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THE BIOLOGICAL ACTIVITY OF SPECIFIC ESSENTIAL OIL CONSTITUENTS

Sammy Tsietsi Seatlholo

A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Master of Science in Medicine

Johannesburg, South Africa, 2007

DECLARATION

I, Sammy Tsietsi Seatlholo declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

……..

……. day of ……, 2007

DEDICATIONS

We become so busy in our everyday life such that everything we have been through becomes a memorable moment in time. This is because people who are very close to our hearts make the true meaning and essence of how life is like. This work is in loving memory of my late, caring, supportive and beloved sister, Ellen Sameeng, who left this world while I was still busy with this wonderful project. Unequivocally, I also dedicate this to the rest of my loving, caring and supportive family, the Seatlholos. These wonderful people never gave up on me a single moment of their lives and always believed that I could. In life we meet people with different wonderful characters whom we associate ourselves with, to lend us their lives to lean on. This special dedication is to my loving and caring fiancée for her love and support which kept me strong and helped me to conquer all the obstacles on my way. What would my life have been like without you people? I am even reminded to thank above all, God our father, who have always been near and taking a lead in my life ever since I was born. Thank you father God very much once more, for when happy or difficult moments come my way I shall not fear, because I know that you are always my strength, wisdom and understanding. A million thanks once again to my people, I love you all.

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS STUDY

PUBLICATIONS

1. van Zyl RL, Seatlholo ST, van Vuuren SF and Viljoen AM. The biological activity of 20 nature identical essential oil constituents. Journal of Essential Oil Research 2006; 18: 129-133.

PRESENTATIONS

1. Seatlholo ST, van Zyl RL, van Vuuren SF and Viljoen AM. The biological activity of specific essential oil constituents. BAASA Conference and SAAB Congress – University of KwaZulu-Natal, Durban, South Africa. 18- 22 January 2004.

ABSTRACT

Twenty essential oil constituents (EOC′s) from seven structural groups were tested for their antimalarial, antimicrobial (both bacterial and fungal), anti-oxidant, anticholinesterase and toxicity properties. To test for their antimalarial property, the tritiated hypoxanthine incorporation assay was used, while the disc diffusion and minimum inhibitory concentration (MIC) microplate assays were employed for the antimicrobial properties. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) method was used to test the anti-oxidant property and their toxicity profile was assessed with the 3-(4,5-dimethyl-2-thiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) cellular viability assay. The anticholinesterase activity was determined using the thin layer chromatography (TLC) bioautographic method. The EOC´s were found to inhibit the growth of *Plasmodium falciparum* with IC_{50} values ranging between 0.9 to 1528.8µM with *E*- and *Z*-(±)-nerolidol, (-)-pulegone, (+)-α-pinene and linalyl acetate being the most active. In combination *p*-cymene (the least active) and *E*- and *Z*-(±)-nerolidol (the most active) displayed the most synergistic interaction (∑FIC = 0.09), with their antimalarial activity comparable to that of the interaction between *E*- and *Z*-(±)-nerolidol and quinine (∑FIC = 0.01). Eugenol had the most favourable safety index and was the only EOC with anti-oxidant activity comparable to vitamin C. Combination studies showed that *E*- and *Z*-(±)-nerolidol and (-)-pulegone or quinine, *p*-cymene and γ-terpinene or (-)-pulegone potentiated each other′s toxicity. The EOC´s inhibited the growth of Gram-positive, Gram-negative bacteria and yeast with MIC values ranging from 1.66 to >238.4mM. When combined, synergism was observed between (+)-β-pinene and carvacrol or γ-terpinene; γ-terpinene and geranyl acetate when tested against *Staphylococcus aureus*, while (+)-β-pinene and (-)-menthone showed antagonism against *C. albicans*. The combinations of EOC′s and a standard antimicrobial resulted in synergistic interactions between carvacrol and ciprofloxacin against *Bacillus cereus*, eugenol and ciprofloxacin against *Eschericia coli*, carvacrol and amphotericin B against *C. albicans*. The *trans*-geraniol and *E*- and *Z*-(±)-nerolidol combination demonstrated an additive interaction against *B. cereus*, while for eugenol and *E*- and *Z*-(±)-nerolidol an indifferent interaction against *E. coli* was noted. These results show that the biological activities of EOC′s can vary when used alone and in combination. They do have the potential to be used as templates for novel drugs and as adjuncts to modern medicines in the combat against drug resistance.

