrane more permeable through contact with the intracellular sites, resulting in cell destruction [112, 113]. The antimicrobial activity of linalyl acetate, an ester, has been shown through the compound's inhibition if spore germination [39].

The antimicrobial activity of terpenoids, specifically phenolic terpenoids, may be attributed to the important function played by hydroxyl groups and the presence of delocalised electrons [114]. The compounds menthol and α-terpinene are both monoterpenes, where menthol is further classified as a monoterpene alcohol. Monoterpene's antibacterial activity is as a result of their ability to increase the permeability of the bacterial plasma membranes through disruption of the lipid portion of the plasma membrane. This results in leakage of intracellular components [115], causing reduction in membrane integrity.

Santalol, a sesquiterpene alcohol, may have its antimicrobial activity attributed to the ability of alcohols to cause protein denaturation and cell membrane damage, as well as disturbance to cell metabolism and ultimately, cell lysis [116]. Thymoquinone, a p-benzoquinone, attributes its antibacterial activity through the formation of ROS (reactive oxygen species) which results in oxidative stress, leading to damage of bacterial proteins, DNA and membranes resulting in cell death [117].

Table 2.1: Minimum inhibitory concentration of compounds against the ESKAPE pathogens

Compound	**Micro-organism**	**MIC value**	**Reference**
Carvacrol	*E. faecium* (Vancomycin-resistant NCTC[1] 12202)	3.95 mM	[118]
	S. aureus (I.P.53126)	0.25 mg/ml	[109]
	S. aureus (clinical strain)	0.70 mg/ml	[40]
	K. pneumoniae (carbapenem resistant-clinical strain)	0.03-0.13 mg/ml	[119]
	K. pneumoniae (ATCC[2] BAA-2473)	1.91 mg/ml	[120]
	K. pneumoniae (clinical strain)	0.70 mg/ml	[40]

Compound	Micro-organism	MIC value	Reference
β-Caryophyllene	*A. baumannii* (ATCC 19606)	0.03 mg/ml	[41]
	P. aeruginosa	0.02 mg/ml	[121]
	P. aeruginosa (clinical strain)	1.41 mg/ml	[40]
	E. coli (I.P.54127)	0.25 mg/ml	[109]
	E. coli (clinical strain)	0.70 mg/ml	[40]
	E. faecium (CIP[3] 103.014)	0.03 mg/ml	[42]
	S. aureus (ATCC 25.923)	> 0.20 mg/ml	[42]
	K. pneumoniae (CIP 52.145)	> 0.20 mg/ml	[42]
	A. baumannii (CIP 70.34)	> 0.20 mg/ml	[42]
	P. aeruginosa (CIP 103.467)	> 0.20 mg/ml	[42]
	E. coli (ATCC 25922)	> 0.20 mg/ml	[42]
Cinnamaldehyde	*S. aureus* (ATCC 25923)	0.30 mg/ml	[43]
	S. aureus (20 clinical strains)	0.0004 mg/ml	[111]
	A. baumannii (four clinical carbapenem resistant strains)	0.88 mg/ml	[44]
	P. aeruginosa (NCTC 6749 and 9027)	0.0014 mg/ml	[45]
	E. coli (NCTC 8196)	< 0.0002 mg/ml	[45]
	E. coli (ATCC 8739)	0.02 mg/ml	[43]
Citral	*S. aureus* (Lab strain number 20)	0.0008 mg/ml	[111]
	K. pneumoniae (UNSW[4] isolate)	0.20% v/v	[47]
	A. baumannii 1567	3.90 µl/ml	[48]
	P. aeruginosa (NCTC 6750)	2.0% v/v	[47]
	P. aeruginosa (NCTC 6749 and 9027)	> 0.02 mg/ml	[45]
	E. coli (NCTC 8196)	0.0005 mg/ml	[45]
p-Cymene	*E. faecium* (three strains)	2.56 mg/ml	[65]
	S. aureus (ATCC 25923)	> 16.00 mM	[122]
	K. pneumoniae (ATCC 10031)	> 15.00 mg/ml	[49]
	A. baumannii (five strains)	128-256 µl/ml	[50]
	P. aeruginosa (ATCC 27853)	4.00 µl/ml	[51]
	E. coli (ATCC 25922)	15.00 mg/ml	[49]

Compound	Micro-organism	MIC value	Reference
Eugenol	*S. aureus* (ATCC 25923)	10.00 µl/ml	[123]
	S. aureus (clinical strain)	5.63 mg/ml	[40]
	K. pneumoniae (ATCC 43816)	0.10 mg/ml	[124]
	K. pneumoniae (four carbapenem resistant strains)	0.20 mg/ml	[124]
	K. pneumoniae (ATCC BAA-2473)	4.14 mg/ml	[120]
	K. pneumoniae (clinical strain)	5.63 mg/ml	[40]
	P. aeruginosa (NCTC 6749 and 9027)	0.02 mg/ml	[45]
	E. coli (NCTC 8196)	0.0006 mg/ml	[45]
	E. coli (clinical strain)	5.63 mg/ml	[40]
Geraniol	*S. aureus* (ATCC 25923)	11.20 mg/ml	[125]
	S. aureus (FDA209P JC-1)	0.08% w/v	[17]
	K. pneumoniae (ATCC BAA-2473)	1.74 mg/ml	[120]
	P. aeruginosa (NCTC 9027 and NCTC 6749)	> 0.02 mg/ml	[45]
	E. coli (NCTC 8196)	0.0005 mg/ml	[45]
	E. coli (ATCC 25922)	5.60 mg/ml	[125]
	E. coli (NIHJ JC-2)	> 0.08% w/v	[17]
Isoeugenol	*S. aureus* (15 clinical isolates)	0.51 mg/ml	[126]
	S. aureus (1199)	0.04 mg/ml	[105]
	S. aureus	0.50 mg/ml	[52]
	P. aeruginosa (clinical strain 13 – septic patient)	0.37 mg/ml	[53]
	P. aeruginosa (clinical strain 20 – gangrene patient)	0.19 mg/ml	[53]
	P. aeruginosa (clinical strain 21 – coma patient)	0.19 mg/ml	[53]
	E. coli	0.50 mg/ml	[52]
	E. coli O157 (DCS^{5} 497)	1000.00 mg/l	[127]

Compound	Micro-organism	MIC value	Reference
R (+)-Limonene	*S. aureus* (ATCC 6538)	20.00 ml/l[7]	[128]
	S. aureus (ATCC 25923)	3.00 mg/ml[7]	[129]
	S. aureus (ATCC 12600)	13.00 mg/ml[8]	[54]
	K. pneumoniae (ATCC 13883)	12.00 mg/ml[8]	[54]
	K. pneumoniae (RSKK[6] 574)	0.008 mg/ml[9]	[58]
	A. baumannii (50 clinical isolates)	> 134.00 mg/ml[7]	[55]
	P. aeruginosa (ATCC 9027)	4.00 mg/ml[8]	[54]
	E. coli (ATCC 11775)	11.00 mg/ml[8]	[54]
Linalool	*E. faecium* (CIP 103.014)	0.10 mg/ml	[42]
	S. aureus (ATCC 25.923)	0.10 mg/ml	[42]
	S. aureus (ATCC 29213)	0.19 % v/v	[64]
	K. pneumoniae (CIP 52.145)	> 0.20 mg/ml	[42]
	K. pneumoniae (ATCC 10031)	12.50 mM	[130]
	K. pneumoniae (ATCC BAA-2473)	6.48 mg/ml	[120]
	A. baumannii (CIP 70.34)	> 0.20 mg/ml	[42]
	P. aeruginosa (CIP 103.467)	> 0.20 mg/ml	[42]
	P. aeruginosa (NCTC 9027 and NCTC 6749)	> 0.02 mg/ml	[45]
	E. coli (NCTC 8196)	0.0005 mg/ml	[45]
	E. coli (ATCC 25922)	> 0.20 mg/ml	[42]
	E. coli (NCTC 8196)	0.097% v/v	[64]
Linalyl acetate	*S. aureus* (ATCC 29213)	0.19% v/v	[64]
	S. aureus (ATCC 6538P)	0.06 mg/ml[10]	[61]
	S. aureus (ATCC 25923)	2.50 mg/ml	[56]
	K. pneumoniae (ATCC 10031)	25.00 mg/ml	[56]
	P. aeruginosa (G 28)	0.60 mg/ml[10]	[61]
	P. aeruginosa (27853)	5.00 mg/ml	[56]
	E. coli (NCTC 8196)	0.19% v/v	[64]
	E. coli (25922)	5.00 mg/ml	[56]
Menthol	*S. aureus* (FDA209P JC-1)	> 0.16% w/v	[17]
	S. aureus (ATCC 29213)	1.00 mg/ml	[57]
	S. aureus (clinical strain)	> 6.00 mg/ml	[40]

Compound	Micro-organism	MIC value	Reference
	K. pneumoniae (RSKK 574)	0.008 mg/ml[11]	[58]
	A. baumannii (clinical isolates 5, 2, 35, 12, 16)	0.004 mg/ml	[131]
	P. aeruginosa	0.03 mg/ml	[132]
	P. aeruginosa 1532	> 7.80 µl/ml	[48]
	E. coli (NIHJ JC-2)	> 0.16% w/v	[17]
	E. coli (clinical strain)	> 6.00 mg/ml	[40]
Nerol	*S. aureus* (ATCC 25923)	≥ 1.03 mg/ml	[59]
	K. pneumoniae (RSKK 574)	0.008 mg/ml	[58]
	A. baumannii 1567	1.95 µl/ml	[48]
	P. aeruginosa 1532	7.80 µl/ml	[48]
	E. coli (ATCC 25923)	0.51 mg/ml	[59]
(+)-α-Pinene	*S. aureus* (ATCC 25923)	20.00 µl/ml	[123]
	S. aureus (CIP)	1.90 µl/ml	[60]
	K. pneumoniae (CIP)	1.20 µl/ml	[60]
	A. baumannii (five strains)	32-64 µl/ml	[50]
	P. aeruginosa (CIP)	> 10.00 µl/ml	[60]
	E. coli (NIHJ JC-2)	> 0.32 mg/ml	[17]
	E. coli (CIP)	6.00 µl/ml	[60]
Santalol	*S. aureus* (ATCC 6538P)	0.60 mg/ml	[61]
	P. aeruginosa (G 28)	0.06 mg/ml	[61]
	E. coli (ATCC 8739)	0.60 mg/ml	[61]
α-Terpinene	*S. aureus* 1199B	≥ 1.03 mg/ml	[62]
	S. aureus (NCTC 6571)	4.00% v/v	[63]
	K. pneumoniae (ATCC 700603)	0.13 mg/ml	[133]
	E. coli (NCTC 10418)	2.00% v/v	[63]
γ -Terpinene	*S. aureus* (ATCC 25923)	> 15.00 mg/ml	[49]
	S. aureus (NCTC 6571)	4.00% v/v	[63]
	E. coli (ATCC 25922)	7.50 mg/ml	[49]
	E. coli (NCTC 10418)	> 8.00% v/v	[63]

Compound	Micro-organism	MIC value	Reference
(+)-Terpinen-4-ol	*S. aureus* (FDA209P JC-1)	0.32 %w/v	[17]
	S. aureus (ATCC 29213)	0.19% v/v[12]	[64]
	A. baumannii (50 clinical isolates)	5.00 mg/ml[12]	[55]
	P. aeruginosa (NCTC 9027)	0.0093 mg/ml	[45]
	P. aeruginosa (NCTC 6749)	0.0047 mg/ml	[45]
	E. coli (NCTC 8196)	0.0012 mg/ml	[45]
	E. coli (NIHJ JC-2)	0.32% w/v	[17]
	E. coli (NCTC 8196)	0.05% v/v[12]	[64]
α-Terpineol	*E. faecium* (CIP 103.014)	> 0.20 mg/ml	[42]
	S. aureus (ATCC 25.923)	0.03 mg/ml	[42]
	S. aureus (ATCC 29213)	0.19% v/v	[64]
	K. pneumoniae (CIP 52.145)	> 0.2 mg/ml	[42]
	A. baumannii (CIP 70.34)	> 0.20 mg/ml	[42]
	A. baumannii (50 clinical isolates)	0.07 mg/ml	[55]
	P. aeruginosa (CIP 103.467)	> 0.20 mg/ml	[42]
	P. aeruginosa (NCTC 9027)	0.02 mg/ml	[45]
	P. aeruginosa (NCTC 6749)	> 0.02 mg/ml	[45]
	E. coli (NCTC 8196)	0.0012 mg/ml	[45]
	E. coli (ATCC 25922)	> 0.20 mg/ml	[42]
	E. coli (NCTC 8196)	0.10% v/v	[64]
Thymol	*E. faecium* (three strains)	0.16 mg/ml	[65]
	S. aureus (ATCC 25923)	0.40 mg/ml	[49]
	S. aureus (ATCC 29213)	0.25 mg/ml	[57]
	K. pneumoniae (ATCC 10031)	1.60 mg/ml	[49]
	A. baumannii (ATCC 19606)	0.05 mg/ml	[41]
	K. pneumoniae (ATCC BAA-2473)	0.78 mg/ml	[120]
	P. aeruginosa (ATCC 85327)	1.60 mg/ml	[49]
	P. aeruginosa (planktonic strain)	0.05 mg/ml	[121]
	E. coli (ATCC 25922)	0.40 mg/ml	[49]
Thymoquinone	*E. faecium* B577	0.26 mg/ml	[66]
	S. aureus (ATCC 9144)	0.003 mg/ml	[117]
	S. aureus (ATCC 25923)	0.008 mg/ml	[67]

Compound	Micro-organism	MIC value	Reference
	S. aureus (ATCC 29213)	0.05 mg/ml	[134]
	K. pneumoniae (clinical strain)	0.0013 mg/ml	[135]
	A. baumannii (ATCC 19606)	0.005 mg/ml	[136]
	P. aeruginosa (ATCC 14886)	0.00156 mg/ml	[117]
	P. aeruginosa (ATCC 27853)	> 0.51 mg/ml	[67]
	E. coli (ATCC 25922)	0.10 mg/ml	[117]
	E. coli (ATCC 35218)	> 0.51 mg/ml	[67]

[1]NCTC - National Collection of Type Cultures; [2]ATCC - American Type Culture Collection; [3]CIP - Institut Pasteur Collection; [4]UNSW - University of New South Wales; [5]DCS - DuPont collection of strains; [6]RSKK - Refik Saydam Central Hygiene Institute-Culture Collection, The Ministry of Health of Republic of Turkey, Ankara; [7]MIC value of limonene, and not R (+)-limonene; [8]MIC value of (+)-limonene, and not R (+)-limonene; [9]MIC value of d-limonene, and not R (+)-limonene; [10]MIC value of racemic linalyl acetate; [11]MIC value of (–)-menthol, and not menthol; [12]MIC value of terpinene-4-ol, and not (+)-terpinen-4-ol.

2.2 Antimicrobial activity of essential oil compounds against *Candida albicans*

Although the focus of this study is on the ESKAPE pathogens, there is an escalating development of resistance of *C. albicans* against antifungals [137], thus this pathogen has been included due to the severity of the issue. **Table 2.2** summarises the studies identified which investigated the antimicrobial activity of the selected compounds against *C. albicans*. The compounds geraniol, (+)-α-pinene, thymol, and thymoquinone are reported to have the most noteworthy activity against *C. albicans*.

The anti-fungal activity of phenolic compounds is attributed to the inhibition of fungal cell-wall biosynthesis as well as cell membrane disruption which causes cell death. This attack on the cell walls may be due to the chitin build up [138, 139]. Phenolic compounds cause membrane lesions and reduce the production of ergosterol which is a sterol specific to fungi which plays an imperative role in fungal membrane and enzyme functioning [140-142]. Ergosterol biosynthetic inhibition by phenolic compounds plays a role in fungal cell death as it causes leakage of cell organelles [141, 143]. Phenolics therefore play an important role in both antibacterial and antifungal activity.

Cinnamaldehyde has been shown to have noteworthy anti-fungal effects through the reduction in ergosterol within the fungal cell wall [144] which is known to be an important element to fungal cell functioning. The compound also forms lesions on fungal cell membranes which

leads to cell death [145]. The anti-fungal activity of geraniol may be attributed to the ability to inhibit fungal biofilm formation [146]. The virulence of *C. albicans* is dependent on the formation of pseudo-hyphae [147, 148] as they prevent fungal phagocytosis, while also allowing fungi to penetrate endothelial and epithelial tissues [148]. Geraniol inhibits the formation of pseudo-hyphae, thus contributing to anti-fungal activity [147]. Geraniol has also been shown to cause potassium leakage from fungal cells which increases membrane permeability and affects membrane integrity, thus compromising fungal development [149].

The compound *p*-cymene's anti-fungal properties are caused by inhibition of the enzyme pectin methyl esterase which controls methyl-esterification of pectin, a major element within the fungal cell wall. Disruption to the pectin component results in changes in pH, plasticity, cellular adhesion, and ionic contents of the cell wall, thus affecting membrane integrity and permeability and ultimately, fungal development [150]. Against plant pathogens, monoterpenoids provide a mechanism of self-defence [151]. The anti-fungal properties of β-caryophyllene are due to the hydrophobic nature which allows an increase in the permeability of the fungi's cell membrane, thus affecting integrity [152]. Thymoquinone is a *p*-benzoquinone. Quinones join irreversibly to the nucleophilic amino acids found in proteins resulting in inactivation of the proteins, and therefore damaging the protein function [151].

Ergosterol biosynthesis is one of the main fungal targets of essential oil compounds such as carvone, linalool, menthol, and menthone [142, 153]. Ergosterol is a sterol specific to fungi which plays an imperative role in the functioning of the fungal membrane and enzyme [140-142]. Essential oil compounds alter membrane integrity of fungi while also inhibiting adhesion, morphogenesis and adhesion of *C. albicans*, as shown by plant terpenoids, which include the compounds citral, geraniol, and thymol [154], as well as several other compounds used in this study.

Table 2.2: Minimum inhibitory concentration of compounds against *C. albicans*

Compound	***C. albicans* strains**	**MIC value**	**Reference**
Carvacrol	ATCC[1] 40042, 13803 and 76485	0.26 mg/ml	[155]
β-Caryophyllene	Strain of human source	> 1.00 mg/ml	[156]
Cinnamaldehyde	Against planktonic growth	0.06 mg/ml	[157]

Compound	*C. albicans* strains	MIC value	Reference
Citral	ATCC 10231	0.03% v/v	[47]
	ATCC 76645	0.03 mg/ml	[158]
p-Cymene	ATCC 10231	> 8.00% v/v	[159]
Eugenol	Against various fluconazole susceptible strains	0.50 mg/ml	[160]
Geraniol	Against nine strains	0.02 mg/ml	[161]
Isoeugenol	ATCC 76485	0.26 mg/ml	[162]
	Against various fluconazole resistant strains	0.17 mg/ml	[163]
Limonene[2]	Strain of human source	1.00 mg/ml	[156]
Linalool	ATCC 14053	8.00 mM	[164]
	ATCC 51501	0.0007 mg/ml	[165]
	ATCC 10231	0.80 mg/ml	[166]
	ATCC 10231	0.50 mg/ml	[56]
Linalyl acetate	ATCC 3153	1.00% v/v	[167]
	ATCC 10231	0.38 mg/ml	[166]
	ATCC 10231	15.00 mg/ml	[56]
Menthol	Clinical isolate	0.13 mg/ml	[168]
Nerol	ATCC 90028	2.00 mg/ml	[154]
(+)-α-Pinene	ATCC 10231	0.003 mg/ml	[169]
Santalol	ATCC 10231	0.60 mg/ml	[170]
α-Terpinene	ATCC 10231	8.00% v/v	[159]
γ-Terpinene	ATCC 10231	> 8.00% v/v	[159]
Terpinen-4-ol[3]	ATCC 10231	0.25% v/v	[159]
α-Terpineol	ATCC 10231	0.12% v/v	[159]
Thymol	CBS 562	0.04 mg/ml	[171]
Thymoquinone	Clinical isolate[4]	0.32 mg/ml	[172]
	ATCC 10231	0.03 mg/ml	[173]
	ATCC 10231	0.01 mg/ml	[134]

[1]ATCC - American Type Culture Collection; [2]Results for limonene, no data for R (+)-limonene; [3]Results for terpinen-4-ol, no data for (+)-terpinen-4-ol; [4]Thymoquinone used in the micro-drug form.

2.3 Antimicrobial activity of carrier oils against ESKAPE pathogens and *C. albicans*

The antimicrobial activity of the selected carrier oils against the ESKAPE and *C. albicans* pathogens is summarised in **Table 2.3**. The carrier oils *A. vera*, *C. officinalis*, *P. armeniaca* and *S. chinensis* showed some antimicrobial activity against selected pathogens, however overall, the carrier oils displayed poor antimicrobial activity, as expected [33].