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5) I would also like to thank even those who told me that I would not make it this far in my studies, believe it or not, they too were a part of my motivation. May God bless those who come against S. Seatlholo or Sammy, the road was long but I made it through, thank you.

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LIST OF ABBREVIATIONS

%	Percent
∑ FIC	The sum of the fractional inhibitory concentrations
°C	Degree celsius
µl	Microlitre
µm	Micrometre
µM	Micromolar
Ach	Acetylcholine
AChE	Acetylcholinesterase
AIDS	Autoimmune deficiency syndrome
AMD	Age-related macular degeneration
An	Antagonism
AREDS	Age-related eye disease study
ATCC	American type culture collection
cAMP	Cyclic adenosine monophosphate
CFU	Colony forming units
CO_2	Carbon dioxide
CPDA	Citrate phosphate dextrose adenine
CPM	Count per minute
CYP	Cytochrome P450 enzyme
dhfr	Dihydrofolate reductase
dhps	Dihydropteroate synthetase
DMAPP	Dimethylallyl diphosphate
DMSO	Dimethyl sulfur oxide
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EDTA	Ethylene diamine tetra acetic acid
EOC	Essential oil constituent
FCR-3	*Falciparum* chloroquine resistant strain
FCS	Foetal calf serum
FDA	Food drug administration
FIC	Fractional inhibitory concentration
FPP	Farnesyl pyrophosphate
g	Gram
G6PD	Glucose-6-phosphate dehydrogenase

GC	Gas chromatography
GC-MS	Gas chromatography coupled to mass spectrometry
GI	Growth index
GPP	Geranyl pyrophosphate
h	Hour
HEPES	N-2-hydroxyethyl-piperazine-N-2-ethane-sulfonic acid
HPLC	High performance liquid chromatography
HRP-2	Histidine rich protein
I	Indifferent
IC_{50}	Concentration that inhibited 50% of parasite or cell growth or biological activity
IFRA	International fragrances research association
INT	*p*-Iodonitrotetrazolium violet
IPP	Isopentyl diphosphate
l	Litre
m	Metre
M	Molar
MIC	Minimum inhibitory concentration
min	Minute
ml	Millilitre
mm	Millimetre
mM	Millimolar
MPO	Myeloperoxide
M_r	Molecular weight
MS	Mass spectrometry
MTT	3-(4,5-Dimethyl-2-thiazol-2yl)-2,5-diphenyl-2H-tetrazolium bromide
N_2	Nitrogen
NHLS	National health laboratory services
nM	Nanomolar
nm	Nanometre
NTCC	National type culture collection
O_2	Oxygen
PBS	Phosphate buffer solution
pH	Potential hydrogen
pLDH	Parasite specific lactate dehydrogenase

ppm	Parts per million
r^2	Correlation coefficient
RBC	Red blood cell
Rf	Relative to the front
ROS	Reactive oxygen species
rpm	Revolution per minute
RPMI-1640	Roswell park memorial institute medium type 1640
R_t	Retention time
S	Synergism
s.d.	Standard deviation of the mean
SABS	South African bureau of standards
TLC	Thin layer chromatography
UV-Vis	Ultraviolet visible
WHO	World health organization

CHAPTER ONE
GENERAL INTRODUCTION

1.1 Plants and essential oils

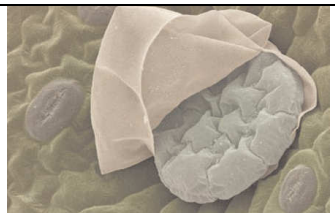
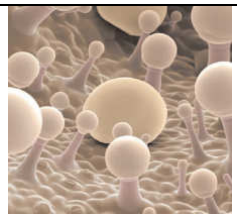
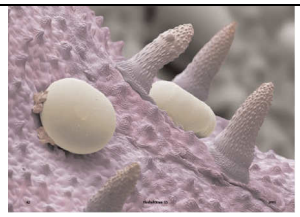
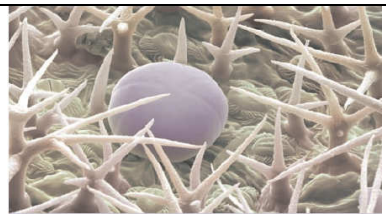
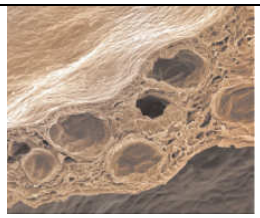
An essential oil is the volatile fraction of an aromatic plant that is borne in that plant within distinctive oil cells. The oil is a concentrated, hydrophobic liquid containing a complex mixture of volatile components of certain secondary plant metabolites. A wide range of plant species including herbs, shrubs and trees synthesise essential oils as secondary metabolites (Table 1.1). The type of essential oil-containing cell is an important characteristic of the plant family which can be used to taxonomically verify a species. The essential oil and its aroma associated with a plant can also be distinctive, e.g. “oil of sandalwood” or “oil of clove” (Lawrence, 2000).