Table 2.3: Minimum inhibitory concentration of carrier oils against ESKAPE pathogens and *C. albicans*

Carrier oil	**Micro-organism**	**MIC value**	**Reference**
Aloe vera	*S. aureus* (ATCC[1] 25924)	10.00 mg/ml	[35]
	P. aeruginosa (ATCC 27858)	1.00 mg/ml	[35]
	P. aeruginosa (clinical isolate)	0.03-0.80 mg/ml[2]	[174]
	E. coli (ATCC 8739)	8.00 mg/ml	[35]
	C. albicans (ATCC 10231)	8.00 mg/ml	[35]
Calendula officinalis	*S. aureus* (ATCC 25924)	4.00 mg/ml	[35]
	S. aureus (CCMI[3] 335)	0.05 mg/ml	[175]
	P. aeruginosa (ATCC 27858)	1.50 mg/ml	[35]
	E. coli (ATCC 8739)	4.00 mg/ml	[35]
	E. coli (CCMI 270)	> 0.20 mg/ml	[175]
	C. albicans (ATCC 10231)	16.00 mg/ml	[35]
Hypericum perforatum	*S. aureus* (ATCC 25924)	4.00 mg/ml	[35]
	P. aeruginosa (ATCC 27858)	1.50 mg/ml	[35]
	E. coli (ATCC 8739)	4.00 mg/ml	[35]
	C. albicans (ATCC 10231)	4.00 mg/ml	[35]
Persea americana	*S. aureus* (ATCC 25924)	4.00 mg/ml	[35]
	P. aeruginosa (ATCC 27858)	1.75 mg/ml	[35]
	E. coli (ATCC 8739)	4.00 mg/ml	[35]
	C. albicans (ATCC 10231)	16.00 mg/ml	[35]

Carrier oil	Micro-organism	MIC value	Reference
Prunus armeniaca	*S. aureus* (ATCC 25924)	2.00 mg/ml	[35]
	S. aureus (CCMM B3)	23.40 mg/ml	[176]
	K. pneumoniae (clinical isolate	11.70 mg/ml	[176]
	P. aeruginosa (ATCC 27858)	1.00 mg/ml	[35]
	P. aeruginosa (ATCC 10240)	11.70 mg/ml	[176]
	E. coli (ATCC 8739)	4.00 mg/ml	[35]
	E. coli (ATCC 8739)	11.70 mg/ml	[176]
	C. albicans (ATCC 10231)	8.00 mg/ml	[35]
Simmondsia chinensis	*S. aureus* (ATCC 25924)	4.00 mg/ml	[35]
	S. aureus (AHRI[4] isolate)	10.10 -24.10 mm[5]	[177]
	P. aeruginosa (ATCC 27858)	1.00 mg/ml	[35]
	P. aeruginosa (AHRI isolate)	0.00 mm[5]	[177]
	E. coli (ATCC 8739)	4.00 mg/ml	[35]
	E. coli (AHRI isolate)	0.00 mm[5]	[177]
	C. albicans (ATCC 10231)	6.00 mg/ml	[35]

[1]ATCC - American Type Culture Collection; [2]MIC of *Aloe vera* gel; [3]CCMI - Culture Collection of Industrial Microbiology LMI (INETI, Lisbon); [4]AHRI - Department of Animal Health Research Institute; [5]Zone of inhibition of *Simmondsia chinensis* wax liquid.

2.4 Toxicity of essential oil compounds

Despite the extensive use of compounds and the plethora of studies reporting the antimicrobial potential of essential oil compounds [3, 4, 15, 178, 179], various essential oil compounds have been highlighted for their toxicity [180-182]. Examples include the compounds carvacrol, eugenol and thymol which all resulted in 100% mortality to selected insects [180, 183]. It is for the same reason that they have gained popularity in agriculture for their use as pesticides. Thymol is a popular monoterpene that is regularly used in agriculture as a bio-insecticide [184, 185]. **Tables 2.4** and **2.5** summarize the toxicity studies of various essential oil compounds. For the purpose of this review, a concentration that inhibited 50% of parasite, cell growth or biological activity (IC_{50}) is considered toxic [39]. Most of the studies referred to their toxicity relative to other compounds which were being investigated in that particular study and thus

would report the result as “least toxic” and “most toxic.” As such, these had to be reinterpreted according to the results obtained and the concentration at which the result was recorded.

Table 2.4 provides a summary of the insecticide and fumigant toxicity assays. The use of essential oil compounds for pest control is popular in agriculture, and as a result this is a highly researched area. This gives an indication of how toxic essential oil compounds are, which could render potential use for humans. The studies mentioned [181, 183, 186-189] found the compound toxicity to be beneficial as the compounds were used as insecticides. Several of the selected compounds displayed toxicity against two or more insect species, therefore suggesting that the toxicity of compounds is not uniform across species. This was seen with linalool, which was highly toxic to *C. felis* [186], toxic to *T. confusum* [190], moderately toxic to *P. xylostella* larvae [191] and showed low toxicity to *I. ricinus* larvae [180]. These studies, however, are not applicable to humans or species of similar biology and thus further research is required.

It was observed that the majority of the studies identified were alternate fumigant and insecticide studies which focused on the use of essential oil compounds as pesticides, and only a handful looked at their potential medicinal properties in humans due to their toxicity. These studies used the MTT and *P. reticulata* assays as their model assays. Further studies focused on the toxicity of essential oils [192, 193], not specifically essential oil compounds. It is important to note that an essential oil compound could be highly toxic to insects, however, less toxic in mammals or mammalian cells. This could be attributed to the chemical constituents of essential oils (compounds) which affect the octopaminergic nervous system in insects, which is absent in mammals thus rendering the oils relatively non-toxic to mammals and fish during toxicity testing [194]. This has been reported with 1,8-cineole, which was recorded as toxic to *Sitophilus granarius* (wheat weevil), however, showed low toxicity in an MTT assay [182, 195]. The toxicity for linalool varied amongst different pests [180, 186, 191] making it difficult to determine whether the compound would be toxic to humans, especially considering the difference in nervous systems between insects and mammals [194].

Table 2.5 provides a summary of the MTT and *Poecilia reticulata* assays. The *Poecilia reticula* (guppy fish), recommended for use in toxicity studies by the Organization for Economical Cooperation and Development (OECD) [196], were grown to two months old and to a mean weight of 0.20 g. The toxicity was measured in terms of mortality and behavioural changes such as paralysis or reduced motility, erratic swimming, and excessive gill movement. The fish

were not fed during the experiment [197]. The disadvantage of this type of assay is similar to that of the zebrafish assay, where it was necessary for the guppy fish to be grown to two months old which is extremely time consuming. The compounds *p*-cymene and limonene do appear to have low toxicity for the studies using MTT assays (**Table 2.5**) and limonene showed low toxicity in both MTT and pest studies. A previous study which investigated potential essential oil use in food, reported that the compounds *p*-cymene and (±)-limonene are considered safe for human consumption [198].

The same toxicity patterns seen in the insect and fumigant toxicity studies were seen in the MTT studies for the compounds limonene and thymol, where limonene resulted in low toxicity and thymol exhibited high toxicity. A high toxicity could be beneficial depending on the use of the essential oil compounds, for example, thymol has been found to have good antiparasitic properties [180, 191, 199, 200]. A previous study [201] states that the hydrophobic characteristic of compounds assists with the compound's antiparasitic activity. The compounds permeate through the cell membrane which disrupts cytoplasmic organelles and metabolic pathways [201]. Eugenol has been found to be hepatotoxic to rats at high concentrations, while other compounds such as limonene and linalool are known to cause allergic contact dermatitis and eczematous reactions, respectively [32, 202]. This is evident by the shortage of studies on human toxicity of essential oil compounds, thus more studies are needed.

The shortage of human relevant toxicity studies on essential oil compounds is a great concern considering the number of products (including baby products) on the market containing these compounds **(Table 2.6)**. Selected commercial products contain carrier oils such as *Calophyllum inophyllum* seed oil or *Helianthus annuus* sunflower seed oil **(Table 2.6)**. The interaction between the essential oil compounds found in the products, such as geraniol, limonene, and menthol, and the *C. inophyllum* and *H. annuus* carrier oils is not known and neither is the potential safety risks or benefit.

If the multiple reports that are available on the toxicity of deodorants and antiperspirants, shampoos, and moisturizers [203-205], are taken into consideration, much of the toxicity has been attributed to specific ingredients such as phthalates or parabens [203-206]. However, it is unknown if a combined effect of ingredients is not a contributing factor to an increase in toxicity or problems coming from these types of products as opposed to single ingredients.

Table 2.4: Insect and fumigant toxicity studies

Compound	Type of assay	Concentration	Results obtained	Interpretation	Reference
Carvacrol	Gastrointestinal nematodes in sheep	8.00 and 1.00 mg/ml	Inhibited larval hatching by 89.30% at 8.00 mg/ml and by 97.70% at 1.00 mg/ml	Highly toxic	[207]
	Blattella germanica (German cockroach)	1.00 µl	Strain S LD_{50}[1]= 0.0075 (0.0047–0.0131) mg/µl and strain D LD_{50}= 0.0065 (0.0041–0.0119) mg/µl	Highly toxic	[208]
	Ixodes ricinus (Castor bean tick) larvae	5.00%	100.00% Mortality	Highly toxic	[180]
Cinnamaldehyde	*Tribolium castaneum* (Flour beetle)	0.06 mg/cm^2	93.33% Mortality	Highly toxic	[183]
Citral	*Callosobruchus maculatus* (Cowpea weevil)	0.89 mg/cm^3	85.00% Mortality during the reproductive cycle of the weevil	Toxic	[209]
p-Cymene	*Frankliniella occidentalis* (Flower Thrips)	10.00 µl	Biologically non-toxic (± 22.00%)	Low toxicity	[210]
	T. castaneum (Red flour beetle), (Herbst), *Sitophilus oryzae*, (L.) (Rice weevil), *Stegobium paniceum* (L.) the drugstore beetle, and *Callosobruchus analis* (F.) (Pulse beetle)	5.00-10.00 µl/ml	*T. castaneum* – 90.00% Mortality	Highly toxic	[181]
			S. oryzae – 92.00% Mortality	Highly toxic	
			S. paniceum – 95.00% Mortality	Highly toxic	
			C. analis – 100.00% Mortality	Highly toxic	
Eugenol	*Blattella germanica* (German cockroach)	1.00 µl	Strain S LD_{50}= 0.02 (0.02–0.03) mg/µl and strain D LD_{50}= 0.03 (0.026–0.035) mg/µl	Toxic	[208]
	T. castaneum	0.013 mg/cm^2	100.00% Mortality	Highly toxic	[183]

Compound	Type of assay	Concentration	Results obtained	Interpretation	Reference
Geraniol	*T. castaneum, S. oryzae, S. paniceum, C. analis*	1.00-10.00 µl/ml	*T. castaneum* – 82.00% Mortality	Toxic	[181]
			S. oryzae – 85.00% Mortality	Toxic	
			S. paniceum – 95.00% Mortality	Highly toxic	
			C. analis – 100.00% Mortality	Highly toxic	
Limonene	*B. germanica*	1.00 µl	Strain SLD_{50}= 0.06 (0.060–0.067) mg/µl and strain DLD_{50}= 0.06 (0.05–0.07) mg/µl	Low toxicity	[208]
Linalool	*Ctenocephalides felis* (Cat flea)	1.00% and 0.50%	1.00% dip = 100.00% Mortality 0.50% dip = 95.00% to 98.00% Mortality	Highly toxic	[186]
	Tribolium confusum (Coleoptera: Tenebrionidae)	7.81, 15.63,31.25 and 62.50 µl/l of air	Young larvae were the most susceptible to toxic effects, LC_{50}^{2} = 14.20 µl/l of air for linalool after 24 h of exposure	Toxic	[190]
	Plutella xylostella (Diamondback moth) larvae	15.00 µg	Exhibited <45.00% mortality in up to 15.00 µg/larva	Moderately toxic	[191]
	I. ricinus larvae	5.00%	Mortality= 0.90± 0.50% at 5.00% concentration	Low toxicity	[180]
Linalyl acetate	*Sitophilus oryzae* (Common rice weevil)	0.10 µl/720.00 ml	*S. oryzae* - 82.50% Mortality	Toxic	[211]
	Rhyzopertha dominica (Wheat weevil)		*R. dominica* - 87.50% Mortality	Toxic	
	T. castaneum		*T. castaneum* – 0.00% Mortality	Low toxicity	
Nerol	*Aedes aegypti* (L.)	0.00-20.00 mg/ml	LC_{50} value - 54.25 µg/cm^2	Toxic	[212]
	Anopheles quadrimaculatus (Say)		LC_{50} value – 108.85 µg/cm^2	Moderately toxic	
α-Pinene	*T. castaneum, S. oryzae, S. paniceum, C. analis*	5.00-10.00 µl/ml	*T. castaneum* – 90.00% Mortality	Highly toxic	[181]
			S. oryzae – 92.00% Mortality	Highly toxic	
			S. paniceum – 98.00% Mortality	Highly toxic	
			C. analis – 100.00% Mortality	Highly toxic	

Compound	Type of assay	Concentration	Results obtained	Interpretation	Reference
Santalol	*Tetranychus urticae* (Acari: Tetranychidae) (Two-spotted spider mite) (TSSM)	0.10%	85.50% Mortality	Toxic	[188]
γ-Terpinene	*Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae) (Egyptian cotton leafworm), *Aphis fabae* L. (Hemiptera: Aphididae) (Black bean aphid)	LD_{25}:LD_{25}[3] (1:1) and/or LC_{25}:LC_{25}[4] (1:1)	The compounds had a synergistic effect in toxic activity, when combined with synthetic insecticides against both insects.	Highly toxic	[187]
Terpinen-4-ol					
	Sitophilus granarius (Wheat weevil)	0.50, 0.75 and 1.00 µl/l	100.00% mortality at all doses after 12 hr exposure	Highly toxic	[182]
Thymol	*T. castaneum*	0.05 mg/cm^2	43.33% Mortality	Low toxicity	[183]
	Spodoptera litura (Fab) (Tobacco cutworms)	15.00-20.00 mg body weight	LD_{50} = 25.40 µg/larva	Highly toxic	[200]
	Sheep gastrointestinal nematodes - egg hatch test	0.50 mg/ml	Inhibited 98.00% of larval hatching	Highly toxic	[199]
	P. xylostella larvae	0.22 µg	LD_{50} of 0.22 µg/larva	Highly toxic	[191]
	I. ricinus larvae	5.00%	100.00% Mortality	Highly toxic	[180]
Thymoquinone	*T. urticae*	0.001–1.00%	LC_{50} = 0.07% w/v	Highly toxic	[189]
	Myzus persicae (Green peach aphid)		LC_{50} = 0.05% w/v	Highly toxic	

[1]LD_{50} - Lethal dose to cause 50% mortality; [2]LC_{50} - Lethal concentration to cause 50% mortality; [3]LD_{25} - Lethal dose to cause 25% mortality; [4]LC_{25} - Lethal concentration to cause 25% mortality.

Table 2.5: MTT and *Poecilia reticulata* (Guppy fish) toxicity studies

Compound	Type of assay	Concentration	Results obtained	Interpretation	Reference
Carvacrol	MTT[1]	10.00% (v/v)	IC_{50}[2] value was 251.80±32.80 µM	Toxic	[7]
ρ-Cymene			IC_{50} value was 673.60±35.40 µM	Low toxicity	
Eugenol			IC_{50} value was 1358.40±13.00 µM	Low toxicity	[39]
R (+)-Limonene			IC_{50} value was 1042.00±41.10 µM		[7]
(±)-Linalool			IC_{50} value was 882.70±25.40 µM		
Linalyl acetate			IC_{50} value was 80.00±2.90 µM	Toxic	
Eugenol	*In vitro* experiment on rat hepatocytes	20 and 30 µg/100g body weight/day	Cell damage and hepatotoxicity was seen in more than 85.00% of cells after 5 hrs of exposure.	Toxic	[213]
trans-Geraniol	MTT	10.00% (v/v)	IC_{50} value was 128.50±4.70 µM	Moderately toxic	[7]
Menthol	*In vitro* experiment on rat hepatocytes	0.00, 200.00, 400.00, 800.00 mg menthol/ kg body wt./day	An increase in liver weight and the forming of vacuoles within/adjacent to hepatocytes was seen. Vacuolisation is associated with liver injury [214].	Toxic	[215]
Thymol	MTT	6400.00 µg/ml	Cytotoxic concentration for 50.00% culture (CC_{50}[3]) for MDBK cells was 1404.32 ± 6.91 µg/ml	Highly toxic	[195]
	P. reticulata	0.00-20.00 mg/l	LC_{50}[4] value for female fish was 12.51 mg/l	Moderately toxic	[197]

[1]MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltretrazolium bromide; [2]IC_{50} - Concentration that inhibited 50% of parasite, cell growth or biological activity; [3]CC_{50} - Cytotoxic concentration for 50% culture; [4]LC_{50} - Lethal concentration to cause 50% mortality.

Thus, this study highlights the interactions that may occur which may result in a potential increase in toxicity.

Table 2.6: Products on the market containing compounds (bold) and/or carrier oils (bold and italics)

Product	Ingredients	Reference
	Water, Cetearyl Alcohol, Coco glucoside, ***Lanolin***, Lactic acid, Parfum, Centrimonium bromide, Citronellol, **Geraniol**, **Linalool**, CI 17200.	[216]
	Aqua, Sodium laureth sulphate, Cocamide DEA, Cocamidopropyl betaine, Sorbitol, Sodium chloride, Decyl glucoside, PEG-120 Methyl glucose dioleate, *Adansonia digitata* fruit extract, *Aspalathus linearis* Leaf extract, Citric acid, Laureth-4, Disodium EDTA, Cocamidopropyl dimethylamine, Salicylic acid, Phenoxyethanol, Caprylyl glycol, Sodium hydroxide, Sodium metabisulphite, Fragrance, Alpha-isomethyl Ionone, **Limonene**, **Linalool**.	[217]

Product	Ingredients	Reference
	Water, Glycerin, Sodium laureth sulfate, Cocamidopropyl betaine, PEG-120 methyl glucose Dioleate, PEG-40 hydrogenated castor oil[1], PEG-7 glyceryl cocoate, Polysorbate 20, Alcohol denat, Sodium chloride, Phenoxyethanol, Panthenol, *Melaleuca alternifolia* leaf oil, Sodium benzoate, ***Calophyllum inophyllum seed oil***, Citric acid, Allantoin, **Menthol**, Butyl methoxydibenzoylmethane, **Limonene**, Sodium hydroxide, *Leptospermum petersonii* oil, Tocopherol, CI 19140/Yellow 5, Caramel, CI 42090/Blue 1.	[218]
	Aqua/Water/Eau, Sodium lauroyl methyl isethionate, Disodium laureth sulfosuccinate, Sodium lauroyl sarcosinate, Cocamidopropyl betaine, Sodium lauryl sulfoacetate, Sodium methyl 2-sulfolaurate, Panthenol, Parfum/Fragrance, Sodium methyl isethionate, Benzyl alcohol, Acrylates/C1030 alkyl acrylate cross polymer, Lauric acid, Ppg26buteth26, Coco glucoside, Phenethyl benzoate, Glyceryl oleate, Peg40 hydrogenated castor oil[1], Sodium benzoate, Glycol distearate, Citric acid, Trisodium ethylenediamine disuccinate, Polyquaternium7, Disodium2sulfolaurate, Sodium hydroxide, Sodium laurate, Amo dimethicone, Guar hydroxypropyltrimonium chloride, C1115pareth7, ***Helianthus annuus sunflower seed oil***, Glyceryl stearate, Glycerin, Laureth9, Citronellol, **Geraniol**, Trideceth12, Bixaorell anaseed extract	[219]

Product	Ingredients	Reference
	Cyclopentasiloxane, Aluminum zirconium Tetrachlorohydrex gly, Stearyl alcohol, C12-15 Alkyl Benzoate, PPG-14 Butyl ether, Phenyl trimethicone, Petrolatum, ***Hydrogenated castor oil***, Talc, Fragrance, PEG-40 Hydrogenated castor oil[1], Silica, Sodium starch octenylsuccinate, Alpha-isomethyl ionone, Benzyl benzoate, Benzyl salicylate, Cinnamyl alcohol, **Citral**, Citronellol, Coumarin, **Geraniol**, Hexyl cinnamal, Hydroxycitronellal, **Limonene**, **Linalool**	[220]

[1]Synthetic.

Although it is believed that they are non-toxic at low concentrations (< 1.00 mg/ml), some of the available literature suggests that essential oil compounds may possess an allergy potential, with side effects such as contact dermatitis [221, 222], and contact stomatitis [32]. Concentrations of < 1.00 mg/ml are considered low since the compounds in this study were toxic when tested singularly at a concentration of 1.00 mg/ml. **Table 2.7** provides examples of several essential oil compounds and their potential side effects. A common topical side effect due to the compounds' toxicity is allergic contact dermatitis [221-223], as the complex chemical structures of the compounds in topical formulations may expose the skin to potential allergens [32].

Table 2.7: Examples of side effects caused by various essential oil compounds

Essential oil compound	Side-effects	Reference
Carvacrol	Skin inflammation, hyperplasia	[224, 225]
Cinnamaldehyde	Allergic contact dermatitis	[221]
Citral	Allergic contact dermatitis	[226]
p-Cymene	Allergic contact dermatitis	[222]
Eugenol	Hepatotoxicity, contact dermatitis, contact stomatitis	[32, 227]
Geraniol	Allergic contact dermatitis	[223]
Isoeugenol	Contact allergy/Allergic contact dermatitis	[227, 228]

Essential oil compound	Side-effects	Reference
Limonene	Allergic contact dermatitis	[202]
Linalool	Dermatitis, eczematous reaction	[32]
Linalyl acetate	Allergic contact dermatitis	[229]
Menthol	Contact dermatitis	[230]
α-Pinene	Erythema[1]	[231]
α-Terpinene	Allergic contact dermatitis	[222]
Terpinen-4-ol	Allergic contact dermatitis[2]	[222]
Thymol	Allergic contact dermatitis	[232]

[1]Side effects of α-pinene, and not (+)-α-pinene; [2]Side effect of terpinene-4-ol, and not (+)-terpinen-4-ol.

2.5 Toxicity of carrier oils

The toxicity of the selected carrier oils from a previous study [35] using the brine-shrimp lethality assay is summarised in **Table 2.8**. The brine-shrimp lethality assay is deemed a convenient assay to test biological activity [233]. The use of the *Artemia* organism within the brine-shrimp lethality assay is seen as valuable due to the genus' adaptability to various testing environments, as well as the simple and inexpensive nature of laboratory rearing of the *Artemia* species [234]. The assay is simple, rapid, and does not require large amounts of test materials [235]. The assay also has good correlation with pesticidal activity [235].

All carrier oils tested in the previous study [35] were non-toxic (< 50.00% mortality) when tested at a concentration of 1.00 mg/ml. In another study [236], *A. vera* latex showed cytotoxicity to human fibroblast cells, however, *A. vera* gel, tested at a 100.00% concentration, was non-toxic when tested in an *in vitro* system mimicking human skin [237]. The carrier oils *C. officinalis* and *S. chinensis* were found to be non-toxic when *C. officinalis*, as part of a lamellar gel phase emulsion, was tested for wound healing properties in rats [238], and when *S. chinensis* wax was injected subcutaneously into mice [239]. The carrier oil *H. perforatum* was reported to be non-toxic to cells [240]. In a toxicogenetic study of the fruit pulp oil of *P. americana*, the oil was non-genotoxic, however, when tested at the highest dose, the oil led to tissue/hepatic damage [241]. The carrier oil *P. armeniaca* is edible, suggesting its non-toxicity [242]. Overall, previous studies show non-toxicity of the carrier oils tested.

Table 2.8: Toxicity of carrier oils

Carrier oil	Toxicity (% mortality)		Reference
	24 hrs	48 hrs	
Aloe vera	0.00	27.08	[35]
Calendula officinalis	0.00	28.35	
Hypericum perforatum	0.63	39.33	
Persea americana	0.00	25.75	
Prunus armeniaca	4.35	10.38	
Simmondsia chinensis	3.04	24.12	

2.6 Time-kill studies on essential oil compounds

The time-kill assay is used to determine whether a sample is bactericidal/fungicidal, or bacteriostatic/fungistatic over time [243-245]. This is achieved by counting the number of a micro-organism's colony forming units present, after interaction with antimicrobial samples, at several time intervals [246]. The inhibitory effects could either be time or concentration dependent [244]. The time-kill activity of several essential oil compounds has been previously reported and is shown in **Table 2.9**. Overall, carvacrol and thymol showed the most rapid bactericidal activity.

Table 2.9: Time-kill effects of essential oil compounds against ESKAPE pathogens and *C. albicans*

Compound	Micro-organism	Durat-ion of study	MIC/ Conc[1]	Result/ Activity	Ref[2]
Carvacrol	*S. aureus* (MRSA[3])	24 hrs	1.22 mg/ml	Bactericidal at 0.003 hrs	[247]
	K. pneumoniae (Carbapenemase producing)	24 hrs	0.61 mg/ml	Bactericidal at 0.50 hrs	[247]
	A. baumannii (Carbapenem-resistant)	24 hrs	1.22 mg/ml	Bactericidal at 0.003 hrs	[247]

Compound	Micro-organism	Durat-ion of study	MIC/ Conc[1]	Result/ Activity	Ref[2]
	E. coli (ATCC[4] 25922) *E. coli* O157:H7 (ATCC 35150)	24 hrs 60 sec	0.3 mg/ml 0.63 mg/ml	Bactericidal at 0.003 hrs Reduced *E. coli* to below detection limit	[247] [248]
β-Caryophyllene	*S. aureus* (ATCC 25923) *E. coli* (ATCC 8739)	24 hrs	MIC: 1.00 mg/ml	Bacteriostatic Bactericidal at 24 hrs	[249]
Cinnamaldehyde	*A. baumannii* (MDR[5])	2 hrs 4 hrs	MIC: 0.01-0.04% v/v	Bacteriostatic	[250]
Citral	*C. albicans* (ATCC 76485)	24 hrs	MIC: 64 µg/ml	Fungicidal at 4 hrs	[251]
p-Cymene	*S. aureus* (NCTC 8325)	1 hr	No value reported	No reduction in CFUs[6].	[252]
Eugenol	*S. aureus* (ATCC 6538) *K. pneumoniae* (ATCC 10031) *P. aeruginosa* (ATCC 10145) *E. coli* (ATCC 7839) *E. coli* O157:H7 (ATCC 35150) *C. albicans*	24 hrs 24 hrs 24 hrs 24 hrs 60 sec 24 hrs	MIC: 6.25 mM MIC: 12.50 mM MIC: 50.00 mM MIC: 25.00 mM 0.63 mg/ml MIC:	Bactericidal at 1 hr Bactericidal at approximately 1.5 hrs Bactericidal at approximately 1.5 hrs Bactericidal at approximately 2.5 hrs Bacteriostatic Fungicidal at	[253] [253] [253] [253] [248] [253]

Compound	Micro-organism	Durat-ion of study	MIC/ Conc[1]	Result/ Activity	Ref[2]
	(ATCC 10231)		25.00 mM	approximately 2.5 hrs	
Geraniol	*S. aureus* (NKUST[7])	24 hrs	MIC_{50}[8]: 0.45 mg/ml	Bacteriostatic after 16 hrs	[96]
	E. coli (NKUST)	24 hrs	MIC_{50}: 0.45 mg/ml	Bacteriostatic after 16 hrs	[96]
	E. coli (ATCC 8739)	24 hrs	MIC: 0.06 mg/ml	Bacteriostatic[9]	[254]
Isoeugenol	*E. coli* (K12)	24 hrs	MIC: 0.60 mg/ml	No reduction in CFUs.	[255]
Limonene	*S. aureus* (NKUST)	24 hrs	MIC: 0.42 mg/ml	Bacteriostatic after 16 hrs	[96]
	E. coli (NKUST)	24 hrs	MIC: 0.42 mg/ml	Bacteriostatic after 16 hrs	[96]
	C. albicans (ATCC 90,028)	12 hrs	MIC: 300 µg/ml	Fungistatic	[256]
Linalool	*S. aureus* (ATCC 6538)	24 hrs	MIC: 100.00 mM	Bacteriostatic	[253]
	K. pneumoniae (ATCC 10031)	24 hrs	MIC: 12.50 mM	Bactericidal at approximately 22 hrs	[253]
	P. aeruginosa (ATCC 10145)	24 hrs	MIC: 50.00 mM	Bactericidal at 24 hrs	[253]
	E. coli (ATCC 7839)	24 hrs	MIC: 50.00 mM	Bacteriostatic	[253]
	C. albicans (ATCC 10231)	24 hrs	MIC: 25.00 mM	Fungicidal at 24 hrs	[253]
	C. albicans	30 min	0.50% v/v	Fungicidal at 30	[167]

Compound	**Micro-organism**	**Durat -ion of study**	**MIC/ Conc[1]**	**Result/ Activity**	**Ref[2]**
	(ATCC 3153)			sec	
Linalyl acetate	*C. albicans* (ATCC 3153)	30 min	2% v/v	Fungistatic	[167]
Menthol	*C. albicans* (ATCC 10231)	48 hrs	MIC_{90}[10]: 0.01 mg/ml	Fungistatic	[257]
Nerol	*S. aureus* (NKUST)	24 hrs	MIC_{50}: 0.44 mg/ml	Bacteriostatic after 16 hrs	[96]
	E. coli (NKUST)	24 hrs	MIC_{50}: 0.44 mg/ml	Bacteriostatic after 16 hrs	[96]
(+)-α-Pinene	*S. aureus* (MRSA 53)	30 hrs	MBC[12]: 0.3% v/v	Bactericidal[13] at approximately 8 hrs	[258]
	S. aureus (TCC[11])	30 hrs	MIC: 0.03%	No reduction in CFU's[13]	[259]
	S. aureus (TCC)	30 hrs	MBC: 0.13%	Bactericidal[13] at 12 hrs	[259]
	S. aureus (ATCC 25923)	24 hrs	MIC: 2.5 µl/ml	Bacteriostatic	[260]
	E. coli (ATCC 35218)	24 hrs	MIC: 0.8 µl/ml	Bactericidal[13] at 12 hrs	[261]
	E. coli (ATCC 25922)	24 hrs	MIC: 1.25 µl/ml	Bactericidal at approximately 2 hrs	[260]
Santalol	No time-kill data found				
α-Terpinene	No time-kill data found				
γ -Terpinene	*S. aureus* (NCTC 8325)	1 hr	No value reported	No reduction in CFU's	[252]
	P. aeruginosa (NCTC 6749)	1 hr	No value reported	Negligible effects	[252]
	E. coli	30 min	No value	No reduction in	[252]

Compound	Micro-organism	Durat-ion of study	MIC/ Conc[1]	Result/ Activity	Ref[2]
	(AG 100)		reported	CFUs	
	C. albicans	30 min	No value reported	Negligible effects	[252]
	(KEM H5)				
	C. albicans	6 hrs	1.00% v/v	Fungistatic	[159]
	(ATCC 10231)				
Terpinen-4-ol	*S. aureus*	2 hrs	MIC:	Bacteriostatic	[262]
	(ATCC 9144)		0.25% v/v		
	C. albicans	6 hrs	MIC:	Rapid killing	[159]
	(ATCC 10231)		0.25% v/v	effects	
α-Terpineol	*S. aureus*	24 hrs	MIC:	Bacteriostatic[14]	[254]
	(ATCC 25923)		0.03 mg/ml		
	S. aureus	24 hrs	MIC_{50}:	Bacteriostatic	[96]
	(NKUST)		0.75 mg/ml	after 16 hrs[14]	
	E. coli	24 hrs	MIC_{50}:	Bacteriostatic	[96]
	(NKUST)		0.75 mg/ml	after 16 hrs[14]	
	C. albicans	6 hrs	MIC:	Negligible effects	[159]
	(ATCC 10231)		0.12% v/v	on viability	
Thymol	*S. aureus*	24 hrs	1 mg/ml	Bactericidal at	[247]
	(MRSA)			0.003 hrs	
	K. *pneumoniae*	24 hrs	0.5 mg/ml	Bactericidal at	[247]
	(Carbapenemase producing)			0.003 hrs	
	A. baumannii	24 hrs	0.25 mg/ml	Bactericidal at	[247]
	(Carbapenem-resistant)			0.17 hrs	
	E. coli	24 hrs	0.25 mg/ml	Bactericidal at	[247]
	(ATCC 25922)			0.003 hrs	
	E. coli O157:H7	60 sec	0.63 mg/ml	Reduced *E. coli*	[248]
	(ATCC 35150)			to below detection limit	

Compound	Micro-organism	Durat -ion of study	MIC/ Conc[1]	Result/ Activity	Ref[2]
Thymoquinone	*S. aureus* (ATCC 29213)	24 hrs	MBC: 50 µg/ml	Bactericidal after 6 hrs	[134]

[1]MIC/Conc - Minimum Inhibitory Concentration/Concentration; [2]Ref - Reference; [3]MRSA - Methicillin-resistant *S. aureus*; [4]ATCC - American Type Culture Collection; [5]MDR – Multi-drug Resistant; [6]CFU - Colony Forming Units; [7]NKUST - National Kaosiung University Science and Technology; [8]MIC_{50} - Minimum Inhibitory Concentration required to inhibit 50% of micro-organism growth; [9]Results of trans-geraniol; [10]MIC_{90} - Minimum Inhibitory Concentration required to inhibit 90% of micro-organism growth; [11]TCC - Type Culture Collection; [12]MBC - Minimum Bactericidal Concentration; [13]Results of α-pinene; [14]Results of terpineol.

2.7 Interpretation of toxicity in relation to antimicrobial activity

If one considers the antimicrobial activity in isolation (without toxicity data), it cannot be known whether the antimicrobial activity is due to toxicity. Thus, it is recommended that the selectivity index (SI) be calculated which determines whether the MIC of the sample is not toxic [263]. The SI has been recommended [263] for use in studies which investigate bioactive preparations from natural sources. This is important in order to identify whether the samples tested are selective by comparing the required concentration to the toxicity, and to determine the potential therapeutic benefits and safety of the samples [263].

It can be noted that the compounds with the lowest MIC values also have multiple reports on toxicity. It is thus imperative that a means of decreasing the toxicity is found to allow for the use of these notable compounds against this scourge of antimicrobial resistance.

2.8 Combinations

If one considers the use of essential oils, they are often used in combination with the intention of achieving therapeutic synergy [264-266]. Essential oil compounds naturally exist within essential oils [12]. Compounds may be combined, at various ratios, with each other as shown in numerous studies [4, 178, 179] investigating the interaction of essential oil compounds in combination. Essential oil compounds have also been combined with antibiotics to investigate possible synergistic interactions [267-269]. However, no studies could be identified where the compounds have been combined with the carrier oils to determine the influence of carrier oils on the antimicrobial activity or toxicity of compounds.

Chapter 3 – The antimicrobial and toxicity influence of six carrier oils on essential oil compounds

molecules

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The Antimicrobial and Toxicity Influence of Six Carrier Oils on Essential Oil Compounds

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Abstract: Essential oil compounds have been identified as alternative antimicrobials; however, their use is limited due to their toxicity on human lymphocytes, skin, and reproduction. Carrier oils can reduce the toxicity of essential oils, which raises the question as to whether such activity would extend to the essential oil compounds. Thus, this study aimed to investigate the antimicrobial and toxicity activity of essential oil compounds in combination with carrier oils. The antimicrobial properties of the essential oil compounds, alone and in combination with carrier oils, were determined using the broth microdilution assay. The toxicity was determined using the brine shrimp lethality assay. Antimicrobial synergy (ΣFIC $\leq$ 0.50) occurred in 3% of the samples when tested against the ESKAPE pathogens. The compound thymoquinone in combination with the carrier oil *Prunus armeniaca* demonstrated broad-spectrum synergistic activity and a selectivity index above four, highlighting this combination as the most favorable. The carrier oils reduced the toxicity of several compounds, with *Calendula officinalis* and *P. armeniaca* carrier oils being responsible for the majority of the reduced toxicity observed. This study provides insight into the interactions that may occur when adding a carrier oil to essential oil compounds.

Keywords: antimicrobial; toxicity; carrier oils; essential oil compound; minimum inhibitory concentration; selectivity index; ESKAPE; percentage mortality; thymoquinone; *Prunus armeniaca*; *Calendula officinalis*

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1. Introduction

Antimicrobial resistance is responsible for a large number of morbidities and mortalities worldwide [1,2]. The World Health Organization published a list of priority micro-organisms known as the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Escherichia coli*) which requires urgent attention with respect to antimicrobial resistance [3]. Essential oils have been identified as alternative antimicrobial options due to their observed antimicrobial properties against a wide range of pathogens, especially against Gram-positive micro-organisms [4–10].

Essential oils display various biological activities that include antimicrobial [11–14], insecticidal [11,15,16], food preservative [12,17–19], anti-oxidative [14,19], anti-inflammatory [14,20] and anticancer [21,22] activities. Essential oils comprise anywhere from 10 to more than 300 compounds belonging to many different chemical classes such as alcohols, oxides or ethers, aldehydes, esters, ketones, amides, amines, heterocycles, phenols, and terpenes [23]. These compounds possess antimicrobial activity, with different compounds such as carvacrol, cinnamaldehyde, eugenol, geraniol, and thymol exhibiting varying degrees of activity against pathogens such as *S. aureus* and *E. coli* [4,6,23–25]. Despite the extensive use of these compounds and the plethora of studies reporting their antimicrobial potential, their use in humans is limited by their toxicity which has been shown against human lymphocytes, hepatocytes, skin, reproduction, and mucous membranes [26–29].

Carrier oils, also known as fixed oils, are made of a number of lipids such as waxes or fatty acids (Omega 3 and 6) as well as vitamins (E and A) and minerals [30]. These are produced by methods of centrifugation, maceration, cold press, or extraction from the fatty component of a plant [30]. Carrier oils have been shown to reduce the toxicity of essential oils [31]. The constituents responsible for the reduction in toxicity is not known; however, the carrier oils often contain vitamin E, and studies reporting the decrease in toxicity of toxic medicines by vitamin E are available [31–33].

A previous study [31], investigated the combinations of carrier oils with essential oils; however, it was not known whether the previous observations would extend to the single compounds that are found within essential oils. The hypothesis of whether the synergy exerted by the carrier oils on essential oils would extend to the essential oil compounds thus arose. Therefore, this study explored both the antimicrobial and toxicity interactions between a selection of essential compounds and carrier oils to determine which combinations would provide the optimum antimicrobial combination with the least toxicity.

It has been recommended that in studies which investigate bioactive preparations from natural sources, the selectivity index should be examined. This is important in order to determine a safe therapeutic dose which is still active [34]. Taking this into consideration, the selectivity index was determined in optimum combinations, providing insight into favorable combinations.

2. Results and Discussion

2.1. Antimicrobial Analysis

Poor antimicrobial activity was mostly observed with the six carrier oils (*Aloe vera, Calendula officinalis, Hypericum perforatum, Persea americana, Prunus armeniaca*, and *Simmondsia chinensis*) selected for the study (Table 1). Some noteworthy activity was displayed against *C. albicans* by *A. vera*, *C. officinalis* and *S. chinensis*. Minimal antimicrobial activity was expected from the carrier oils due to their consisting of vitamins, minerals, and free fatty acids, which are not known for antimicrobial activity [30]. The lack of antimicrobial activity of the carrier oils has also been previously noted [31]. It was, however, important to document the minimum inhibitory concentration (MIC) values as it forms an important starting point for determining the combined fractional inhibitory concentration index (ΣFIC) values.

Table 1. Antimicrobial activity (MIC in mg/mL with standard deviation (SD) in parentheses) of carrier oils (n = 3) against all pathogens.

Carrier Oil	Micro-Organism						
	Gram-Positive Bacteria		Gram-Negative Bacteria				Yeast
	E. faecium ATCC 27270	*S. aureus* ATCC 25923	*K. pneumoniae* ATCC 13883	*A. baumannii* ATCC 17606	*P. aeruginosa* ATCC 27853	*E. coli* ATCC 8729	*C. albicans* ATCC 10231
Aloe vera Mill.	16.00 (±0.00)	2.00 (±0.00)	2.00 (±0.00)	3.43 (±0.90)	1.75 (±0.43)	3.00 (±1.00)	**1.00 (±0.00)** [1]
Calendula officinalis L.	16.00 (±0.00)	2.00 (±0.00)	2.00 (±0.00)	3.60 (±0.80)	2.25 (±1.09)	2.50 (±0.87)	**1.00 (±0.00)**
Hypericum perforatum L.	16.00 (±0.00)	2.67 (±0.94)	2.00 (±0.00)	3.60 (±0.80)	1.75 (±0.43)	2.50 (±0.87)	1.50 (±0.50)
Persea americana Mill.	4.00 (±0.00)	2.50 (±0.87)	2.00 (±0.00)	3.00 (±1.00)	2.50 (±0.87)	2.50 (±0.87)	3.00 (±1.00)
Prunus armeniaca Blanco.	4.00 (±0.00)	2.50 (±0.87)	2.00 (±0.00)	2.00 (±0.00)	2.67 (±0.94)	3.50 (±0.87)	2.00 (±0.00)
Simmondsia chinensis C.K. Schneid.	4.00 (±0.00)	3.00 (±1.00)	2.00 (±0.00)	4.00 (±0.00)	3.33 (±0.94)	3.50 (±0.87)	**1.00 (±0.00)**
Positive control [2]	1.56 [2.1]	0.73 [2.1]	0.20 [2.1]	0.52 [2.1]	1.25 [2.1]	0.50 [2.1]	0.94 [2.2]
Negative control	> 8.00	3.30	> 8.00	3.00	>8.00	> 8.00	2.00
Culture control	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00

[1] Noteworthy MIC values in bold; [2] Ciprofloxacin [2.1] /Nystatin [2.2] (µg/mL).

Several compounds tested displayed noteworthy antimicrobial activity (≤1.00 mg/mL) against all pathogenic reference strains tested (Table 2). These compounds included: carvacrol, cinnamaldehyde, isoeugenol, and thymol. This highlights the antimicrobial importance of these compounds, since they were able to maintain broad-spectrum, noteworthy antimicrobial activity across all the strains tested. Previous studies have also reported on the antimicrobial activities of carvacrol, cinnamaldehyde, isoeugenol, and thymol against several of the pathogens [35–46]. Phenolic compounds (carvacrol and thymol) could be

potential agents to fight against antimicrobial resistance as data has shown that phenolics inhibit resistant strains [47–49].

Table 2. Antimicrobial activity (MIC in mg/mL and SD in parentheses) of the essential oil compounds (n = 3).

Essential Oil Compound	Micro-Organism						
	Gram-Positive Bacteria		Gram-Negative Bacteria				Yeast
	E. faecium ATCC 27270	*S. aureus* ATCC 25923	*K. pneumoniae* ATCC 13883	*A. baumannii* ATCC 17606	*P. aeruginosa* ATCC 27853	*E. coli* ATCC 8729	*C. albicans* ATCC 10231
Carvacrol	**0.67 (±0.24)** [1]	**0.42 (±0.12)**	**0.25 (±0.00)**	**0.83 (±0.24)**	**0.50 (±0.18)**	**0.63 (±0.22)**	**0.25 (±0.00)**
β-Caryophyllene	4.00 (±0.00)	3.00 (±1.00)	2.00 (±0.00)	3.33 (±0.94)	2.67 (±0.94)	3.33 (±0.94)	2.00 (±0.00)
Cinnamaldehyde	**0.50 (±0.00)**	**0.13 (±0.00)**	**0.33 (±0.12)**	**0.42 (±0.12)**	**0.38 (±0.13)**	**0.38 (±0.13)**	**0.03 (±0.00)**
Citral	1.33 (±0.47)	**0.25 (±0.72)**	**1.00 (±0.00)**	**1.00 (±0.00)**	1.75 (±0.43)	**1.00 (±0.00)**	**0.50 (±0.00)**
p-Cymene	3.33 (±0.94)	2.50 (±0.87)	**1.00 (±0.00)**	3.33 (±0.94)	3.00 (±1.00)	3.00 (±1.00)	1.50 (±0.50)
Eugenol	**1.00 (±0.00)**	1.25 (±0.43)	**0.50 (±0.00)**	**1.00 (±0.00)**	**1.00 (±0.00)**	**1.00 (±0.00)**	**0.38 (±0.13)**
Geraniol	1.67 (±0.47)	1.25 (±0.43)	**0.83 (±0.24)**	**1.00 (±0.00)**	1.25 (±0.43)	1.20 (±0.40)	**0.25 (±0.00)**
Isoeugenol	**0.83 (±0.24)**	**0.33 (±0.12)**	**0.63 (±0.22)**	**0.58 (±0.19)**	**0.55 (±0.24)**	**1.00 (±0.00)**	**0.25 (±0.00)**
R(+)-Limonene	3.00 (±1.00)	3.33 (±0.94)	3.00 (±1.00)	4.00 (±0.00)	2.25 (±1.09)	5.33 (±1.89)	**1.00 (±0.00)**
Linalool	3.33 (±0.94)	1.40 (±1.07)	**1.00 (±0.00)**	2.00 (±0.00)	1.75 (±0.43)	3.00 (±1.00)	**1.00 (±0.00)**
Linalyl acetate	4.00 (±0.00)	2.50 (±0.87)	1.67 (±0.47)	2.00 (±0.00)	1.75 (±0.43)	3.00 (±1.00)	**1.00 (±0.00)**
Menthol	2.67 (±0.94)	**1.00 (±0.00)**	1.67 (±0.47)	2.00 (±0.00)	2.00 (±0.00)	2.00 (±0.00)	**0.50 (±0.00)**
Nerol	**1.00 (±0.00)**	1.33 (±0.47)	1.50 (±0.50)	1.50 (±0.50)	1.33 (±0.47)	1.67 (±0.47)	**0.50 (±0.00)**
(+)-α-Pinene	3.00 (±1.00)	2.00 (±0.00)	3.00 (±1.00)	3.00 (±1.00)	2.00 (±0.00)	6.00 (±2.00)	**1.00 (±0.00)**
Santalol	**0.25 (±0.00)**	**0.19 (±0.06)**	4.00 (±0.00)	**1.00 (±0.00)**	4.00 (±0.00)	**0.50 (±0.00)**	**1.00 (±0.00)**
α-Terpinene	7.00 (±1.73)	2.00 (±0.00)	3.00 (±1.00)	2.00 (±0.00)	2.67 (±0.94)	3.33 (±0.94)	**1.00 (±0.00)**
γ-Terpinene	3.33 (±0.94)	2.67 (±0.94)	3.67 (±3.09)	4.00 (±2.83)	4.00 (±2.45)	4.00 (±0.00)	2.00 (±0.00)
(+)-Terpinen-4-ol	4.00 (±0.00)	3.00 (±1.00)	1.50 (±0.50)	3.00 (±1.00)	2.00 (±0.00)	2.50 (±0.87)	2.00 (±0.00)
α-Terpineol	2.00 (±0.00)	1.33 (±0.47)	**1.00 (±0.00)**	2.00 (±0.00)	2.00 (±0.00)	2.00 (±0.00)	**0.75 (±0.25)**
Thymol	**0.67 (±0.24)**	**0.75 (±0.25)**	**0.25 (±0.00)**	**1.00 (±0.00)**	**0.25 (±0.00)**	**0.75 (±0.25)**	**0.50 (±0.00)**
Thymoquinone	**0.01 (±0.00)**	**0.00 (±0.00)**	**1.00 (±0.00)**	**0.02 (±0.01)**	1.50 (±0.50)	**0.13 (±0.00)**	**0.08 (±0.05)**
Positive control [2]	1.09 [2.1]	0.50 [2.1]	0.08 [2.1]	0.66 [2.1]	0.36 [2.1]	0.88 [2.1]	1.25 [2.2]
Negative control (water in acetone)	>8.00	3.33	4.67	>8.00	4	5	8
Culture control	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00

[1] Noteworthy MIC values in bold; [2] Ciprofloxacin [2.1] /Nystatin [2.2] (μg/mL).