Table 1.1: Distinctive oil cells are found in various aromatic medicinal plant families (Lawrence, 2000).

Essential oil containing structures	Specific examples
Vittae (long secretory ducts) in fruits and roots	• *Adansonia* (baobab fruit) • *Coriandrum* (corriander) • *Foeniculum* (fennel)
Oil ducts in flowers, leaves and stems (associated with vascular bundles)	• *Salvia* (sage) • *Chamaemelum* (chamomile)
Protruding multicelled glandular trichomes	• *Lavandula* (lavender) • *Mentha* (peppermint)
Large oil cells in stem barks or large mesophyllic oil cells in leaves	• *Papaver* (opium poppy) • *Sassafras* (Ague tree)
Large round secretory oil glands or cavities	• *Chamaemelum* (chamomile) • Myrtaceae family
	• Citrus family
Large rectangular- shaped oil cells in testa (seed coat), just within the thin epidermis of the seed.	• *Myristica* (nutmeg)
	• *Zingiber* (ginger)

Essential oils are synthesised and stored in specialised secretory structures (known as glands or trichomes) either on the surface of the plant or within the plant tissue of the leaves, flower calyces, fruits and roots (Table 1.2) (Lahlou, 2004a).

Table 1.2: Essential oil secretory glands of medicinal plants (Svoboda *et al*., 2001).

Plant family	Species and common name	Plant structure and essential oil cells	
Asteraceae	*Chamaemelum nobile* (L.) [Roman chamomile]		
Lamiaceae	*Origanum vulgare* (L.) [oregano]		
	Salvia sclarea (L.) [Clary sage]		
	Mentha x *piperita* (L.) [Peppermint]		
	Lavandula angustifolia Mill. [English lavender]		
Myrtaceae	*Syzygium aromaticum* (L.) [clove]		
Zingiberaceae	*Zingiber officinale* (L.) [ginger]		

There are various types of secretory structures where the oil content, larger size and thick cuticularised lining differentiate them from adjacent non-secretory cells. These cell types are found for example in ginger (*Zingiber officinale*) and nutmeg (*Myristica fragrance*). In contrast to secretory cavities which consist of spherical structures lined with secretory cells that produce the essential oil. Included in this group are the fruits and leaves from the citrus and eucalyptus family and flower buds of cloves (Table 1.2) (Svoboda *et al*., 2001). Secretory ducts, found in the Apiaceae and Asteraceae families, are formed once the cavities between adjacent cells fuse. Cells in these cavities differentiate to form secretory epithelial cells. Glandular trichomes found in lavender, oregano and mint are modified epidermal hairs with a toughened cuticle that covers the trichome and encapsulates the essential oils (Table 1.2).

1.2 Use of essential oils in plants

The biological role of these secondary metabolites are still not fully understood, but they are known to protect the plants against diseases, attract or repel insects, birds and other animals via complex chemical mimicry (Halcon, 2002). For example, certain plants appear to protect themselves from overgrazing by producing sesquiterpene lactones which are known to be potent antifeedants. If eaten by herbivores, these substances affect the normal gastrointestinal flora and interfere with digestion (Onawunmi *et al*., 1984). Corniferous plants secrete a complex mixture of monoterpenes and sesquiterpenes in response to attack by insects and predators (Mahmoud and Croteau, 2002).