Combinations

The MIC values of the 21 compounds in combination with six carrier oils against seven pathogens (882 combinations in total) were determined, and the results are shown in Tables 3–9. In summary, most of the combinations resulted in indifference (56%), followed by 37% additive interactions. There was a total of 3% synergistic (23 combinations) and 4% antagonistic interactions noted.

The combined use of *A. vera*, *C. officinalis*, and *H. perforatum* with α-terpinene resulted in the most synergy against *E. faecium* (ΣFIC value of 0.41) (Table 3). The compound α-terpinene was present in 75% of the synergistic interactions against *E. faecium*. The combined use of thymoquinone with *H. perforatum* resulted in the most antagonistic interaction against *E. faecium* (ΣFIC value of 12.01), and the compound thymoquinone was present in 83% of the antagonistic interactions against *E. faecium*.

Against *S. aureus*, the combination of thymoquinone with *H. perforatum* resulted in the most synergistic interaction (ΣFIC value of 0.13), and the compound thymoquinone was present in most of the synergistic interactions (46%), displaying synergy when combined with all six carrier oils tested (Table 4). The combination of santalol with *A. vera* displayed the highest antagonistic interaction against *S. aureus* (ΣFIC value of 8.75).

None of the compound: carrier oil combinations displayed synergistic interactions against *K. pneumoniae*, and one combination (santalol with *P. americana*) displayed antagonism (ΣFIC value of 6.00) (Table 5).

Table 3. Antimicrobial activity (MIC in mg/mL and ΣFIC) of the essential oil compound: carrier oil combinations against *Enterococcus faecium* ATCC 27270 (n = 3).

Compounds	Carrier Oils											
	A. vera		*C. officinalis*		*H. perforatum*		*P. americana*		*P. armeniaca*		*S. chinensis*	
	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC
Carvacrol	1.50	1.17	1.50	1.17	**1.00**	0.78	**1.00**	0.88	**1.00**	0.88	**1.00**	0.88
β-Caryophyllene	6.00	0.94	4.00	0.63	6.00	0.94	8.00	2.00	4.00	1.00	4.00	1.00
Cinnamaldehyde	**1.00** [1]	1.03	**1.00**	1.03	**0.83**	0.86	**1.00**	1.13	**1.00**	1.13	**1.00**	1.13
Citral	2.00	0.81	3.00	1.22	2.00	0.81	2.00	1.00	2.00	1.00	2.00	1.00
p-Cymene	16.00	2.90	16.00	2.90	7.00	1.27	4.00	1.10	16.00	*4.40* [3]	4.00	1.10
Eugenol	4.00	2.13	2.00	1.06	2.00	1.06	2.00	1.25	2.00	1.25	2.00	1.25
Geraniol	2.00	0.66	2.00	0.66	2.00	0.66	2.00	0.85	2.00	0.85	2.00	0.85
Isoeugenol	2.00	1.26	2.00	1.26	1.50	0.95	1.50	1.09	2.00	1.45	**1.00**	0.73
R(+)-Limonene	4.00	0.79	4.00	0.79	4.00	0.79	4.00	1.17	4.00	1.17	4.00	1.17
Linalool	4.00	0.73	4.00	0.73	4.00	0.73	4.00	1.10	4.00	1.10	4.00	1.10
Linalyl acetate	16.00	2.50	4.00	0.63	4.00	0.63	4.00	1.00	4.00	1.00	4.00	1.00
Menthol	2.00	***0.44*** [2]	8.00	1.75	4.00	0.88	3.00	0.94	4.00	1.25	2.00	0.63
Nerol	2.00	1.06	2.00	1.06	3.00	1.59	3.00	1.88	2.00	1.25	2.00	1.25
(+)-α-Pinene	4.00	0.79	4.00	0.79	4.00	0.79	4.00	1.17	4.00	1.17	4.00	1.17
Santalol	1.50	3.05	1.50	3.05	**0.50**	1.02	**0.50**	1.06	**0.50**	1.06	**0.50**	1.06
α-Terpinene	4.00	***0.41***	4.00	***0.41***	4.00	***0.41***	8.00	1.57	16.00	3.14	4.00	0.79
γ-Terpinene	16.00	2.90	4.00	0.73	6.00	1.09	4.00	1.10	4.00	1.10	4.00	1.10
(+)-Terpinen-4-ol	4.00	0.63	4.00	0.63	4.00	0.63	4.00	1.00	4.00	1.00	4.00	1.00
α-Terpineol	4.00	1.13	4.00	1.13	4.00	1.13	4.00	1.50	4.00	1.50	4.00	1.50
Thymol	2.00	1.56	2.00	1.56	2.00	1.56	3.00	2.63	2.00	1.75	2.00	1.75
Thymoquinone	**0.08**	*5.00*	**0.13**	*8.00*	**0.19**	*12.01*	**0.13**	*8.02*	**0.03**	2.00	**0.13**	*8.02*
Positive control [3]	1.15		1.25		1.09		1.25		1.15		2.50	
Negative control	>8.00		>8.00		>8.00		>8.00		>8.00		>8.00	
Culture control	>8.00		>8.00		>8.00		>8.00		>8.00		>8.00	

[1] Noteworthy MIC values in bold; [2] synergy in bold and italics; [3] antagonism in italics.

Table 4. Antimicrobial activity (MIC in mg/mL and ΣFIC) of the essential oil compound: carrier oil combinations against *Staphylococcus aureus* ATCC 25923 (n = 3).

Compounds	Carrier Oils											
	A. vera		*C. officinalis*		*H. perforatum*		*P. americana*		*P. armeniaca*		*S. chinensis*	
	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC
Carvacrol	1.33	1.93	1.00	1.25	1.33	1.85	0.38	0.53	0.50	0.70	0.50	0.68
β-Caryophyllene	1.67	0.69	1.33	0.56	4.00	1.42	2.00	0.73	3.00	1.10	2.00	0.67
Cinnamaldehyde	1.33	*5.67* [3]	**1.00**	*4.25*	**1.00**	*4.19*	**0.25**	1.05	**0.25**	1.05	**0.13**	0.52
Citral	3.00	*6.75*	3.33	*7.50*	2.00	*4.37*	**0.50**	1.10	**0.50**	1.10	**0.38**	0.81
p-Cymene	2.00	0.90	8.00	3.60	16.00	*6.20*	2.67	1.07	3.33	1.33	2.67	0.98
Eugenol	2.00	1.30	2.00	1.30	3.33	1.96	**1.00**	0.60	**0.50**	***0.30***	**0.50**	***0.28***
Geraniol	**1.00** [1]	0.65	1.33	0.86	2.00	1.17	**0.75**	***0.45***	**0.75**	***0.45***	**0.75**	***0.43***
Isoeugenol	2.00	3.50	**1.00**	1.75	2.00	3.37	**0.50**	0.85	**0.50**	0.85	**0.50**	0.83
R(+)-Limonene	2.67	1.07	2.67	1.07	2.67	0.90	2.00	0.70	2.67	0.93	4.00	1.27
Linalool	4.00	2.43	4.00	2.43	4.00	2.18	1.50	0.84	2.67	1.49	2.67	1.40
Linalyl acetate	5.33	2.40	4.00	1.80	3.00	1.16	3.33	1.33	2.00	0.80	2.67	0.98
Menthol	4.00	3.00	2.00	1.50	3.00	2.06	**1.00**	0.70	**1.00**	0.70	2.00	1.33
Nerol	1.50	0.94	2.67	1.67	2.67	1.50	2.67	1.53	1.50	0.86	2.67	1.44
(+)-α-Pinene	2.00	1.00	2.00	1.00	3.00	1.31	2.00	0.90	2.00	0.90	2.00	0.83
Santalol	3.00	*8.75*	2.00	*5.83*	**1.00**	2.85	**0.75**	2.15	**1.00**	2.87	**0.50**	1.42
α-Terpinene	4.00	2.00	3.33	1.67	16.00	*7.00*	3.00	1.35	2.00	0.90	2.00	0.83
γ-Terpinene	2.00	0.88	4.00	1.75	8.00	3.00	2.00	0.78	2.00	0.78	3.00	1.06
(+)-Terpinen-4-ol	2.00	0.83	2.00	0.83	2.00	0.71	3.00	1.10	3.00	1.10	2.00	0.67
α-Terpineol	4.00	2.50	4.00	2.50	4.00	2.25	1.50	0.86	**1.00**	0.58	2.00	1.08
Thymol	**1.00**	0.92	**0.75**	0.69	**0.75**	0.64	**0.50**	***0.43***	**0.50**	***0.43***	**0.75**	0.63
Thymoquinone	**0.001**	***0.19*** [2]	**0.003**	***0.38***	**0.001**	***0.13***	**0.003**	***0.38***	**0.001**	***0.19***	**0.002**	***0.25***
Positive control	1.88		1.88		1.88		1.46		1.46		1.46	
Negative control	4.00		>8.00		>8.00		>8.00		>8.00		>8.00	
Culture control	>8.00		>8.00		>8.00		>8.00		>8.00		>8.00	

[1] Noteworthy MIC values in bold; [2] synergy in bold and italics; [3] antagonism in italics.

Table 5. Antimicrobial activity (MIC in mg/mL and ΣFIC) of the essential oil compound: carrier oil combinations against *Klebsiella pneumoniae* ATCC 13883 (n = 3).

Compounds	Carrier Oils											
	A. vera		*C. officinalis*		*H. perforatum*		*P. americana*		*P. armeniaca*		*S. chinensis*	
	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC
Carvacrol	**1.00** [1]	2.25	**0.50**	1.13	**0.75**	1.69	0.75	1.69	**1.00**	2.25	**0.38**	0.84
β-Caryophyllene	2.50	1.25	3.00	1.50	2.00	1.00	2.00	1.00	2.00	1.00	2.33	1.17
Cinnamaldehyde	**0.50**	0.88	**0.50**	0.88	**0.50**	0.88	0.50	0.88	**0.50**	0.88	**0.50**	0.88
Citral	2.00	1.50	2.00	1.50	2.00	1.50	3.00	2.25	2.00	1.50	2.00	1.50
p-Cymene	2.00	1.50	2.00	1.50	2.00	1.50	3.00	2.25	2.00	1.50	2.00	1.50
Eugenol	**1.00**	1.25	**1.00**	1.25	1.50	1.88	**1.00**	1.25	**1.00**	1.25	**1.00**	1.25
Geraniol	**1.00**	0.85	1.50	1.28	1.50	1.28	**1.00**	0.85	**1.00**	0.85	**1.00**	0.85
Isoeugenol	**1.00**	1.05	**1.00**	1.05	**1.00**	1.05	**1.00**	1.05	**1.00**	1.05	**1.00**	1.05
R(+)-Limonene	2.00	0.83	2.00	0.83	2.00	0.83	2.00	0.83	2.00	0.83	2.00	0.83
Linalool	3.00	2.25	1.50	1.13	2.00	1.50	2.00	1.50	2.00	1.50	2.00	1.50
Linalyl acetate	2.00	1.10	2.00	1.10	2.00	1.10	2.00	1.10	2.00	1.10	3.00	1.65
Menthol	2.00	1.10	1.50	0.83	2.00	1.10	2.00	1.10	1.50	0.83	1.50	0.83
Nerol	2.00	1.17	2.00	1.17	3.00	1.75	2.00	1.17	2.00	1.17	**1.00**	0.58
(+)-α-Pinene	2.00	0.83	3.00	1.25	1.50	0.63	2.00	0.83	2.00	0.83	2.00	0.83
Santalol	3.00	1.13	3.00	1.13	2.00	0.75	16.00	*6.00* [2]	2.00	0.75	2.00	0.75
α-Terpinene	2.00	0.83	2.00	0.83	2.00	0.83	2.00	0.83	2.00	0.83	2.00	0.83
γ-Terpinene	2.00	0.77	2.00	0.77	2.00	0.77	2.00	0.77	2.00	0.77	1.50	0.58
(+)-Terpinen-4-ol	2.00	1.17	3.00	1.75	1.50	0.88	2.00	1.17	2.00	1.17	1.50	0.88
α-Terpineol	**1.00**	0.75	**1.00**	0.75	2.00	1.50	2.00	1.50	2.00	1.50	**1.00**	0.75
Thymol	**1.00**	2.25	**1.00**	2.25	**1.00**	2.25	**1.00**	2.25	**0.75**	1.69	**1.00**	2.25
Thymoquinone	**1.00**	0.75	**1.00**	0.75	2.00	1.50	2.00	1.50	4.00	3.00	2.00	1.50
Positive control	0.08		0.08		0.10		0.10		0.10		0.08	
Negative control	>8.00		>8.00		>8.00		>8.00		>8.00		>8.00	
Culture control	>8.00		>8.00		>8.00		>8.00		>8.00		>8.00	

[1] Noteworthy MIC values are shown in bold; [2] antagonism in italics.

Table 6. Antimicrobial activity (MIC in mg/mL and ΣFIC) of the essential oil compound: carrier oil combinations against *Acinetobacter baumannii* ATCC 17606 (n = 3).

Compounds	Carrier Oils											
	A. vera		*C. officinalis*		*H. perforatum*		*P. americana*		*P. armeniaca*		*S. chinensis*	
	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC
Carvacrol	**1.00** [1]	0.75	**1.00**	0.74	2.00	1.48	**1.00**	0.77	1.50	1.28	**1.00**	0.73
β-Caryophyllene	4.00	1.18	16.00	*4.62* [2]	4.00	1.16	6.67	2.11	4.00	1.60	4.00	1.10
Cinnamaldehyde	**0.50**	0.67	**0.75**	1.00	**0.75**	1.00	**1.00**	1.37	**0.75**	1.09	**0.75**	0.99
Citral	2.00	1.29	**1.00**	0.64	1.50	0.96	1.50	1.00	1.50	1.13	2.00	1.25
p-Cymene	4.00	1.18	4.00	1.16	4.00	1.16	16.00	*5.07*	4.00	1.60	4.00	1.10
Eugenol	2.00	1.29	2.00	1.28	2.00	1.28	2.00	1.33	2.00	1.50	2.00	1.25
Geraniol	2.00	1.29	1.50	0.96	2.00	1.28	2.00	1.33	2.00	1.50	2.00	1.25
Isoeugenol	1.50	1.50	2.00	1.99	2.00	1.99	**1.00**	1.02	1.50	1.66	**1.00**	0.98
R(+)-Limonene	4.00	1.08	4.00	1.06	4.00	1.06	4.00	1.17	8.00	3.00	4.00	1.00
Linalool	3.00	1.19	2.00	0.78	4.80	1.87	2.00	0.83	4.00	2.00	6.00	2.25
Linalyl acetate	4.00	1.58	3.00	1.17	4.00	1.56	4.00	1.67	4.00	2.00	4.00	1.50
Menthol	4.00	1.58	2.00	0.78	4.00	1.56	2.00	0.83	3.33	1.67	4.00	1.50
Nerol	2.00	0.96	3.00	1.42	3.00	1.42	4.00	2.00	3.00	1.75	2.00	0.92
(+)-α-Pinene	3.00	0.94	4.00	1.22	4.00	1.22	4.00	1.33	16.00	*6.67*	4.00	1.17
Santalol	**1.00**	0.65	3.00	1.92	4.00	2.56	4.00	2.67	6.00	*4.50*	3.00	1.88
α-Terpinene	4.00	1.58	4.00	1.56	3.00	1.17	16.00	*6.67*	4.00	2.00	4.00	1.50
γ-Terpinene	5.33	1.44	3.33	0.88	4.00	1.06	2.00	0.58	4.00	1.50	4.00	1.00
(+)-Terpinen-4-ol	4.00	1.25	4.00	1.22	4.00	1.22	4.00	1.33	4.00	1.67	4.00	1.17
α-Terpineol	3.00	1.19	4.00	1.56	2.00	0.78	3.33	1.39	3.00	1.50	4.00	1.50
Thymol	2.00	1.29	2.00	1.28	2.00	1.28	2.00	1.33	2.00	1.50	2.00	1.25
Thymoquinone	**0.05**	1.01	**0.03**	0.67	**0.05**	1.01	**0.03**	0.67	**0.03**	0.67	**0.03**	0.67
Positive control	0.53		0.43		0.72		0.72		0.63		0.63	
Negative control	>8.00		>8.00		>8.00		>8.00		>8.00		>8.00	
Culture control	>8.00		>8.00		>8.00		>8.00		>8.00		>8.00	

[1] Noteworthy MIC values in bold; [2] antagonism in italics.

Table 7. Antimicrobial activity (MIC in mg/mL and ΣFIC) of the essential oil compound: carrier oil combinations against *Pseudomonas aeruginosa* ATCC 27853 (n = 3).

Compounds	Carrier Oils											
	A. vera		*C. officinalis*		*H. perforatum*		*P. americana*		*P. armeniaca*		*S. chinensis*	
	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC
Carvacrol	**0.67** [1]	0.86	**1.00**	1.22	**0.50**	0.64	**0.67**	0.80	**0.67**	0.79	**0.67**	0.77
β-Caryophyllene	2.00	0.95	3.00	1.23	2.00	0.95	2.00	0.78	3.00	1.12	3.00	1.01
Cinnamaldehyde	**0.50**	0.81	**0.50**	0.78	**0.67**	1.08	**0.50**	0.77	**0.50**	0.76	**0.50**	0.74
Citral	1.67	0.95	3.00	1.52	3.00	1.71	1.67	0.81	1.67	0.79	1.67	0.73
p-Cymene	16.00	*7.24* [2]	2.67	1.04	2.00	0.90	1.67	0.61	1.67	0.59	4.00	1.27
Eugenol	1.33	1.05	1.33	0.96	1.33	1.05	1.33	0.93	1.33	0.92	1.33	0.87
Geraniol	1.33	0.91	1.67	1.04	1.67	1.14	1.33	0.80	1.83	1.08	1.67	0.92
Isoeugenol	**1.00**	1.19	**0.83**	0.94	**0.83**	1.00	**0.83**	0.92	**0.83**	0.91	**1.00**	1.06
R(+)-Limonene	6.00	3.05	2.67	1.19	1.67	0.85	2.00	0.84	3.00	1.23	1.67	0.62
Linalool	1.67	0.95	4.00	2.03	3.00	1.71	1.67	0.81	3.00	1.42	1.67	0.73
Linalyl acetate	1.67	0.95	2.50	1.27	3.33	1.90	2.00	0.97	1.67	0.79	3.33	1.45
Menthol	3.00	1.61	3.00	1.42	4.00	2.14	2.00	0.90	3.00	1.31	2.00	0.80
Nerol	1.67	1.10	1.67	1.00	1.67	1.10	1.67	0.96	1.67	0.94	1.67	0.88
(+)-α-Pinene	2.00	1.07	2.00	0.94	2.00	1.07	2.00	0.90	2.00	0.87	2.00	0.80
Santalol	4.00	1.64	2.00	0.69	2.00	0.82	3.00	0.98	2.00	0.62	2.00	0.55
α-Terpinene	4.00	1.89	2.00	0.82	2.00	0.95	5.33	2.07	2.00	0.75	3.00	1.01
γ-Terpinene	3.00	1.23	2.00	0.69	8.00	3.29	3.00	0.98	3.00	0.94	4.00	1.10
(+)-Terpinen-4-ol	2.00	1.07	2.00	0.94	2.00	1.07	2.00	0.90	2.00	0.87	4.00	1.60
α-Terpineol	3.00	1.61	2.00	0.94	2.00	1.07	4.00	1.80	2.00	0.87	2.00	0.80
Thymol	1.00	2.29	0.50	1.11	0.50	1.14	0.50	1.10	0.75	1.64	0.75	1.61
Thymoquinone	2.00	1.24	2.00	1.11	2.00	1.24	2.00	1.07	2.00	1.04	2.00	0.97
Positive control	0.53		0.53		0.65		0.65		0.65		0.65	
Negative control	>8.00		3.33		>8.00		>8.00		>8.00		>8.00	
Culture control	>8.00		>8.00		>8.00		>8.00		>8.00		>8.00	

[1] Noteworthy MIC values in bold; [2] antagonism in italics.

Table 8. Antimicrobial activity (MIC in mg/mL and ΣFIC) of the essential oil compound: carrier oil combinations against *Escherichia coli* ATCC 8739 (n = 3).