1.3 History of essential oils

Essential oils have been used since antiquity by different cultures in a variety of ways as medicines, in ritual worship and as perfumes (Halcon, 2002; Nakatsu *et al.*, 2000). They were very precious in the ancient world and were traded for gold, silver and even slaves. The first nation to use essential oils were the Egyptians who created fragrances for personal use as well as for ritual and ceremonial use in the temples and pyramids (Gollin, 1999). Some oils, particularly frankincense, are cited repeatedly in religious texts of Judeo-Christians and Muslims (Inouye *et al*., 2001). In 1817, an 870 foot long Ebers Papyrus dating back to 1500 B.C. was discovered listing over 800 herbal prescriptions and remedies (Jirovertz *et al*., 1990). Many listed herbals contained oils which were used for embalming due to their effective antibacterial properties and it is reported that the corpses of the Pharaohs were embalmed with these oils (Gollin, 1999). Other oils such as lotus and sandalwood were also widely used in ancient Egyptian purification and embalming rituals. The importance of these essential oils is best displayed by the discovery of 350 litres of essential oils sealed and

preserved in alabaster jars found in Tutankhamen′s tomb when opened in 1922 (Inouye *et al*., 2001). Even 3000 years after being encased in the jars, there was a faint odour of lotus oil and frankincense.

The "Father of Modern Medicine," Hippocrates (460BC) stated that "a daily aromatic bath followed by a scented massage is a proper way to good health" (Lis-Balchin, 1997). This practice was also employed by the Romans and Greeks who in addition to using aromatic oils in their bath houses, diffused essential oils in their temples and political buildings. Physicians of Greece including Hippocrates attended the Egyptian school of Cas to learn about the oils. Amongst the medicinal properties taught were the antiseptic properties of clove and lemon essential oils, hundreds of years prior to the discovery of modern antimicrobials used today (Jirovertz *et al*., 1990).

The ancient Arabian people researched the chemical properties of essential oils and further developed and refined the distillation process. Arabian perfumes were first discovered by western man and brought back to Europe by the crusaders, where they became well known and fashionable with the aristocracy (Buchbauer, 1993). During the Middle Ages the antiseptic properties of essential oils became popular to the doctors of that time, such that they carried these antiseptic aromatic oils in the handles of their walking sticks, which they held to their noses during their visits to patients (Buchbauer, 1993).

The rediscovery of the antiseptic properties of essential oils is attributed to French cosmetic chemist, René-Maurice Gattefossé, as a consequence of his personal experiences (Halcon, 2002). It was in July of 1910 when a laboratory explosion set him aflame. "It is reported that, after extinguishing the flames his hands were quickly developing gas gangrene". However, "one rinse with lavender essential oil stopped this process and the next day healing began". This incident motivated Gattefossé to investigate the healing components of essential oils. The research on essential oils prompted their clinical use on wounded World War II soldiers, and wounds responded better to the oils than to the antibiotics of that time (Jirovertz *et al*., 1990). Around this time another medical scientist Royal R. Rife discovered that essential oils could effectively treat various bacterial diseases such as coughs and flu (Jirovertz *et al*., 1990).

1.4 Isolation procedures of essential oils from plants

For an essential oil to be a true essential oil, it must only be isolated by physical means (Nakatsu *et al*., 2000). The methods of isolating the essential oils from plants significantly affect the chemical constituents and composition of the essential oil (Lahlou, 2004b). For instance, α-pinene and β-pinene can be isomerized during the process and analysis, forming camphene and possibly other monoterpenes in the process (Geron *et al*., 2000). Lawrence (2000) stated that the most appropriate and convenient method to concentrate the essential oil should be selected. If the activity is based on the mixture, and not on a single compound, then all components should be concentrated from the isolates (Lawrence, 2000). Generally most essential oil constituents are small, volatile and lipophilic compounds, thus a key consideration is the need to separate them from aqueous plant materials (Lahlou, 2004b; Lawrence, 2000; Nakatsu *et al*., 2000).

The physical methods used include distillation (hydrodistillation, steam distillation and hydrodiffusion) (Figure 1.1), cold pressing (expression), maceration/distillation and microwave irradiation (Lahlou, 2004b). In the hydrodistillation process, the plant material is heated in two or three times its weight of water with indirect steam from outside the still. In contrast, in steam distillation the plant material is isolated by direct steam produced in the still, or by indirect steam produced outside and fed into the still. In hydrodiffusion, low pressure steam (0.1 bar) replaces the volatile oil from the intact plant material by an osmotic action (Lahlou, 2004b; Lawrence, 2000).