Compounds	Carrier Oils											
	A. vera		*C. officinalis*		*H. perforatum*		*P. americana*		*P. armeniaca*		*S. chinensis*	
	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC
Carvacrol	1.33	1.29	1.80	1.80	1.33	1.33	1.33	1.33	1.33	1.26	1.33	1.26
β-Caryophyllene	4.00	1.27	4.00	1.40	4.00	1.40	2.00	0.70	4.00	1.17	16.00	*4.69*
Cinnamaldehyde	0.67	1.00	**0.67** [1]	1.02	**0.67**	1.02	**0.83**	1.28	**0.67**	0.98	**0.67**	0.98
Citral	2.00	1.33	2.00	1.40	1.33	0.93	1.67	1.17	2.00	1.29	**1.00**	0.64
p-Cymene	4.00	1.33	4.00	1.47	4.00	1.47	2.67	0.98	4.00	1.24	16.00	*4.95*
Eugenol	2.00	1.33	2.00	1.40	2.67	1.87	2.00	1.40	2.00	1.29	2.00	1.29
Geraniol	2.00	1.17	1.67	1.03	1.67	1.03	2.00	1.23	2.00	1.12	1.67	0.93
Isoeugenol	1.33	0.89	1.33	0.93	1.33	0.93	1.33	0.93	1.67	1.07	**1.00**	0.64
R(+)-Limonene	4.00	1.04	4.00	1.18	4.00	1.18	4.00	1.18	4.00	0.95	4.00	0.95
Linalool	3.00	1.00	2.67	0.98	2.67	0.98	2.50	0.92	4.00	1.24	16.00	*4.95*
Linalyl acetate	4.00	1.33	3.33	1.22	3.33	1.22	16.00	5.87	4.00	1.24	4.00	1.24
Menthol	2.00	0.83	2.00	0.90	3.00	1.35	3.00	1.35	2.00	0.79	12.00	*4.71*
Nerol	2.00	0.93	3.33	1.67	3.33	1.67	2.67	1.33	2.00	0.89	2.00	0.89
(+)-α-Pinene	4.00	1.00	4.00	1.13	4.00	1.13	4.00	1.13	4.00	0.90	4.00	0.90
Santalol	3.00	3.50	4.00	*4.80* [3]	4.00	*4.80*	2.00	2.40	2.00	2.29	2.00	2.29
α-Terpinene	6.00	1.90	3.33	1.17	4.00	1.40	2.00	0.70	16.00	*4.69*	4.00	1.17
γ-Terpinene	4.00	1.17	2.00	0.65	3.33	1.08	3.00	0.98	4.00	1.07	4.00	1.07
(+)-Terpinen-4-ol	4.00	1.47	2.00	0.80	2.67	1.07	4.00	1.60	4.00	1.37	4.00	1.37
α-Terpineol	3.00	1.25	2.00	0.90	3.00	1.35	2.00	0.90	3.00	1.18	3.00	1.18
Thymol	2.00	1.67	1.50	1.30	2.00	1.73	2.00	1.73	1.50	1.21	2.00	1.62
Thymoquinone	**0.02**	***0.09*** [2]	**0.13**	0.51	**0.13**	0.51	**0.09**	***0.38***	**0.13**	***0.50***	**0.09**	***0.37***
Positive control	1.88		0.98		5.23		1.05		1.05		1.29	
Negative control	5.33		>8.00		5.33		5.33		5.33		>8.00	
Culture control	>8.00		>8.00		>8.00		>8.00		>8.00		>8.00	

[1] Noteworthy MIC in bold; [2] synergy in bold and italics; [3] antagonism in italics.

Table 9. Antimicrobial activity (MIC in mg/mL and ΣFIC) of the essential oil compound: carrier oil combinations against *Candida albicans* ATCC 10231 (n = 3).

Compounds	Carrier Oils											
	A. vera		*C. officinalis*		*H. perforatum*		*P. americana*		*P. armeniaca*		*S. chinensis*	
	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC
Carvacrol	**1.00** [1]	2.50	**1.00**	2.50	**0.75**	1.75	**1.00**	2.17	**0.50**	1.13	**0.75**	1.88
β-Caryophyllene	1.50	1.13	2.00	1.50	**1.00**	0.58	2.00	0.83	**1.00**	*0.50*	1.50	1.13
Cinnamaldehyde	**0.13**	2.15	**0.13**	2.15	**0.13**	2.13	**0.13**	2.10	**0.13**	2.11	**0.13**	2.15
Citral	**0.50**	0.75	**0.38**	0.56	**0.50**	0.67	**0.50**	0.58	**0.50**	0.63	**0.50**	0.75
p-Cymene	**1.00**	0.83	**1.00**	0.83	**0.75**	***0.50*** [2]	2.00	1.00	2.00	1.17	**1.00**	0.83
Eugenol	**1.00**	1.83	**0.75**	1.38	**0.50**	0.83	**0.50**	0.75	**0.50**	0.79	**0.50**	0.92
Geraniol	**0.75**	1.88	**1.00**	2.50	**0.50**	1.17	**0.50**	1.08	**0.50**	1.13	**0.50**	1.25
Isoeugenol	**1.00**	2.50	**0.50**	1.25	**0.50**	1.17	**0.50**	1.08	**0.50**	1.13	**0.50**	1.25
R(+)-Limonene	**1.00**	1.00	**1.00**	1.00	**1.00**	0.83	2.00	1.33	2.00	1.50	1.50	1.50
Linalool	**1.00**	1.00	**1.00**	1.00	3.00	2.50	**1.00**	0.67	1.50	1.13	**1.00**	1.00
Linalyl acetate	**1.00**	1.00	**1.00**	1.00	3.00	2.50	3.33	2.22	2.00	1.50	2.00	2.00
Menthol	**1.00**	1.50	**1.00**	1.50	2.00	2.67	**1.00**	1.17	**1.00**	1.25	**1.00**	1.50
Nerol	**1.00**	1.50	**1.00**	1.50	**1.00**	1.33	**1.00**	1.17	**1.00**	1.25	**1.00**	1.50
(+)-α-Pinene	**1.00**	1.00	**1.00**	1.00	2.00	1.67	1.50	1.00	1.50	1.13	**1.00**	1.00
Santalol	1.50	1.50	**1.00**	1.00	2.00	1.67	**1.00**	0.67	2.00	1.50	**1.00**	1.00
α-Terpinene	**1.00**	1.00	1.50	1.50	1.50	1.25	3.00	2.00	**1.00**	0.75	**1.00**	1.00
γ-Terpinene	3.00	2.25	**1.00**	0.75	**1.00**	0.58	3.00	1.25	3.00	1.50	**1.00**	0.75
(+)-Terpinen-4-ol	1.25	0.94	**1.00**	0.75	2.00	1.17	2.00	0.83	2.00	1.00	**1.00**	0.75
α-Terpineol	**1.00**	1.17	**1.00**	1.17	1.50	1.50	1.50	1.25	**1.00**	0.92	**1.00**	1.17
Thymol	**0.50**	0.75	**0.50**	0.75	**1.00**	1.33	**1.00**	1.17	**0.75**	0.94	**0.50**	0.75
Thymoquinone	**0.13**	0.86	**0.25**	1.73	**0.19**	1.26	**0.13**	0.82	**0.13**	0.83	**0.13**	0.90
Positive control	12.50		12.50		10.00		0.94		0.94		6.25	
Negative control	>8.00		>8.00		2.00		>8.00		>8.00		2.00	
Culture control	>8.00		>8.00		>8.00		>8.00		>8.00		>8.00	

[1] Noteworthy MIC values in bold; [2] synergy in bold and italics.

None of the compound: carrier oil combinations resulted in synergy against *A. baumannii* (Table 6). Antagonism was apparent for combinations of α-terpinene with *P. americana*, and (+)-α-pinene with *P. armeniaca* (ΣFIC values of 6.67). The carrier oils *P. americana* and *P. armeniaca* were present most often in antagonistic interactions (40%).

None of the compound: carrier oil interactions displayed synergistic interactions against *P. aeruginosa* (Table 7). The combination of *p*-cymene with *A. vera* was the only combination which resulted in antagonism (ΣFIC value of 7.24).

The combination demonstrating the most synergy against *E. coli* was thymoquinone and *A. vera* (ΣFIC value of 0.09) (Table 8). Thymoquinone was present in all of the synergistic interactions observed. The combinations which showed the most antagonism was *p*-cymene combined with *S. chinensis* and linalool combined with *S. chinensis* (ΣFIC values of 4.95). The compound santalol (29%) and carrier oil *S. chinensis* (57%) were present most often in antagonistic interactions.

The combination of *p*-cymene with *H. perforatum* and β-caryophyllene with *P. armeniaca* resulted in synergistic interactions against *C. albicans* (ΣFIC values of 0.50) (Table 9). None of the combinations resulted in antagonism.

In summary (Figure 1), the compound thymoquinone and the carrier oil *P. armeniaca* were present in the majority of the synergistic combinations. The carrier oil *H. perforatum* and the compound santalol were present most frequently in the antagonistic combinations.

Of the four compounds (carvacrol, cinnamaldehyde, isoeugenol, and thymol) which showed broad-spectrum, noteworthy antimicrobial activity against all reference strains tested, only thymol produced some synergistic antimicrobial activity when combined with carrier oils. This suggests that noteworthy antimicrobial activity of a compound by itself does not necessarily correlate to synergy when combined with carrier oils. In fact, the compounds cinnamaldehyde, citral, santalol, and thymoquinone, which showed noteworthy MIC values when tested alone, were present in several antagonistic combinations when combined with carrier oils. Thymoquinone and santalol in particular were present repeat-

edly in antagonistic combinations against more than one reference strain. The influence of the carrier oil on the antimicrobial activity of a compound differed according to the reference strain tested.

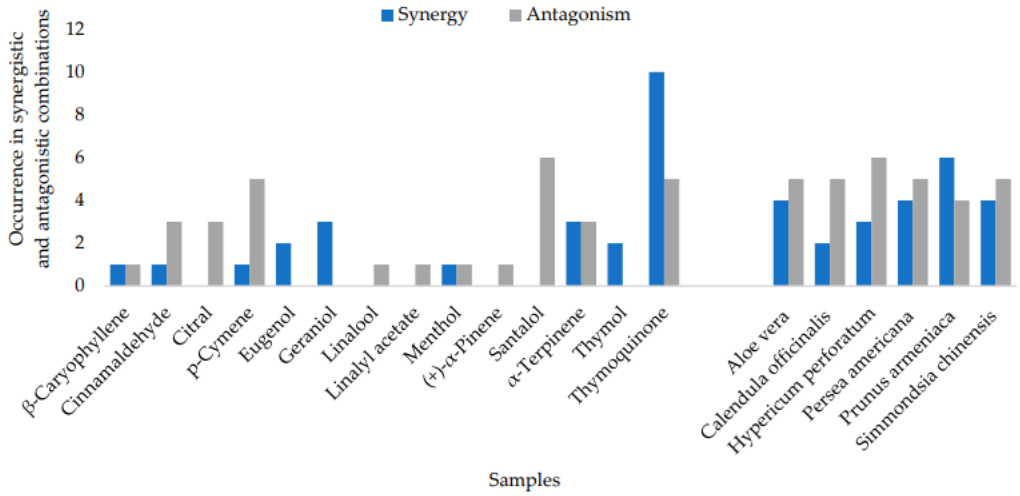

Figure 1. Summary of compounds and carrier oils occurring in synergistic and antagonistic interactions.

When observing the interactive profiles of the compound: carrier oil combinations against the various reference strains tested, it was noted that the combinations tested against the Gram-positive bacteria displayed the highest synergy (6%) as well as the highest antagonism (6%). The combinations tested against the Gram-negative bacteria displayed the least synergy (1%), and combinations tested against the yeast reference strain displayed the second highest synergy (2%) and the least antagonism (0%). Thymoquinone was the compound most commonly observed in the synergistic antimicrobial interactions.

In another study [31] where essential oils were combined with carrier oils against skin pathogens, most of the synergistic interactions also occurred against the Gram-positive bacteria. The enhanced susceptibility of the Gram-positive bacteria may be due to the susceptibility of the outer membrane where the structure is less complex than that of the Gram-negative micro-organisms. The structure consists of a membrane weaker than that of the Gram-negative bacteria and consists only of a thick peptidoglycan wall which is not adequate to prevent the entry of antimicrobial compounds [50,51].

The antimicrobial enhancing properties of the carrier oils present in the synergistic combinations could be attributed to their free fatty acids [31]. Free fatty acids, such as oleic and linoleic acid, have been reported to show antimicrobial activity against the Gram-positive micro-organism *S. aureus* when tested at high concentrations [52]. The antimicrobial activity of free fatty acids could be attributed to their ability to cause cell lysis, disruption to nutrient uptake, inhibition of enzyme activity, and formation of auto-oxidation products, as well as their ability to alter pH levels, thus causing disturbance to the bacterial membrane [53,54].

In a previous study [31], combinations of essential oils with the carrier oils *A. vera*, *H. perforatum*, *P. americana*, *P. armeniaca* and *S. chinensis* resulted in synergy against *C. albicans*. In this study, combinations of two compounds with *H. perforatum* and *P. armeniaca* resulted in synergy. The two compounds which showed synergy against *C. albicans* were present as major compounds in the essential oils *Kunzea ericoides* A.Rich. Joy Tomps. (kanuka) and *Lavandula angustifolia* Mill. (lavender) which also resulted in synergy with the carrier oils [31]. This demonstrates that there were instances where the synergy observed for a single compound: carrier oil combination correlated with the synergistic interaction observed by the neat essential oil: carrier oil combination. As an example, *K. ericoides* essential oil, containing *p*-cymene (11.9%) as a major compound, resulted in synergy when

combined with *A. vera*, and *p*-cymene combined with *A. vera* resulted in synergy in this study. This was also observed with *L. angustifolia* essential oil, containing linalyl acetate (35.6%), which resulted in synergy when combined with *C. officinalis;* and in this study, the combination of linalyl acetate with *C. officinalis* also resulted in synergy.

Very few synergistic interactions were observed against the Gram-negative bacteria previously [31], and in this study. The combinations which did result in synergy against Gram-negative bacteria were thymoquinone combined with *A. vera, P. americana, P. armeniaca,* and *S. chinensis.* Antagonism was seen most frequently against the Gram-negative bacteria by the compound santalol and the carrier oils *P. americana* and *S. chinensis.*

2.2. Toxicity Analysis

All six carrier oils tested were non-toxic at both 24 and 48 h (Table 10). The least toxic of the carrier oils was *P. americana*. These results are congruent with a previous carrier oil study [31].

Table 10. Toxicity (% mortality) and standard deviation (SD) at 24 and 48 h.

Sample	% Mortality (±SD)	
	24 h	48 h
A. vera	**1.13 (±1.60)** [1]	**3.10 (±0.70)**
C. officinalis	**1.14 (±0.81)**	**1.14 (±0.81)**
H. perforatum	**0.69 (±0.98)**	**4.78 (±1.24)**
P. americana	**0.00 (±0.00)**	**0.95 (±1.35)**
P. armeniaca	**1.44 (±1.34)**	**3.38 (±1.67)**
S. chinensis	**2.27 (±3.21)**	**15.29 (±15.09)**
Carvacrol	100.00 (±0.00)	100.00 (±0.00)
β-Caryophyllene	**19.52 (±0.88)**	**41.65 (±1.66)**
Cinnamaldehyde	100.00 (±0.00)	100.00 (±0.00)
Citral	100.00 (±0.00)	100.00 (±0.00)
p-Cymene	**18.93 (±1.63)**	**38.24 (±7.42)**
Eugenol	100.00 (±0.00)	100.00 (±0.00)
Geraniol	100.00 (±0.00)	100.00 (±0.00)
Isoeugenol	100.00 (±0.00)	100.00 (±0.00)
R(+)-Limonene	**48.84 (±15.25)**	74.60 (±29.73)
Linalool	100.00 (±0.00)	100.00 (±0.00)
Linalyl acetate	**22.31 (±3.87)**	**27.73 (±3.08)**
Menthol	100.00 (±0.00)	100.00 (±0.00)
Nerol	100.00 (±0.00)	100.00 (±0.00)
(+)-α-Pinene	85.32 (±9.99)	94.70 (±3.53)
Santalol	100.00 (±0.00)	100.00 (±0.00)
α-Terpinene	57.19 (±7.30)	65.75 (±6.31)
γ-Terpinene	**9.86 (±11.23)**	**26.52 (±9.59)**
(+)-Terpinen-4-ol	100.00 (±0.00)	100.00 (±0.00)
α-Terpineol	100.00 (±0.00)	100.00 (±0.00)
Thymol	100.00 (±0.00)	100.00 (±0.00)
Thymoquinone	100.00 (±0.00)	100.00 (±0.00)
Potassium dichromate (positive control)	100.00 (±0.00)	100.00 (±0.00)
2% DMSO (negative control)	**0.41 (±0.57)**	**1.69 (±1.60)**
20% Diluted acetone (negative control) [2]	**1.85 (±0.41)**	**7.53 (±1.60)**
50% Diluted acetone (negative control)	**1.90(±0.19)**	**15.04(±3.97)**
Salt water (negative control)	**3.45 (±2.53)**	**6.51 (±1.22)**

[1] Bold values represent biological non-toxicity; shaded area shows carrier oils and non-shaded area shows compounds. [2] Although acetone is known as a toxic agent to brine shrimp, it was the only solvent that allowed dilution of several insoluble compounds; thus, diluted acetone was used and included as a negative control.

At 24 h, 24% of the compounds showed non-toxic results and 19% of the compounds showed non-toxic results at 48 h. At both 24 and 48 h, the compounds β-caryophyllene, p-cymene, linalyl acetate, and γ-terpinene were non-toxic, and R (+)-limonene was non-toxic only at 24 h. The compounds p-cymene (at 24 h), linalyl acetate, and γ-limonene showed

non-toxicity in previous studies [55–57]. The other compounds (76% and 81%) showed toxicity to the brine shrimp either at 24 h or, at both 24 and 48 h, showing the highly toxic nature of the compounds by themselves, even when diluted to a concentration of 1.00 mg/mL.

The compounds carvacrol, citral, eugenol, cinnamaldehyde, geraniol, linalool, menthol, nerol, α-pinene, santalol, γ-terpinene, terpinene-4-ol, thymol, and thymoquinone have previously demonstrated various biological toxicities [58–69]. This study, together with the literature, shows that the essential oil compounds tested are predominantly toxic, and unless a means of decreasing their toxicity is found, their application for humans is limited.

Combinations

After combining the 21 compounds with all six carrier oils (Tables 11–16), it was found that in several instances the toxicity of the compounds was reduced. At 24 h, the combinations containing *C. officinalis*, *H. perforatum*, and *P. armeniaca* resulted in the most reduction in compound toxicity, and at 48 h, *H. perforatum* resulted in the most reduction in compound toxicity. The carrier oil *H. perforatum* would therefore be a suitable option to be combined with compounds tested for the purpose of reducing toxicity.

Table 11. Mean toxicity (% mortality), standard deviation (SD), ΣFPM (fractional percentage mortality index), and interpretation of essential oil compound: *Aloe vera* combinations (n = 3).

Essential Oil Compound	*Aloe vera*							
	24 h				48 h			
	% Mortality (±SD)	Incr/Decr Toxicity [1]	ΣFIC	Int [2]	% Mortality (±SD)	Incr/Decr Toxicity	ΣFIC	Int
Carvacrol	100.00 (±0.00)	Equal	*44.75*	Ant	100.00 (±0.00)	Equal	*16.63*	Ant
β-Caryophyllene	**3.85 (±3.23)** [3]	5-fold decr	1.80	Ind	**38.17 (±9.09)**	1-fold decr	*6.61*	Ant
Cinnamaldehyde	100.00 (±0.00)	Equal	*44.75*	Ant	100.00 (±0.00)	Equal	*16.63*	Ant
Citral	100.00 (±0.00)	Equal	*44.75*	Ant	100.00 (±0.00)	Equal	*16.63*	Ant
p-Cymene	**0.61 (±0.86)**	31-fold decr	***0.28***	**Syn**	**6.90 (±6.73)**	6-fold decr	1.20	Ind
Eugenol	100.00 (±0.00)	Equal	*44.75*	Ant	100.00 (±0.00)	Equal	*16.63*	Ant
Geraniol	100.00(±0.00)	Equal	*44.75*	Ant	100.00 (±0.00)	Equal	*16.63*	Ant
Isoeugenol	**38.96 (±10.70)**	3-fold decr	*17.44*	Ant	75.88 (±13.85)	1-fold decr	*12.62*	Ant
R(+)-Limonene	81.25 (±9.56)	2-fold incr	*36.78*	Ant	92.25 (±1.51)	1-fold incr	*15.5*	Ant
Linalool	63.17 (±5.09)	2-fold decr	*28.27*	Ant	90.32 (±1.78)	1-fold decr	*15.02*	Ant
Linalyl acetate	**1.67 (±2.36)**	13-fold decr	0.77	Add	**4.01 (±1.81)**	7-fold decr	0.72	Add
Menthol	100.00 (±0.00)	Equal	*44.75*	Ant	100.00 (±0.00)	Equal	*16.63*	Ant
Nerol	94.62 (±2.57)	1-fold decr	*42.34*	Ant	99.42 (±0.83)	1-fold decr	*16.53*	Ant
(+)-α-Pinene	95.88 (±0.37)	1-fold incr	*42.99*	Ant	97.85 (±1.70)	1-fold incr	*16.30*	Ant
Santalol	**18.74 (±10.75)**	5-fold decr	*8.39*	Ant	69.29 (±11.64)	1-fold decr	*11.52*	Ant
α-Terpinene	**0.81 (±1.15)**	71-fold decr	***0.37***	**Syn**	**2.88 (±2.09)**	23-fold decr	***0.49***	**Syn**
γ-Terpinene	100.00 (±0.00)	10-fold incr	*49.32*	Ant	100.00 (±0.00)	4-fold incr	*18.01*	Ant
(+)-Terpinen-4-ol	90.38 (±6.46)	1-fold decr	*40.44*	Ant	96.33 (±3.81)	1-fold decr	*16.02*	Ant
α-Terpineol	100.00 (±0.00)	Equal	*44.75*	Ant	100.00 (±0.00)	Equal	*16.63*	Ant
Thymol	100.00 (±0.00)	Equal	*44.75*	Ant	100.00 (±0.00)	Equal	*16.63*	Ant
Thymoquinone	100.00 (±0.00)	Equal	*44.75*	Ant	100.00 (±0.00)	Equal	*16.63*	Ant

[1] Incr/decr toxicity (increase/decrease toxicity)—the increase or decrease in toxicity of the compound from when tested alone to when combined with the carrier oil. [2] Int (interpretation)—the interpretation of ΣFIC values, whether antagonistic (Ant) (italics), synergistic (Syn) (bold and italics), additive (Add), or indifferent (Ind). [3] Bold values represent biological non-toxicity.

The combination of *p*-cymene with *A. vera* resulted in the most favorable synergistic interaction at 24 h (ΣFIC value of 0.28) (Table 11), and α-terpinene when combined with *A. vera* resulted in the only synergistic interaction observed at 48 h. The highest antagonistic ΣFIC values at 24 and 48 h resulted from the combination of γ-terpinene with *A. vera* (ΣFIC values of 49.32 and 18.01 respectively).

At 24 h, when R (+)-limonene and γ-terpinene were combined with *C. officinalis*, a complete reduction in toxicity was observed (Table 12). γ-Terpinene combined with *C. officinalis* resulted in the only synergistic interaction observed at 48 h (ΣFIC value of 0.25). Several compound: carrier oil combinations resulted in the most antagonistic interactions observed at both 24 and 48 h (ΣFIC value of 44.36).

Table 12. Mean toxicity (% mortality), standard deviation (SD), ΣFPM, and interpretation of essential oil compound: *Calendula officinalis* combinations (n = 3).

Essential Oil Compound	*Calendula officinalis*							
	24 h				48 h			
	% Mortality (±SD)	Incr/Decr Toxicity [1]	ΣFIC	Int [2]	% Mortality (±SD)	Incr/Decr Toxicity	ΣFIC	Int
Carvacrol	100.00 (±0.00)	Equal	*44.36*	Ant	100.00 (±0.00)	Equal	*44.36*	Ant
β-Caryophyllene	**5.70 (±5.22)** [3]	3-fold decr	2.65	Ind	**40.49 (±22.73)**	1-fold decr	*18.25*	Ant
Cinnamaldehyde	100.00 (±0.00)	Equal	*44.36*	Ant	100.00 (±0.00)	Equal	*44.36*	Ant
Citral	100.00 (±0.00)	Equal	*44.36*	Ant	100.00 (±0.00)	Equal	*44.36*	Ant
p-Cymene	**37.12 (±2.59)**	2-fold incr	*17.26*	Ant	83.69 (±14.40)	2-fold incr	*37.80*	Ant
Eugenol	100.00 (±0.00)	Equal	*44.36*	Ant	100.00 (±0.00)	Equal	*44.36*	Ant
Geraniol	100.00(±0.00)	Equal	*44.36*	Ant	100.00 (±0.00)	Equal	*44.36*	Ant
Isoeugenol	**21.40 (±6.96)**	5-fold decr	*9.49*	Ant	89.86 (±13.05)	1-fold decr	*39.86*	Ant
R(+)-Limonene	**0.00 (±0.00)**	Complete decr	**NV** [4]	**Syn**	**8.15 (±3.98)**	9-fold decr	3.63	Ind
Linalool	**6.66 (±1.39)**	15-fold decr	2.95	Ind	70.46 (±17.83)	1-fold decr	*31.26*	Ant
Linalyl acetate	**0.69 (±0.50)**	32-fold decr	***0.32***	**Syn**	**8.34 (±1.76)**	3-fold decr	3.81	Ind
Menthol	100.00 (±0.00)	Equal	*44.36*	Ant	100.00 (±0.00)	Equal	*44.36*	Ant
Nerol	95.25 (±0.94)	1-fold decr	*42.25*	Ant	100.00 (±0.00)	Equal	*44.36*	Ant
(+)-α-Pinene	**4.36 (±3.11)**	20-fold decr	1.94	Ind	79.14 (±9.01)	1-fold decr	*35.13*	Ant
Santalol	78.13 (±0.50)	1-fold decr	*34.66*	Ant	99.61 (±0.55)	1-fold decr	*44.19*	Ant
α-Terpinene	**24.58 (±5.37)**	2-fold decr	*11.00*	Ant	65.62 (±8.55)	1-fold decr	*29.28*	Ant
γ-Terpinene	**0.00 (±0.00)**	Complete decr	**NV**	**Syn**	**0.56 (±0.79)**	47-fold decr	***0.25***	**Syn**
(+)-Terpinen-4-ol	**0.48 (±0.67)**	208-fold decr	***0.21***	**Syn**	**15.26 (±3.89)**	7-fold decr	*6.77*	Ant
α-Terpineol	100.00 (±0.00)	Equal	*44.36*	Ant	100.00 (±0.00)	Equal	*44.36*	Ant
Thymol	100.00 (±0.00)	Equal	*44.36*	Ant	100.00 (±0.00)	Equal	*44.36*	Ant
Thymoquinone	100.00 (±0.00)	Equal	*44.36*	Ant	100.00 (±0.00)	Equal	*44.36*	Ant

[1] Incr/decr toxicity (increase/decrease toxicity)—the increase or decrease in toxicity of the compound from when tested alone to when combined with the carrier oil. [2] Int (interpretation)—the interpretation of ΣFIC values, whether antagonistic (Ant) (italics), synergistic (Syn) (bold and italics), additive (Add), or indifferent (Ind). [3] Bold values represent biological non-toxicity. [4] NV—no value could be calculated due to the carrier oil's toxicity being 0.00%.

Table 13. Mean toxicity (% mortality), standard deviation (SD), ΣFPM, and interpretation of essential oil compound: *Hypericum perforatum* combinations (n = 3).

Essential Oil Compound	*Hypericum perforatum*							
	24 h				48 h			
	% Mortality (±SD)	Incr/Decr Toxicity [1]	ΣFIC	Int [2]	% Mortality (±SD)	Incr/Decr Toxicity	ΣFIC	Int
Carvacrol	100.00 (±0.00)	Equal	*72.96*	Ant	100.00 (±0.00)	Equal	*10.96*	Ant
β-Caryophyllene	**4.57 (±1.55)** [3]	4-fold decr	3.43	Ind	**25.18 (±3.52)**	2-fold decr	2.94	Ind
Cinnamaldehyde	100.00 (±0.00)	Equal	*72.96*	Ant	100.00 (±0.00)	Equal	*10.96*	Ant
Citral	100.00 (±0.00	Equal	*72.96*	Ant	100.00 (±0.00)	Equal	*10.96*	Ant
p-Cymene	**22.42 (±9.18)**	1-fold incr	*16.84*	Ant	61.16 (±18.45)	2-fold incr	*7.20*	Ant
Eugenol	100.00 (±0.00)	Equal	*72.96*	Ant	100.00 (±0.00)	Equal	*10.96*	Ant
Geraniol	100.00 (±0.00)	Equal	*72.96*	Ant	100.00 (±0.00)	Equal	*10.96*	Ant
Isoeugenol	**46.84 (±20.84)**	2-fold decr	*34.17*	Ant	91.62 (±4.65)	1-fold decr	*10.04*	Ant
R (+)-Limonene	**3.09 (±3.73)**	16-fold decr	2.27	Ind	**4.04 (±3.41)**	18-fold decr	***0.45***	**Syn**
Linalool	**18.59 (±6.49)**	5-fold decr	*13.57*	Ant	86.17 (±3.85)	1-fold decr	*9.44*	Ant
Linalyl acetate	**1.79 (±1.75)**	12-fold decr	1.34	Ind	**7.12 (±3.64)**	4-fold decr	0.87	Add
Menthol	100.00 (±0.00)	Equal	*72.96*	Ant	100.00 (±0.00)	Equal	*10.96*	Ant
Nerol	96.48 (±1.63)	1-fold decr	*70.4*	Ant	100.00 (±0.00)	Equal	*10.96*	Ant
(+)-α-Pinene	71.13 (±1.77)	1-fold decr	*51.96*	Ant	98.66 (±0.14)	1-fold incr	*10.84*	Ant
Santalol	**0.63 (±0.89)**	159-fold decr	***0.46***	**Syn**	**27.44 (±5.18)**	4-fold decr	3.01	Ind
α-Terpinene	**11.15 (±4.76)**	5-fold decr	*8.18*	Ant	**33.33 (±8.26)**	2-fold decr	3.74	Ind
γ-Terpinene	**0.68 (±0.96)**	15-fold decr	0.53	Add	**3.42 (±1.07)**	8-fold decr	***0.42***	**Syn**
(+)-Terpinen-4-ol	**1.98 (±1.67)**	51-fold decr	1.44	Ind	**13.49 (±2.97)**	7-fold decr	1.48	Ind
α-Terpineol	100.00 (±0.00)	Equal	*72.96*	Ant	100.00 (±0.00)	Equal	*10.96*	Ant
Thymol	100.00 (±0.00)	Equal	*72.96*	Ant	100.00 (±0.00)	Equal	*10.96*	Ant
Thymoquinone	100.00 (±0.00)	Equal	*72.96*	Ant	100.00 (±0.00)	Equal	*10.96*	Ant

[1] Incr/decr toxicity (increase/decrease toxicity)—the increase or decrease in toxicity of the compound from when tested alone to when combined with the carrier oil. [2] Int (interpretation)—the interpretation of ΣFIC values, whether antagonistic (Ant) (italics), synergistic (Syn) (bold and italics), additive (Add), or indifferent (Ind). [3] Bold values represent biological non-toxicity.

Table 14. Mean toxicity (% mortality), standard deviation (SD), ΣFPM, and interpretation of essential oil compound: *Persea americana* combinations (n = 3).

Essential Oil Compound	*Persea americana*							
	24 h				48 h			
	% Mortality (±SD)	Incr/Decr Toxicity [1]	ΣFIC	Int [2]	% Mortality (±SD)	Incr/Decr Toxicity	ΣFIC	Int
Carvacrol	100.00 (±0.00)	Equal	- [4]	-	100.00 (±0.00)	Equal	*53.13*	Ant
β-Caryophyllene	**10.96 (±9.23)** [3]	2-fold decr	-	-	50.12 (±14.10)	1-fold incr	*26.98*	Ant
Cinnamaldehyde	100.00 (±0.00)	Equal	-	-	100.00 (±0.00)	Equal	*53.13*	Ant
Citral	100.00 (±0.00)	Equal	-	-	100.00 (±0.00)	Equal	*53.13*	Ant
p-Cymene	**37.79 (±5.23)**	2-fold incr	-	-	**48.19 (±1.08)**	1-fold incr	*25.99*	Ant
Eugenol	100.00 (±0.00)	Equal	-	-	100.00 (±0.00)	Equal	*53.13*	Ant
Geraniol	100.00 (±0.00)	Equal	-	-	100.00 (±0.00)	Equal	*53.13*	Ant
Isoeugenol	**47.69 (±21.04)**	2-fold decr	-	-	82.80 (±10.15)	1-fold decr	*44.00*	Ant
R(+)-Limonene	**48.76 (±4.70)**	1-fold decr	-	-	66.84 (±7.72)	1-fold decr	*35.63*	Ant
Linalool	**17.20 (±10.04)**	6-fold decr	-	-	89.16 (±4.60)	1-fold decr	*47.37*	Ant
Linalyl acetate	**6.40 (±2.73)**	3-fold decr	-	-	**22.59 (±7.30)**	1-fold decr	*12.30*	Ant
Menthol	100.00 (±0.00)	Equal	-	-	100.00 (±0.00)	Equal	*53.13*	Ant
Nerol	100.00 (±0.00)	Equal	-	-	100.00 (±0.00)	Equal	*53.13*	Ant
(+)-α-Pinene	79.20 (±5.30)	1-fold decr	-	-	98.37 (±1.36)	1-fold incr	*52.29*	Ant
Santalol	**1.38 (±1.12)**	72-fold decr	-	-	**2.78 (±1.97)**	36-fold decr	1.48	Ind
α-Terpinene	**3.12 (±3.46)**	18-fold decr	-	-	62.95 (±18.31)	1-fold decr	*33.61*	Ant
γ-Terpinene	100.00 (±0.00)	10-fold incr	-	-	100.00 (±0.00)	4-fold incr	*54.52*	Ant
(+)-Terpinen-4-ol	**3.69 (±2.50)**	27-fold decr	-	-	**48.41 (±7.13)**	2-fold decr	*25.72*	Ant
α-Terpineol	100.00 (±0.00)	Equal	-	-	100.00 (±0.00)	Equal	*53.13*	Ant
Thymol	100.00 (±0.00)	Equal	-	-	100.00 (±0.00)	Equal	*53.13*	Ant
Thymoquinone	100.00 (±0.00)	Equal	-	-	100.00 (±0.00)	Equal	*53.13*	Ant

[1] Incr/decr toxicity (increase/decrease toxicity)—the increase or decrease in toxicity of the compound from when tested alone to when combined with the carrier oil. [2] Int (interpretation)—the interpretation of ΣFIC values, whether antagonistic (Ant) (italics), synergistic (Syn) (bold and italics), additive (Add), or indifferent (Ind). [3] Bold values represent biological non-toxicity. [4] Value could not be calculated due to the carrier oil's toxicity being 0.00%.

Table 15. Mean toxicity (% mortality), standard deviation (SD), ΣFPM, and interpretation of essential oil compound: *Prunus armeniaca* combinations (n = 3).

Essential Oil Compound	*Prunus armeniaca*							
	24 h				48 h			
	% Mortality (±SD)	Incr/Decr Toxicity [1]	ΣFIC	Int [2]	% Mortality (± SD)	Incr/Decr Toxicity	ΣFIC	Int
Carvacrol	100.00 (±0.00)	Equal	*35.22*	Ant	100.00 (±0.00)	Equal	*15.29*	Ant
β-Caryophyllene	**13.56 (±11.70)** [3]	1-fold decr	*5.06*	Ant	**42.37 (±20.55)**	1-fold incr	*6.78*	Ant
Cinnamaldehyde	100.00 (±0.00)	Equal	*35.22*	Ant	100.00 (±0.00)	Equal	*15.29*	Ant
Citral	100.00 (±0.00)	Equal	*35.22*	Ant	100.00 (±0.00)	Equal	*15.29*	Ant
p-Cymene	52.07 (±11.54)	3-fold incr	*19.46*	Ant	65.88 (±8.12)	2-fold incr	*10.61*	Ant
Eugenol	100.00 (±0.00)	Equal	*35.22*	Ant	100.00 (±0.00)	Equal	*15.29*	Ant
Geraniol	100.00 (±0.00)	Equal	*35.22*	Ant	100.00 (±0.00)	Equal	*15.29*	Ant
Isoeugenol	**9.31 (±8.72)**	11-fold decr	3.28	Ind	58.40 (±5.45)	2-fold decr	*8.93*	Ant
R(+)-Limonene	**0.55 (±0.77)**	89-fold decr	***0.20***	**Syn**	**1.26 (±0.91)**	59-fold decr	***0.19***	**Syn**
Linalool	**26.64 (±3.99)**	4-fold decr	*9.38*	Ant	90.97 (±5.88)	1-fold decr	*13.91*	Ant
Linalyl acetate	**0.78 (±1.11)**	29-fold decr	***0.29***	**Syn**	**1.99 (±1.99)**	14-fold decr	***0.33***	**Syn**
Menthol	100.00 (±0.00)	Equal	*35.22*	Ant	100.00 (±0.00)	Equal	*15.29*	Ant
Nerol	100.00 (±0.00)	Equal	*35.22*	Ant	100.00 (±0.00)	Equal	*15.29*	Ant
(+)-α-Pinene	**32.97 (±17.92)**	3-fold decr	*11.64*	Ant	68.70 (±20.45)	1-fold decr	*10.53*	Ant
Santalol	**0.00 (±0.00)**	Complete decr	**NV** [4]	**Syn**	**1.26 (±0.89)**	79-fold decr	***0.19***	**Syn**
α-Terpinene	**5.98 (±3.09)**	10-fold decr	2.13	Ind	71.18 (±18.94)	1-fold incr	*11.07*	Ant
γ-Terpinene	**5.64 (±2.83)**	2-fold decr	2.25	Ind	**20.61 (±1.75)**	1-fold decr	3.44	Ind
(+)-Terpinen-4-ol	**7.51 (±3.83)**	13-fold decr	2.64	Ind	65.88 (±4.68)	2-fold decr	*10.08*	Ant
α-Terpineol	100.00 (±0.00)	Equal	*35.22*	Ant	100.00 (±0.00)	Equal	*15.29*	Ant
Thymol	100.00 (±0.00)	Equal	*35.22*	Ant	100.00 (±0.00)	Equal	*15.29*	Ant
Thymoquinone	100.00 (±0.00)	Equal	*35.22*	Ant	100.00 (±0.00)	Equal	*15.29*	Ant

[1] Incr/decr toxicity (increase/decrease toxicity)—the increase or decrease in toxicity of the compound from when tested alone to when combined with the carrier oil. [2] Int (interpretation)—the interpretation of ΣFIC values, whether antagonistic (Ant) (italics), synergistic (Syn) (bold and italics), additive (Add), or indifferent (Ind). [3] Bold values represent biological non-toxicity. [4] NV—no value could be calculated as the % mortality of the combination was 0.00.

Table 16. Mean toxicity (% mortality), standard deviation (SD), ΣFPM, and interpretation of essential oil compound: *Simmondsia chinensis* combinations (n = 3).

Essential Oil Compound	*Simmondsia chinensis*							
	24 h				48 h			
	% Mortality (±SD)	Incr/Decr Toxicity [1]	ΣFIC	Int [2]	% Mortality (±SD)	Incr/Decr Toxicity	ΣFIC	Int
Carvacrol	100.00 (±0.00)	Equal	*22.53*	Ant	100.00 (±0.00)	Equal	3.77	Ind
β-Caryophyllene	**16.26 (±4.95)** [3]	1-fold decr	*4.00*	Ant	68.47 (±5.74)	2-fold incr	3.06	Ind
Cinnamaldehyde	100.00 (±0.00)	Equal	*22.53*	Ant	100.00 (±0.00)	Equal	3.77	Ind
Citral	100.00 (±0.00)	Equal	*22.53*	Ant	100.00 (±0.00)	Equal	3.77	Ind
p-Cymene	86.56 (±2.28)	5-fold incr	*21.35*	Ant	92.48 (±3.14)	2-fold incr	*4.23*	Ant
Eugenol	100.00 (±0.00)	Equal	*22.53*	Ant	100.00 (±0.00)	Equal	3.77	Ind
Geraniol	100.00 (±0.00)	Equal	*22.53*	Ant	100.00 (±0.00)	Equal	3.77	Ind
Isoeugenol	70.84 (±17.88)	1-fold decr	*15.96*	Ant	96.16 (±2.83)	1-fold decr	3.63	Ind
R(+)-Limonene	67.02 (±24.02)	1-fold incr	*15.45*	Ant	85.67 (±13.21)	1-fold incr	3.38	Ind
Linalool	100.00 (±0.00)	Equal	*22.53*	Ant	100.00 (±0.00)	Equal	3.77	Ind
Linalyl acetate	88.41 (±16.40)	4-fold incr	*21.45*	Ant	96.38 (±5.12)	3-fold incr	*4.89*	Ant
Menthol	85.71 (±5.73)	1-fold decr	*19.31*	Ant	100.00 (±0.00)	Equal	3.77	Ind
Nerol	100.00 (±0.00)	Equal	*22.53*	Ant	100.00 (±0.00)	Equal	3.77	Ind
(+)-α-Pinene	100.00 (±0.00)	1-fold incr	*22.61*	Ant	100.00 (±0.00)	1-fold incr	3.80	Ind
Santalol	100.00 (±0.00)	Equal	*22.53*	Ant	100.00 (±0.00)	Equal	3.77	Ind
α-Terpinene	72.51 (±14.26)	1-fold incr	*16.61*	Ant	87.89 (±10.59)	1-fold incr	3.54	Ind
γ-Terpinene	71.27 (±9.04)	7-fold incr	*19.31*	Ant	79.51 (±5.71)	3-fold incr	*4.10*	Ant
(+)-Terpinen-4-ol	98.92 (±1.52)	1-fold decr	*22.28*	Ant	100.00 (±0.00)	Equal	3.77	Ind
α-Terpineol	100.00 (±0.00)	Equal	*22.53*	Ant	100.00 (±0.00)	Equal	3.77	Ind
Thymol	100.00 (±0.00)	Equal	*22.53*	Ant	100.00 (±0.00)	Equal	3.77	Ind
Thymoquinone	100.00 (±0.00)	Equal	*22.53*	Ant	100.00 (±0.00)	Equal	3.77	Ind

[1] Incr/decr toxicity (increase/decrease toxicity)—the increase or decrease in toxicity of the compound from when tested alone to when combined with the carrier oil. [2] Int (interpretation)—the interpretation of ΣFIC values, whether antagonistic (Ant) (italics), synergistic (Syn) (bold and italics), additive (Add), or indifferent (Ind). [3] Bold values represent biological non-toxicity.

The combined use of santalol and *H. perforatum* was the only synergistic interaction at 24 h, and at 48 h γ-terpinene and *H. perforatum* resulted in the most synergistic interaction (ΣFIC value of 0.42) (Table 13). Several compound: carrier oil combinations resulted in antagonistic ΣFIC values at 24 and 48 h.

The toxicity of *P. americana* alone at 24 h was 0.00% and so ΣFIC values at 24 h could not be calculated; however, it could be noted that the toxicity of the compounds isoeugenol, linalool, santalol, α-terpinene, and (+)-terpinen-4-ol reduced from toxic levels to non-toxic levels when combined with *P. americana* at 24 h. None of the compound: carrier oil combinations displayed synergy at 48 h (Table 14). The combination of γ-terpinene and *H. perforatum* was the most antagonistic (ΣFIC value of 54.52).

At 24 h, the combination of santalol and *P. armeniaca* resulted in a complete decrease in toxicity, followed by R (+)-limonene and *P. armeniaca* which resulted in the second most synergistic interaction with an ΣFIC value of 0.20. At 48 h, santalol or R (+)-limonene combined with *P. armeniaca* resulted in the most synergistic interactions (ΣFIC values of 0.19). At 24 and 48 h, several combinations were antagonistic (Table 15).

All of the compounds with *S. chinensis* resulted in antagonism at 24 h (Table 16). Less antagonism was observed at 48 h, where the most antagonistic ΣFIC value resulted from linalyl acetate combined with *S. chinensis* (ΣFIC value of 4.89).