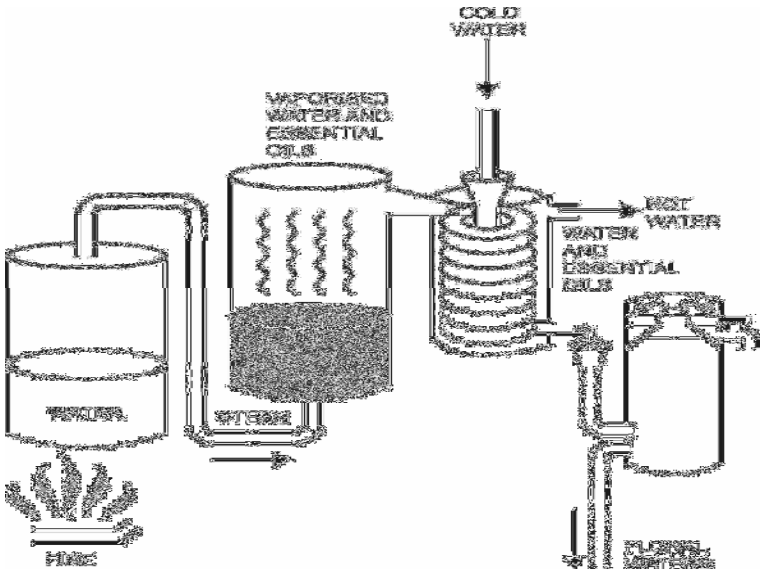


Figure 1.1: A schematic diagram showing a distillation apparatus used for the isolation of essential oils (adapted from Nakatsu *et al*., 2000).

Cold pressing (Figure 1.2) is a technique used to isolate citrus peel oil and many other essential oils from seeds, grains, kernels and fruits (Inouye *et al.*, 2001; Nakatsu *et al.*, 2000). During this process, machines score the fruit/seed releasing the oil. Nakatsu *et al*. (2000) mentioned that this method has recently been used to great advantage in isolating oxygen-containing terpenes, which tend to degrade or rearrange when heat is applied.



Figure 1.2: An industrial machine used for isolating essential oils during cold pressing process (Lahlou, 2004b).

During maceration/distillation the plant material is macerated in warm water to release the enzyme-bound essential oil. Examples of essential oils produced by this technique are garlic, wintergreen and bitter almond (Carson and Riley, 1995). Microwave irradiation is a modern technique using microwaves to excite water molecules in the plant tissues, thus causing the plant cells to rupture and release the essential oils trapped in the extracellular tissues of the plant (Lahlou, 2004b).

Solvent isolation is used to isolate essential oils from those plants that cannot withstand the process of distillation and heat (e.g. jasmine). A solvent such as hexane or supercritical CO_2 can be used to isolate the essential oil and is then removed with alcohol and distilled to obtain the required amount of essential oil (Lahlou, 2004b).

1.5 The chemistry of essential oils

Various factors influence the quantity and composition of the essential oil synthesised by plants. These include soil conditions, geographical and seasonal variations, climate (rainfall) and altitude; as well as harvest method, isolation technique and which part of the plant is used (Lahlou, 2004b). In addition, each plant can produce several different chemotypes with biochemical variations that change the composition and ratio of essential oil constituents and ultimately influence its therapeutic efficacy (Viljoen *et al*., 2005).

Essential oils are volatile, complex mixtures, composed of numerous terpene hydrocarbons and oxygenated compounds (Griffin *et al*., 1999; Lis-Balchin, 1997). These constituents are not only volatile, but also lipophilic (Griffin *et al*., 1999). All terpenes are synthesized through the condensation of isopentyl diphosphate (IPP) and its allylic isomer, dimethylallyl diphosphate (DMAPP as seen in Figure 1.3) (Mahmoud and Croteau, 2002).

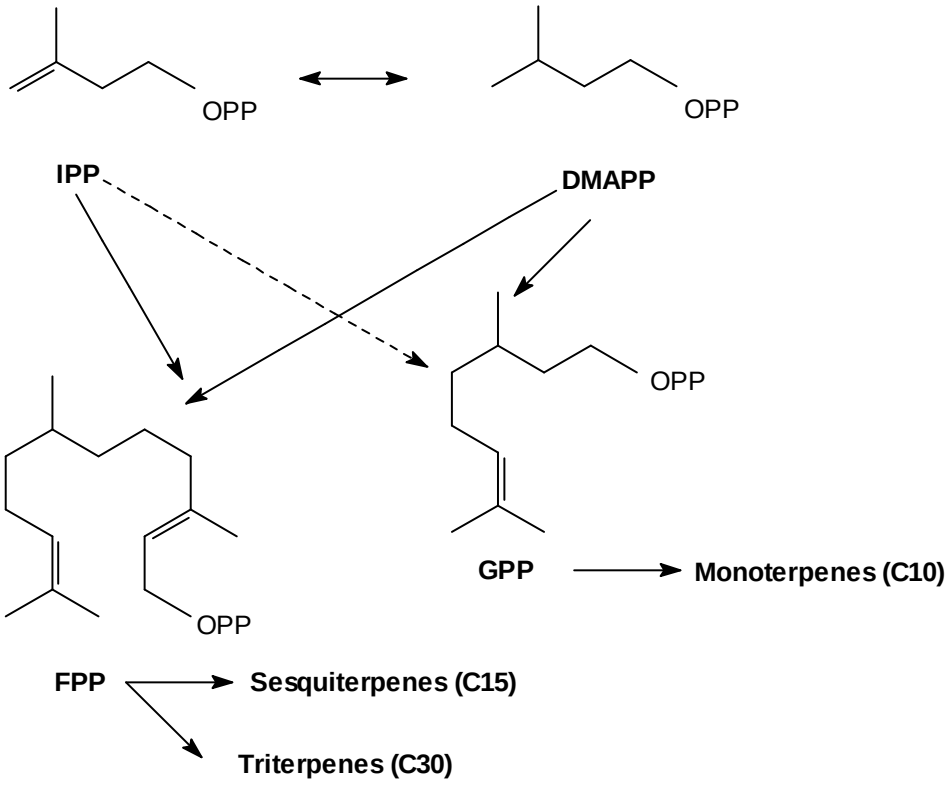
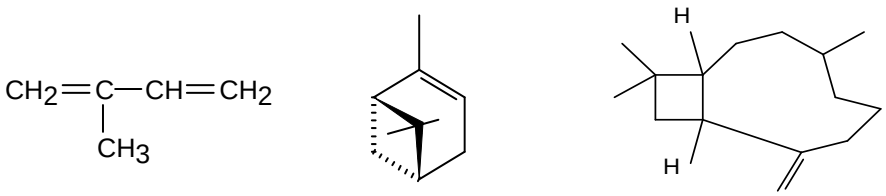


Figure 1.3: An overview of isoprenoids biosynthesis pathway in plants (adapted from Mahmoud and Croteau, 2002). Where IPP = isopentyl diphosphate; GPP = geranyl pyrophosphate; FPP = farnesyl pyrophosphate and DMAPP = dimethylallyl diphosphate.

1.5.1 Terpenes

Terpenes are hydrocarbons that usually contain one or more C=C double bonds (Griffin *et al*., 1999). They are derived from the condensation of isoprene units (Figure 1.4a) and are categorised according to the number of these units in the carbon skeleton. They can be divided into two subcategories, monoterpenes (C_{10}) (e.g. α-pinene) and sesquiterpenes (C_{15}) (e.g. β-caryophyllene) (Figure 1.4b and c, respectively). Besides being linear (acyclic) (e.g. α-myrcene; Figure 1.5i), terpenes can be monocyclic or bicyclic molecules, for example: limonene (Figure 1.5c) and β-caryophyllene (Figure 1.4c), respectively.



(a) (b) (c)

Figure 1.4: The chemical structures of (a) an isoprene unit (b) α-pinene and (c) β-caryophyllene (adapted from Nakatsu *et al*., 2000).