Table 17 provides a summary of the toxicity percentage of the interactions of each carrier oil in combination with the essential oil compounds. Synergy indicates that the carrier oil was able to quench the toxicity of the essential oil compounds, rendering it non-toxic. The carrier oil *P. armeniaca* resulted in the most synergy in its respective combinations with the compounds at 48 h. A constituent of *P. armeniaca*, vitamin E [31], may be the contributing factor to the carrier oil's favorable toxicity quenching abilities as it was previously reported that vitamin E was able to reduce the toxic effect of the medicine digoxin in rabbits [32] and acute mercury toxicity in rats [70].

Table 17. Interactions (%) of each carrier oil within its respective compound–carrier oil combinations.

Carrier Oil	24 h				48 h			
	% Syn [1]	% Ant [2]	% Ind [3]	% Add [4]	% Syn	% Ant	% Ind	% Add
A. vera	10	80	5	5	5	85	5	5
C. officinalis	19	67	14	0	5	85	10	0
H. perforatum	5	71	19	5	10	66	19	5
P. americana	-	-	-	-	0	95	5	0
P. armeniaca	14	72	19	0	14	81	5	0
S. chinensis	0	100	0	0	0	14	86	0

[1] % synergy; [2] % antagonism; [3] % indifference; [4] % additive.

At 24 h, the carrier oil *S. chinensis* resulted in the most antagonism within its combinations, and *C. officinalis* (responsible for majority of the synergistic interactions) showed the least antagonism. Therefore, at 24 h, *C. officinalis* would be the most favorable carrier oil choice to be combined with the compounds used in this study to reduce their toxicity. At 48 h, the carrier oil *P. americana* was responsible for the majority of the antagonistic interactions, and *S. chinensis* showed the least.

The compounds that most commonly quenched toxicity and therefore resulted in synergistic interactions when combined with the carrier oils at both 24 and 48 h were α-terpinene; linalyl acetate; γ-terpinene; R (+)-limonene; and santalol. The compound R (+)-limonene quenched toxicity the most.

To the best of our knowledge, to date there have been no previous studies conducted on the toxicity of the combined use of essential oil compounds with carrier oils; however, there has been a study on the combined use of essential oils with the same carrier oils as carried out in this study [31]. The synergy was consistent for several of the compounds and essential oils across the two studies. This could be observed for the synergistic combination of *p*-cymene with *A. vera*. At 24 h, the essential oils *K. ericoides* and *Melaleuca alternifolia* Cheel (tea tree), containing the compound *p*-cymene (11.9% and 9.6%, respectively), showed synergy when combined with *A. vera* [31]. This could suggest a correlation between the synergistic activity seen with the essential oil: carrier oil combinations and the synergistic activity seen with the essential oil compound: carrier oil combinations.

The previous study also found the carrier oils *A. vera* and *S. chinensis* to reduce the toxicity of the essential oils at 24 h and *A. vera* and *P. armeniaca* to cause the most reduction in toxicity at 48 h. *Aloe vera* was present in most synergistic essential oil–carrier oil combinations over 24 and 48 h [31]. Some differences in the results between this study and the previous one suggests that the toxicity patterns shown by the combined use of carrier oils and essential oils cannot always be generalized to predict which carrier oil would be most advantageous in decreasing the toxicity of the compounds. The essential oil *L. angustifolia*, containing linalyl acetate (35.6%), linalool (32.8%), and β-caryophyllene (10.2%) as its major compounds, resulted in synergy when combined with *C. officinalis* [31], and in this study, linalyl acetate resulted in synergy with *C. officinalis* whereas β-caryophyllene and linalool did not. This observed difference may also be due to the mixture of compounds in the neat essential oil reacting differently when compared to examining combinations with single compounds.

2.3. Selectivity Index

The selectivity index for all the combinations which showed antimicrobial synergy was calculated (Table 18). Various interpretations exist, however, this study considers a selectivity index of >4 as being acceptable, when the antimicrobial benefit is not lost due to the toxicity [71]. A selectivity index below four indicates that the toxicity of the compound: carrier oil combination is too high and the antimicrobial activity is most likely attributed to the toxicity of the sample and not the interaction [71]. Of the 23 synergistic combinations, 10 at 24 h and 9 at 48 h had SI values of >4, with thymoquinone being the main compound present in these combinations.

Table 18. Selectivity index (SI) of synergistic combinations found in the antimicrobial studies.

Carrier Oil	Compound	SI at 24 h	SI at 48 h
	E. faecium		
A. vera	Menthol	0	0
A. vera	α-Terpinene	**15** [1]	4
C. officinalis	α-Terpinene	1	0
H. perforatum	α-Terpinene	1	0
	S. aureus		
A. vera	Thymoquinone	**500**	**500**
C. officinalis	Thymoquinone	**167**	**167**
H. perforatum	Thymoquinone	**500**	**500**
P. americana	Geraniol	1	1
P. americana	Thymol	1	1
P. americana	Thymoquinone	**167**	**167**
P. armeniaca	Eugenol	1	1
P. armeniaca	Geraniol	1	1
P. armeniaca	Thymol	1	1
P. armeniaca	Thymoquinone	**500**	**500**
S. chinensis	Eugenol	1	1
S. chinensis	Geraniol	1	1
S. chinensis	Thymoquinone	**250**	**250**
	E. coli		
A. vera	Thymoquinone	**25**	**25**
P. americana	Thymoquinone	**6**	**6**
P. armeniaca	Thymoquinone	4	4
S. chinensis	Thymoquinone	**6**	**6**
	C. albicans		
H. perforatum	p-Cymene	3	1
P. armeniaca	β-Caryophyllene	4	1

[1] Bold values represent acceptable SI values.

3. Materials and Methods

3.1. Sample Selection and Preparation

A selection of 21 essential oil compounds (Sigma-Aldrich, Johannesburg, South Africa) were selected based on their previously reported noteworthy antimicrobial activity [2,4,31,72–76]. All carrier oils were obtained from Escentia (Johannesburg, South Africa) and Scatters Oils (Johannesburg, South Africa) and consisted of *Aloe vera* (Aloe vera); *Calendula officinalis* (Calendula); *Hypericum perforatum* (St John's wort); *Persea americana* (Avocado); *Prunus armeniaca* (Apricot kernel); and *Simmondsia chinensis* (Jojoba). The carrier oil selection was based on their frequent use in aromatherapy and relevance to application on the skin.

3.2. Culture Preparation

The micro-organisms selected for this study included the ESKAPE pathogens and one yeast pathogen. Selection was based on their importance in contributing towards antimicrobial resistance [2,77]. The investigated bacteria included *Enterococcus faecium* (ATCC 27270), *Staphylococcus aureus* (ATCC 25923), *Klebsiella pneumoniae* (ATCC 13883), *Acinetobacter baumannii* (ATCC 17606), *Pseudomonas aeruginosa* (ATCC 27858), and *Escherichia coli* (ATCC 8739). The pathogen reference strain *Candida albicans* (ATCC 10231) was selected as a yeast representative. The micro-organisms were cultured in Tryptone Soya broth (TSB) (Oxoid), and Tryptone Soya agar (TSA) and were incubated at 37 °C for 24 h (bacteria) and at 37 °C for 48 h (yeast). The purity of the micro-organisms was confirmed by streaking each culture onto an agar plate and ensuring growth of single colonies, as well as checking colony morphology with visual standards within the microbiology laboratory.

3.3. Sample Preparation

For the broth microdilution assay, the samples were diluted to a concentration of 32.00 mg/mL in acetone. For the brine shrimp lethality assay, all selected samples were prepared in 2% dimethyl sulfoxide (DMSO) or 20–50% acetone at a concentration of 2.00 mg/mL depending on solubility.

3.4. Antimicrobial Analysis

The broth microdilution method using a 96-well microtiter plate, as described in a previous study [5], was used to quantify the inhibitory activity of the compounds and carrier oils. Preparation of the microtiter plates involved the aseptic addition of 100.00 µL of TSB into each of the wells of the microtiter plate. The samples were then added, at a volume of 100.00 µL, to the first row of the plate. When testing the combinations, a modification was made where 50.00 µL of the compound and 50.00 µL of the carrier oil were placed in the first row of wells (to make up 100 µL of sample) of the plate. A volume of 100.00 µL of a positive, negative, and culture control were included for each strain studied. The positive control (0.01 mg/mL ciprofloxacin for bacteria or 0.1 mg/mL nystatin for yeast) was used to ensure microbial susceptibility. The negative control (32.00 mg/mL water in acetone) was included to rule out whether the antimicrobial activity was attributed to the solvent. A culture control in TSB was included to ensure the broth supported growth of the reference strains. The samples were then serially diluted down the rows in concentrations of 8.00; 4.00; 2.00; 1.00; 0.50; 0.25; 0.13; and 0.06 mg/mL. After the preparation of an approximate inoculum concentration of 1×10^6 colony-forming units (CFU)/mL for each reference strain, 100.00 µL was added to each of the wells. A sterile adhesive sealing film was used to seal the microtiter plate to prevent loss of the samples through evaporation. Incubation of the microtiter plates occurred at 37 °C for 24 h for bacteria and 37 °C for 48 h for the yeast. A volume of 40.00 µL of *p*-iodonitrotetrazolium violet solution (INT) (Sigma-Aldrich), at a concentration of 0.04 mg/mL, was then added to each well after incubation. The lowest concentration with no colour change was taken as the minimum inhibitory concentration (MIC) for that sample. All samples were tested in triplicate. The average of the samples was calculated and the standard deviation (SD) determined using Microsoft Excel (Microsoft Office Home and Student 2016). Results were considered noteworthy if the MIC value was $\leq$1.00 mg/mL [5].

3.5. Toxicity Studies

The brine shrimp lethality assay [78] was used to determine the toxicity of 21 compounds and six carrier oils alone and in combination. Artificial seawater was prepared by dissolving 16.00 g of Tropic Marine® sea salt in 500.00 mL of distilled water. This solution was transferred into a bottomless, inverted receptacle. Dried brine shrimp (*Artemia franciscana*) eggs, from Ocean Nutrition™, were added to the salt water. Aeration of the water with a rotary pump was included to ensure a high brine shrimp hatch rate. A constant source of light and warmth, from a 220 to 240 V lamp, was used to assist with the hatching process. The eggs were incubated at 25 °C for 24–48 h. For the assay, a 48-well microtiter plate was prepared by adding 400.00 µL of salt water containing 40–60 live brine shrimp to each well. A volume of 400.00 µL of sample was added to each well. For the combinations, a 1:1 ratio of 200 µL each of each sample (carrier oil: compound) was prepared prior to being added to the well containing the shrimp. The assay included a negative, non-toxic control of 32.00 g/L of artificial seawater to ensure the promotion of growth and survival of the brine shrimp. The positive control in the assay consisted of 1.60 mg/mL of potassium dichromate, a highly toxic compound. At 0, 24 and 48 h, the dead brine shrimp were viewed and counted under a light microscope (Olympus) at 40× magnification. A lethal dose of acetic acid (Saarchem; 100% (*v/v*); 50.00 µL) was added to each well and a final count of dead brine shrimp taken [79]. Then, the percentage mortality was calculated using Equation (1). Biological toxicity was considered for a percentage mortality of 50% or greater [80].

All studies were carried out in triplicate. The average percentage mortality of the brine shrimp was recorded on Microsoft Excel (Microsoft Office Home and Student 2016).

$$\% \text{ Mortality} = \frac{\text{Dead shrimp at } \frac{24}{48}\text{h (before acetic acid)} - \text{Dead shrimp (time} = 0)}{\text{Dead shrimp (after acetic acid)} \times 100} \quad (1)$$

3.6. Interactive Profiles of Combinations

The interactive profiles of the combinations for the antimicrobial and toxicity assays were undertaken, and the fractional inhibitory concentration index (ΣFIC antimicrobial) or the fractional percentage mortality (ΣFPM toxicity) was calculated, respectively, according to Equation (2).

The (a*) represents the essential oil compound in combination and (b*) represents the carrier oil.

$$\begin{gathered} \text{FIC or FPM (i)} = \frac{\text{(a*)combined with (b*)}}{\text{(a) independently}} \\ \text{FIC or FPM (ii)} = \frac{\text{(b) combined with (a)}}{\text{(b) independently}} \\ \Sigma\text{FIC} = \text{FIC (i)} + \text{FIC (ii)} \text{ or } \Sigma\text{FPM} = \text{FPM (i)} + \text{FPM (ii)} \end{gathered} \quad (2)$$

The interactive profile was interpreted as follows: an ΣFIC or ΣFPM value of $\leq$0.5 represented synergy, >0.5–1.0 indicated additive interactions, >1.0–$\leq$4.0 demonstrated indifference, and a value > 4.0 indicated antagonism [81]. For antimicrobial studies, a synergistic combination is regarded as having increased antimicrobial activity and an antagonistic combination is regarded as having decreased antimicrobial activity. Where MIC values of >8.00 mg/mL were determined, they were recorded as 16.00 mg/mL for the purpose of calculating an ΣFIC value. For toxicity studies, synergy is due to a decrease in toxicity of the compounds.

3.7. Selectivity Index (SI)

The selectivity index indicates the ratio of toxicity to antimicrobial activity of a sample and was calculated using Equation (3).

$$\text{SI} = \frac{\text{LC}_{50}}{\text{MIC}} \quad (3)$$

4. Conclusions

This study investigated the antimicrobial and toxicity effects of carrier oils in combination with essential oil compounds. When looking at the antimicrobial activity of 882 combinations, 3% of combinations were synergistic and 4% were antagonistic. The compound thymoquinone and the carrier oil *P. armeniaca* were present in the majority of the antimicrobial synergistic combinations, and the compound santalol and carrier oil *H. perforatum* were found in the majority of the antagonistic combinations.

When investigating the toxicity interactions of 105 combinations at 24 h, 10% of the combinations were synergistic, and 77% were antagonistic. When investigating the toxicity of 126 combinations at 48 h, 6% of the combinations were synergistic and 71% were antagonistic. These antagonistic interactions warrant caution when combining equal ratios of compound to carrier oil. The carrier oil *C. officinalis* was present in the majority of the antagonistic toxicity combinations at 24 h, and the carrier oil *P. armeniaca* was present in the majority of the synergistic toxicity combinations at 48 h. The selectivity index demonstrated thymoquinone to be the most favorable compound in combination with carrier oils because it was present in the majority of combinations that had an SI value of >4.

Future studies investigating varying ratios may provide a more optimal toxicity profile. It may also be beneficial to investigate the various constituents of the carrier oils themselves, such as the separate free fatty acids and the vitamins, to determine their influence on the essential oil compound toxicity and antimicrobial activity. Nonetheless, this study provides

valuable insight into the antimicrobial and toxicity effects of carrier oils when combined with essential oil compounds.

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Chapter 4 - Additional studies: Time-kill assay

4.1 Introduction

The compound-carrier oil combination which showed synergy in both the antimicrobial and toxicity assays was further investigated using the time-kill assay against the Gram-positive micro-organism, *E. faecium* (ATCC 27270). The reference strain *E. faecium* was used as the combination of *A. vera* and α-terpinene showed a synergistic interaction against *E. faecium*, as observed in the antimicrobial studies (**Chapter 3**). The aim was to determine whether the combination had bacteriostatic or cidal effects over time.

4.2 Materials and method

A time-kill method conducted in a previous study [270] was adapted and used as depicted in **Figure 4.1**. The assay was performed using 48-well microtiter plates (Thermo Fisher Scientific, Denmark). A volume of 100.00 µl of 0.90% sodium chloride (NaCl) (Merck, Modderfontein) solution was added into the wells of the first row of the plate. The NaCl solution was prepared using water as a solvent. After the preparation of an approximate inoculum concentration of $1x10^6$ colony forming units (CFU)/ml for the reference strain (*E. faecium*), 100.00 µl was added to the first row of wells. A volume of 100.00 µl of the compound, carrier oil or combination thereof was also added to the first row of wells. The first row of wells therefore contained a total volume of 300.00 µl (NaCl solution, culture, and compound/carrier oil/combination). The remaining rows were filled with 900.00 µl of the 0.90% NaCl solution. Each well of the first row was then mixed, and a volume of 100.00 µl of each well of the first row was transferred into the well below it which contained the 0.90% NaCl solution. This was performed for each column from wells A to F. Thereafter, aliquots of 50.00 µl from wells B to F of every column of the plate were transferred onto a Tryptone Soya agar (TSA) plate and spread using a sterile spreader (time 0 hrs). The agar spread plates were incubated at 37°C for 24 hrs. The microtiter plate was sealed to prevent evaporation and incubated at 37°C for 6 hrs. The 6-hr incubation period covers the initial growth kinetics of the pathogen. The microtiter plate was removed from the incubator every hour for 6 hrs where subsequent aliquots of 50.00 µl from wells B to F of every column of the plate were spread onto agar plates and incubated. After the 24-hour incubation of all spread plates, the number of colonies formed on each plate was counted and

recorded in Microsoft Excel (Microsoft Office Home and Student 2016). The logarithm of CFU/ml present in the original well (well A) was calculated using **Equation 4.1**.

$$\text{Log CFU/ml} = \text{Log}_{10}(\text{CFU} \times 0.05 \times 10^{\wedge *}) \qquad \textbf{Equation 4.1}$$

*1 if counting colonies of well B; 2, if counting colonies of well C; 3, if counting colonies of well D; 4, if counting colonies of well E; 5, if counting colonies of well F.

A positive antibiotic control, ciprofloxacin, was used at a concentration of 0.1 mg/ml to ensure microbial susceptibility. A culture control was also used to ensure growth of the reference strain. All tests were conducted in triplicate. A graph of log CFU/ml was plotted against time. Bactericidal activity is depicted by a line which reaches the base point of the graph, whereas bacteriostatic activity is depicted by a line which continues straight on without reaching the basepoint of the graph. Synergy, indifferent, and additive values will always be dependent on the sample which is not in combination and thus cannot be used for comparison.

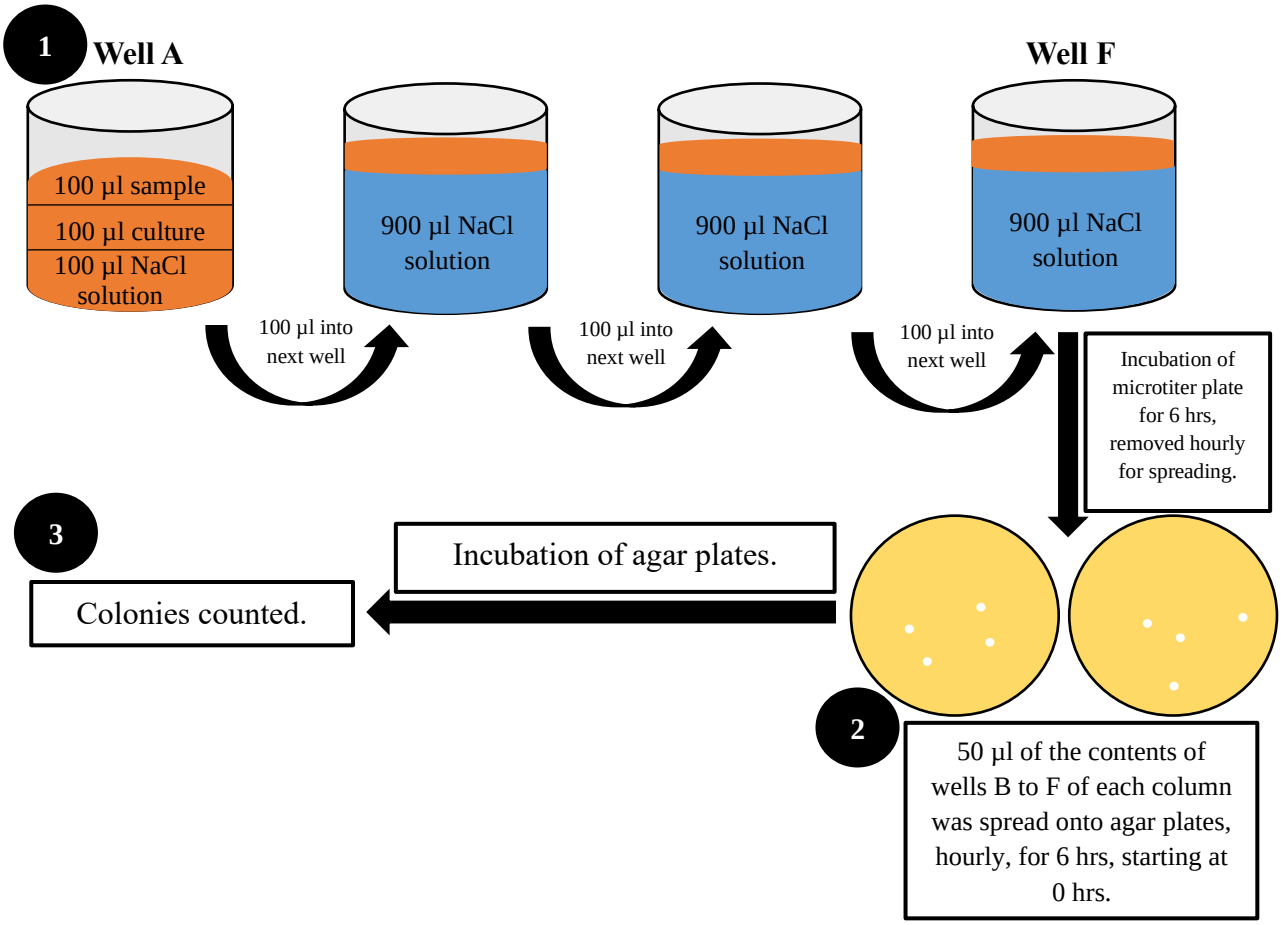

Figure 4:1: Time-kill assay methodology

4.3 Results and discussion

After conducting the time-kill assay on the combination of *A. vera* with α-terpinene (**Figure 4.2**), it was observed that the antimicrobial activity of the carrier oil, *A. vera*, was steady over time, without causing any reduction in colony forming units. This was expected due to the carrier oil's poor antimicrobial activity seen in this study (**Chapter 3**) as well as in a previous study [35]. The combination of *A. vera* with α-terpinene resulted in the growth of 6.55% less colony forming units than the compound α-terpinene, demonstrating little improvement over time. A noteworthy observation is that both the combination and compound showed better bacteriostatic activity than the antibiotic control, ciprofloxacin, thus showing superior antimicrobial activity. The positive control, ciprofloxacin, resulted in bacteriostatic over time. The culture control showed increased growth of the reference strain *E. faecium*, over time, as expected.

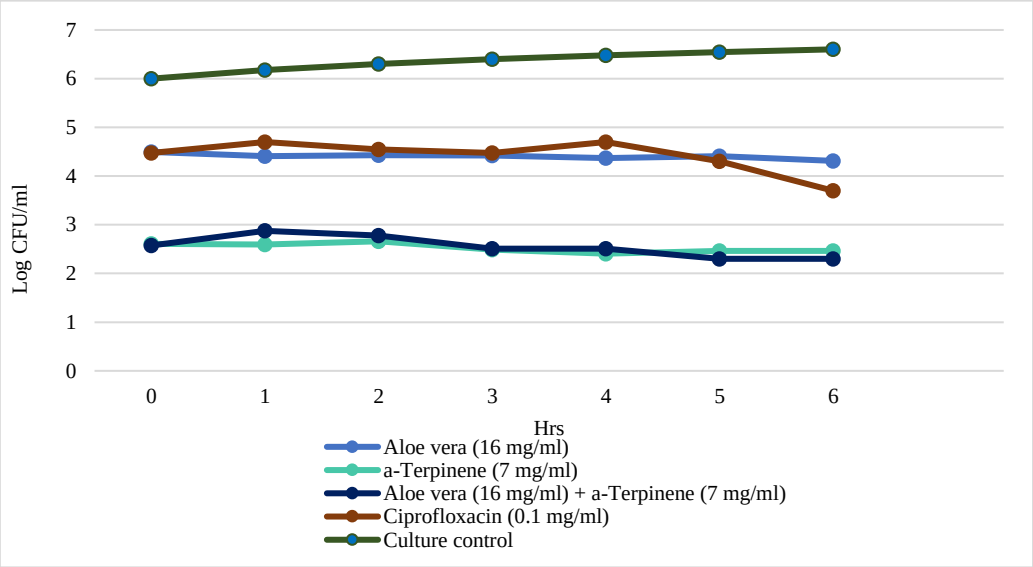

Figure 4:2: Time-kill assay of *Aloe vera* + α-terpinene against *E. faecium*

Previous studies were found on the time-kill activity of essential oils which contain α-terpinene as a constituent as well the time-kill activity of other compounds [252, 260, 262], however, none were found on the time-kill effects of the compound α-terpinene, alone, or in combination with carrier oils.

Chapter 5 - Conclusion

This study aimed to investigate the antimicrobial and toxicity effects of carrier oils in combination with essential oil compounds.

5.1 Study highlights

Objectives one to four were successfully completed within the publication (**Chapter 3**). Objective five was investigated and discussed in the bridging chapter (**Chapter 4**).

After the investigation of 21 compounds and six carrier oils alone, and 882 compound: carrier oil: pathogen reference strain combinations within the antimicrobial studies, the following highlights were observed;

- While the carrier oils tested did not display any noteworthy antimicrobial activity as expected, the carrier oils *A. vera, C. officinalis* and *S. chinensis* showed noteworthy (1.00 mg/ml) activity against *C. albicans*, a yeast reference strain.

- The compounds carvacrol, cinnamaldehyde, isoeugenol, and thymol showed noteworthy MIC values (0.50 mg/ml – 0.83 mg/ml) against all reference strains tested, indicating their broad-spectrum antimicrobial activity.

- When investigating the antimicrobial combination studies, indifferent interactions of the compound: carrier oil combinations predominated in this study (56%), whereas additive (37%) antagonistic (4%) and synergistic (3%) interactions were observed less frequently.

- The compound: carrier oil combinations tested against the Gram-positive bacteria displayed the highest amount of synergy (6%).

- The compound thymoquinone and the carrier oil *P. armeniaca* were present in the majority of the synergistic combinations. The use of *P. armeniaca* in essential oil therapy where thymoquinone is the major compound would therefore be favourable.

- The compound: carrier oil combinations tested against the Gram-positive bacteria displayed the highest amount of antagonism (6%) and combinations tested against the yeast pathogen resulted in no antagonism.

- The carrier oil *H. perforatum* and the compound santalol were present most often in antagonistic combinations, cautioning against the use of *H. perforatum* and essential oils containing a large proportion of santalol, within aromatherapy.

After the investigation of 21 compounds and six carrier oils alone, and 126 compound: carrier oils combinations within the toxicity studies, the following highlights were observed;

- The brine-shrimp lethality assay showed that all six of the carrier oils were non-toxic.

- At 24 and 48 hrs, 76% and 81% of the essential oil compounds were toxic.

- At both 24 and 48 hrs, the compounds β-caryophyllene, *p*-cymene, linalyl acetate, and y-terpinene were non-toxic, and R (+)-limonene was non-toxic only at 24 hrs.

- The compound y-terpinene was the least toxic at both 24 and 48 hrs.

- From the compound tested, 67% showed 100% toxicity at both 24 and 48 hrs.

- The toxicity of several compounds such as R (+)-limonene, santalol and γ- terpinene, was reduced to non-toxic levels when combined with carrier oils.

- Overall, at 48 hrs, 6% of compound: carrier oil combinations resulted in synergy. Antagonism was predominant (71%).

- At 48 hrs, combinations of compounds with *P. armeniaca* resulted in the most amount of synergy.

- Combinations of compounds with *P. americana* resulted in the most amount of antagonism.

- Combinations with *P. americana* and *S. chinensis* resulted in the least amount of synergy.

- Combinations of compounds with *Simmondsia chinensis* resulted in the least amount of antagonism.

- The carrier oil *P. armeniaca* would be a favourable choice to combine with compounds.

- For use in aromatherapy, the carrier oil *P. armeniaca* would be a favourable choice to combine with essential oil compounds, whereas *P. americana* would not be.

When considering the relationship between toxicity and antimicrobial activity;

- The compound thymoquinone was present in majority (> 50%) of the combinations which had an SI value > 4, showing that the antimicrobial activity of the compound: carrier oil combination was not due to toxicity.

- The combinations with an SI value < 4 show that the toxicity of the combinations was too toxic and that the combination's antimicrobial activity was most likely due to the toxicity.

When considering the time-kill assay;

- The carrier oil, *A. vera*, did not reduce the growth of CFU's over time.

- The combination of *A. vera* and α-terpinene reduced the growth of CFU's slightly more than the compound, α-terpinene.

- The combination and compound showed bacteriostatic activity over 6 hrs, greater than that of the antibiotic control, ciprofloxacin.

5.2 Limitations and future considerations

This study investigated the antimicrobial activity and toxicity of only a selected number of compounds and carrier oils. Future research should include the compounds showing antimicrobial activity such as benzyl acetate [52], 1,8-cineole [271], and β-citronellol [272]; and other carrier oils such as *H. annuus* (sunflower seed oil) and *Vitis vinifera* (grapeseed oil) [33] that were not investigated in this study and which are present in commonly used cosmetic products.

The ΣFIC value was calculated to determine the interaction between the essential oil compounds and carrier oils. In some instances, the ΣFIC value could not be calculated due to the sample having no toxicity and was thus represented as 0.00%. This did not allow for the interaction type to be determined. Future studies could consider the feasibility of samples prepared at higher starting concentrations. Lower concentrations were more favourable in the current study in order to achieve the lowest toxicity and most noteworthy antimicrobial activity of the compound: carrier oil combinations.

Further exploration of varied ratios of the carrier oils with the compounds is also recommended as this study only focused on the 1:1 ratio. In the practice of aromatherapy, the essential oil is always diluted in a carrier oil before applied to the skin [273, 274]. A much greater proportion of carrier oil is mixed with the essential oil, where the essential oil should make up 1-3% of the essential oil: carrier oil mix [266, 273, 275, 276], therefore, the compound: carrier oil combinations should be further investigated using these ratios.

The antimicrobial investigation of the highlighted synergistic combinations (**Table 5.1**) should be further explored against clinical strains. Anti-quorum sensing, and antibiofilm studies could also be included to allow for an in-depth investigation of the contribution these combinations may make to fighting antimicrobial resistance [277].

Table 5.1: Synergistic compound: carrier oil combinations observed in the antimicrobial and toxicity studies

Antimicrobial studies	
Micro-organism	**Synergistic compound: carrier oil combinations**
E. faecium	Menthol + *A. vera*
	α-Terpinene + *A. vera*
	α-Terpinene + *C. officinalis*
	α-Terpinene + *H. perforatum*
S. aureus	Eugenol + *P. armeniaca*
	Eugenol + *S. chinensis*
	Geraniol + *P. americana*
	Geraniol + *P. armeniaca*
	Geraniol + *S. chinensis*
	Thymol + *P. americana*
	Thymol + *P. armeniaca*
	Thymoquinone + *A. vera*
	Thymoquinone + *C. officinalis*
	Thymoquinone + *H. perforatum*
	Thymoquinone + *P. americana*
	Thymoquinone + *P. armeniaca*
	Thymoquinone + *S. chinensis*
E. coli	Thymoquinone + *A. vera*
	Thymoquinone + *P. americana*
	Thymoquinone + *P. armeniaca*
	Thymoquinone + *S. chinensis*
C. albicans	β-caryophyllene + *P. armeniaca*
	p-Cymene + *H. perforatum*
Toxicity studies	
Carrier oil	**Compound combined with carrier oil**
A. vera	α-Terpinene

Carrier oil	Compound combined with carrier oil
C. officinalis	γ-Terpinene
H. perforatum	R (+)-Limonene
	γ-Terpinene
P. armeniaca	R (+)-Limonene
	Linalyl acetate
	Santalol

Further studies investigating the constituents of carrier oils, such as the vitamins and free fatty acids [38], in combination with the essential oil compounds, should be considered. This will determine which constituents are responsible for the positive effects of quenching toxicity and thus add to enhancing of the antimicrobial activity of the compounds.

This study did not include a mechanism of action component and this should be further explored as a previous study [278] states the antimicrobial mechanism of action differs according to the type of essential oil used. A previous study [76] investigated the correlation between acaricidal activity and the chemical structure of compounds. These studies could be further extended to investigate whether the mechanisms change according to the class of compound. Furthermore, the combination can be compared to when tested independently in terms of changes in mechanistic activity. Proteomics studies could be investigated to understand the mechanism of action driving the antimicrobial activity of essential oil compounds [279], as well as pharmacokinetic and pharmacodynamic studies on the compounds.

Permeability studies should be considered. It is known that the antimicrobial contribution of the essential oil compounds is often linked to their membrane permeability [109, 280]. A study [281] identified that the log P value of the compounds is an indicator of whether a compound will be absorbed by plant, human, or animal tissue (due to lipophilicity), or whether it will be easily distributed by water (hydrophilic) [281]. A positive log P value indicates that a compound is lipophilic and will be absorbed into tissue [282]. Compounds which have a log P value of ≤ 5 have good membrane permeability [280], as well as cause membrane disintegration, resulting in cell death [109]. A summary of the log P values of the selected compounds is given in **Table 5.2**.

Table 5.2: Log P values of selected compounds

Compound	Log P value	Reference
Carvacrol	3.52	[109]
β-Caryophyllene	4.32	[283]
Cinnamaldehyde	2.12	[284]
Citral	3.45	[285]
p-Cymene	4.10	[81]
Eugenol	3.04	[286]
Geraniol	3.90	[287]
Isoeugenol	2.50	[288]
R (+)-Limonene	3.40	[289]
Linalool	2.80	[290]
Linalyl acetate	4.10	[291]
Menthol	3.10	[292]
Nerol	3.02	[59]
(+)-α-Pinene	4.83	[293]
α-Santalol[1]	4.50	[294]
α-Terpinene	4.25	[83]
γ-Terpinene	4.50	[84]
(+)-Terpinen-4-ol	2.60	[295]
α-Terpineol	2.98	[296]
Thymol	3.30	[297]
Thymoquinone	2.20	[298]

[1]Log P value available only for α-Santalol.

A log P value of ≤ 5 has been observed for all 21 compounds suggesting good membrane permeability of the compounds. The log P values of the carrier oils and combinations involving the compounds is not known and would be an interesting area for further exploration.

5.3 Final remarks

This study identified favourable combinations such as *A. vera* and α-terpinene, *P. armeniaca* and thymoquinone, *P. americana* and thymoquinone, and *H. perforatum* and *p*-cymene, against

selected pathogens. This is the first study to investigate the interactions of compounds with carrier oils, where the antimicrobial activity of 882 compound: carrier oil: reference strain combinations, and the toxicity of 126 compound: carrier oil combinations was tested. This study has highlighted several synergistic and beneficial interactions exerted by the carrier oils on the essential oil compounds. The toxicity of several compounds was decreased in this study prompting the investigation of ways in which the compounds could be made viable for human use. This study demonstrates how essential oil compounds and carrier oils interact biologically, and for selected synergistic combinations where synergistic interactions are predominant, explains the requisite to combine for a better outcome.

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Appendix A1 - Abstract for presentation

The antimicrobial and toxicity properties of essential oil compounds combined with carrier oils.

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Purpose: Essential oils contain a number of biologically active compounds that have been identified as alternative antimicrobials, however, their use is often limited due to their toxic nature. Carrier oils can reduce the toxicity of essential oils, which raises the question as to whether such activity would extend to the essential oil compounds. Thus, this study aimed to investigate the toxicity and the antimicrobial activity of 21 essential oil compounds in combination with six carrier oils against the ESKAPEE pathogen group.

Methods: The antimicrobial properties of the essential oil compounds, alone and in combination with carrier oils, were determined using the broth microdilution assay to determine the minimum inhibitory concentration (MIC) against *Enterococcus faecium* (ATCC 27270), *Staphylococcus aureus* (ATCC 25923), *Klebsiella pneumoniae* (ATCC 13883), *Acinetobacter baumannii* (ATCC 17606), *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 8739) reference strains. A yeast reference strain, *Candida albicans* (ATCC 10231), was also included. The toxicity was determined using the brine shrimp lethality assay. The interactive profiles of the combinations of the compounds and carrier oils was determined by calculating the fractional inhibitory concentration index (ΣFIC) (MIC studies) and the fractional percentage mortality index (ΣFPM) (toxicity studies). The time-kill effects of the essential oil compound: carrier oil combination that was synergistic in the MIC assay and that demonstrated reduced toxicity, was further evaluated.

Results: Of the combinations tested in the broth microdilution assay, 3% resulted in synergy (ΣFIC $\leq$ 0.50), with the compound thymoquinone and the carrier oil *Prunus armeniaca* demonstrating broad-spectrum synergistic activity. The carrier oils reduced the toxicity of the compounds, where at 24 and 48 hrs, the combinations showed 8% and 6% synergy, respectively. *Calendula officinalis* and *P. armeniaca* carrier *oils* were responsible for majority of the reduced toxicity observed. The combination of *Aloe vera* with α-terpinene demonstrated synergy in the broth-microdilution assay (ΣFIC value of 0.41), as well as reduced toxicity (ΣFIC value of 0.49) and was thus evaluated in the Time-kill assay. The combination provided bacteriostatic activity over 6 hrs.

Conclusion: This study provides evidence of the essential oil compound: carrier oil interactions where favourable combinations such as *A. vera* and α-terpinene, *P. armeniaca* and thymoquinone, *P. americana* and thymoquinone and *H. perforatum* and *p*-cymene, could be incorporated into novel antimicrobial formulations. Furthermore, the interactions demonstrate the added value of a carrier oils in combination with essential oil compounds.

Appendix A2 - Poster

The antimicrobial and toxicity properties of essential oil compounds combined with carrier oils

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Introduction and aim

Essential oil compounds (constituents of the essential oil) are responsible for the antimicrobial activity that has been observed for essential oils. However, their use is limited due to toxicity. A previous study investigated the effects of carrier oils on the antimicrobial activity and toxicity of essential oils.(1) This study reported a decrease in the toxicity of several of the essential oils when combined with carrier oils, while the antimicrobial activity was either maintained or enhanced. This raised the question as to whether this effect would extend to the essential oil compounds. **This study aimed to investigate whether the antimicrobial efficacy and toxicity of essential oil compounds are influenced by the addition of carrier oils.**

Methodology

Antimicrobial activity: The broth microdilution assay(2) was used to quantify the minimum inhibitory concentration (MIC) of the compounds and carrier oils, alone and in combination. An MIC value of ≤ 1.00 mg/ml was considered noteworthy.
Toxicity: The brine-shrimp lethality assay(3) was used to determine the toxicity levels of the compounds and carrier oils, alone and in combination. The percentage mortality was calculated where a % Mortality ≥ 50% was considered biologically toxic, and a % Mortality < 50% was considered biologically non-toxic.(4)
The compounds and carrier oils were tested in a 1:1 ratio in both the antimicrobial and toxicity studies.

Results and discussion

Antimicrobial studies

The antimicrobial activity of the compounds and carrier oils were tested alone and in combination (**Figure 1**). The yeast reference strain, *Candida albicans*, was found to be most susceptible to the microbial inhibition of the compounds and carrier oils, alone and in combination. The compounds carvacrol, cinnamaldehyde, isoeugenol and thymol showed noteworthy activity (≤ 1.00 mg/ml) against all reference strains tested.

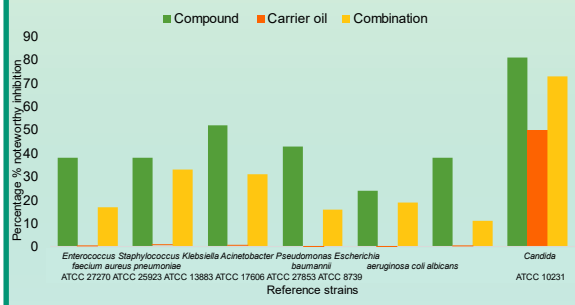

Figure 1: Percentage of essential oil compounds, carrier oils and compound: carrier oil combinations that inhibited the reference strains at ≤ 1.00 mg/ml .

The compound-carrier oil combinations predominantly resulted in indifference (56%), followed by 37% additive interactions, 4% antagonism, and 3% synergy. The compound thymoquinone and carrier oil *P. armeniaca* were present in the majority of the synergistic combinations and the compound santalol and carrier oil *H. perforatum* were present in the majority of the antagonistic combinations (**Figure 2**).

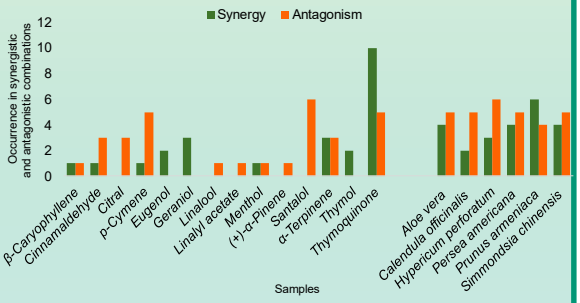

Figure 2: Occurrence of compounds and carrier oils in synergistic and antagonistic interactions in the study.

Toxicity studies

All carrier oils were non-toxic at 24 and 48 hrs. The toxicity of the compounds tested alone was reduced when combined with the carrier oils **(Figure 3)**. At 24 hrs, the compound-carrier oil combinations resulted in 10% synergistic, 2% additive, and 11% indifferent interactions. Antagonism was predominant (77%). At 48 hrs, 6% synergistic, 2% additive and 21% indifferent interactions occurred. Antagonism was predominant (71%). The carrier oils which were present most often in synergistic and antagonistic combinations were identified **(Table 1)**. In some instances, the carrier oils reduced the toxicity of the compounds p-cymene, R(+)-limonene, linalyl acetate, santalol, α-terpinene, γ-terpinene and (+)-terpinen-4-ol, resulting in synergistic combinations. Further studies on varied ratios, where the carrier oil is in a higher ratio to the compound, should be done to determine the effect on toxicity.

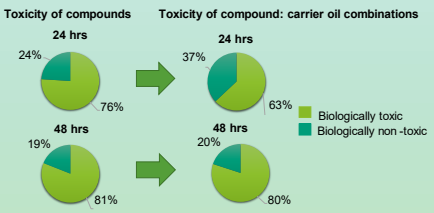

Figure 3: Compound and compound: carrier oil combination toxicity.

Table 1: % Interactions of each carrier oil within its respective compound -carrier oil combinations.

Carrier oils	Interaction			
	24 hrs		48 hrs	
	% Synergy	% Antagonism	% Synergy	% Antagonism
Aloe vera	10	80	5	85
Calendula officinalis	19*	67	5	85
Hypericum perforatum	5	71	10	66
Persea americana	-	-	0	95
Prunus armeniaca	14	67	14	81
Simmondsia chinensis	0	100	0	14

*The carrier oils which occurred most often in synergistic and antagonistic combinations are outlined in red.

Conclusion

This study provides evidence of the essential oil compound-carrier oil interactions. Favourable combinations such as *A. vera* with α-terpinene, *P. armeniaca* with thymoquinone, *P. americana* with thymoquinone and *H. perforatum* with p-cymene, were identified. The interactions demonstrate the ability of the carrier oils to enhance the antimicrobial properties and reduce the toxicity of several essential oil compounds, when in combination.

References: (1) Orchard, A., Kamatou, G., Viljoen, A. M., Patel, N., Mawela, P. & Vuuren, S. F. V. 2019. The influence of carrier oils on the antimicrobialactivity and cytotoxicity of essential oils. *Evidence-Based Complementary and Alternative Medicine*, 2019. (2) Orchard, A., Sandasi, M., Kamatou, G., Viljoen, A. & Van Vuuren, S. 2017. The in vitro antimicrobial activity and chemometric modelling of 59 commercial essential oils against pathogens of dermatological relevance. *Chemistry & Biodiversity*, 14, e1600218. (3) Hübsch, Z., Van Vuuren, S. & Van Zyl, R. 2014. Can rooibos (aspalathus linearis) tea have an effect on conventional antimicrobial therapies? *South African Journal of Botany*, 93, 148-156.(4) Bussmann, R., Malca, G., Glenn, A., Sharon, D., Nilsen, B., Parris, B., Dubose, D., Ruiz, D., Saleda, J. & Martinez, M. 2011. Toxicity of medicinal plants used in traditional medicine in northern Peru. *Journal of Ethnopharmacology*, 137, 121-140.

Appendix B - Ethics waiver

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research & Post Graduate Affairs)

12/04/2021

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TO WHOM IT MAY CONCERN

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

Investigator: Ms S Moola
Student No. (if appropriate): 1645450
Staff No. (if appropriate):

Supervisor: Professor S van Vuuren

School: Therapeutic Sciences
Department: Pharmancy and Pharmacology
Medical School
University

Project title: *The antimicrobial and toxicity properties of essential oil compounds combined with carrier oils*

Reason: Laboratory study
No human participants will be involved in the study

Dr CB Penny
Chairperson: Human Research Ethics Committee (Medical)

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