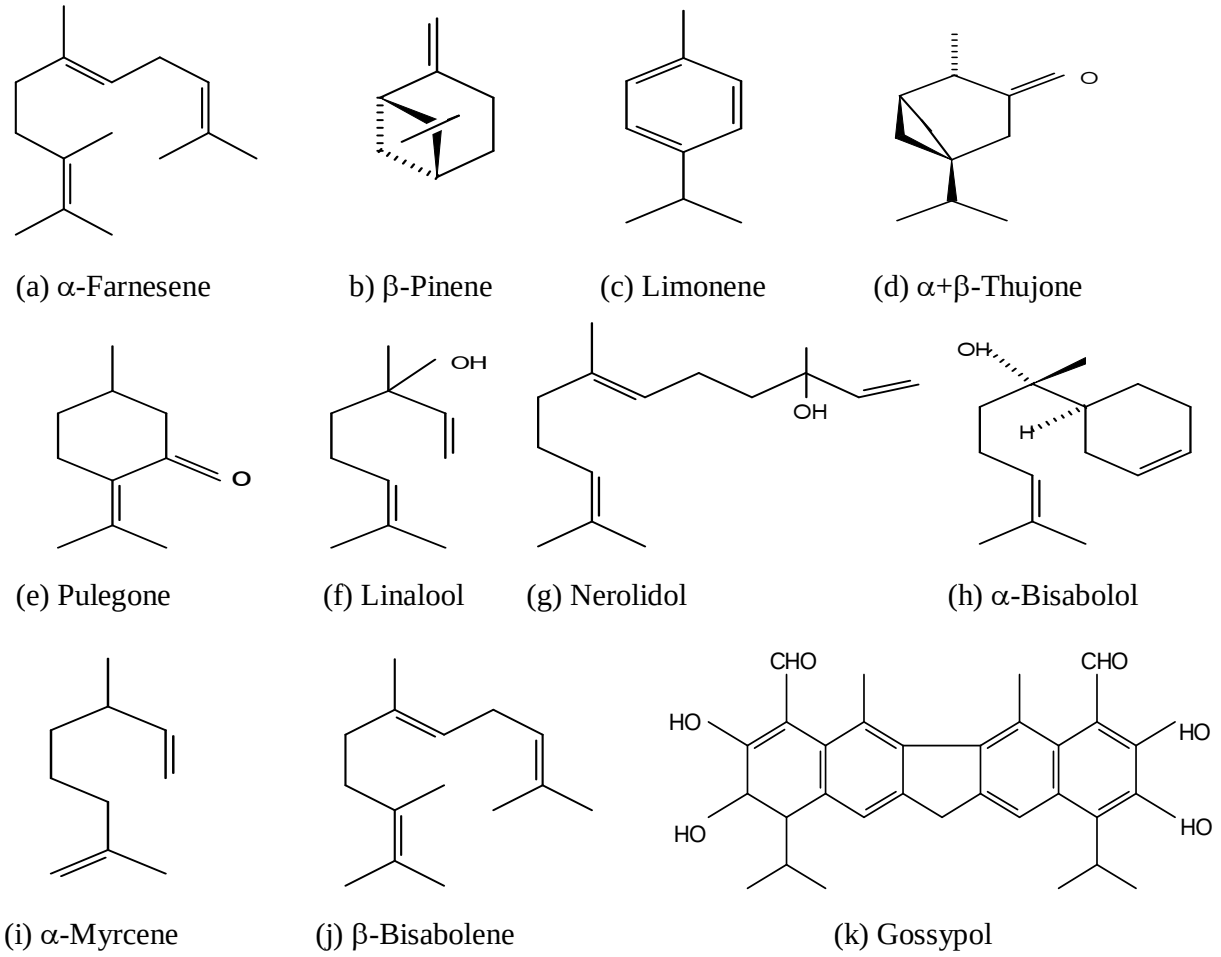
These monoterpenes and sesquiterpenes are widely distributed in various medicinal and aromatic plants. Spikenard (*Nardostachys jatamansi*) is almost entirely composed of sesquiterpenes (Lahlou, 2004a). The sesquiterpene group also constitutes secondary metabolites, some exhibiting stress-induced compounds (e.g. gossypol; Figure 1.5k) formed as a result of disease or injury to the plant.

1.5.1.1 Monoterpenes

These compounds are found in nearly all essential oils and are biogenetically derived from geranyl pyrophosphate (GPP) (Figure 1.3), and have 10 carbon atoms derived from two isoprene units. Monoterpenes react readily to air (i.e. oxidize readily) and heat sources. Common examples are limonene (Figure 1.5c) and β-pinene (Figure 1.5b).

1.5.1.2 Sesquiterpenes

They consist of 15 carbon atoms and are biogenetically derived from farnesyl pyrophosphate (FPP) (Figure 1.3) and can also be linear (e.g. α-farnesene; Figure 1.5a), monocyclic (e.g. β-bisabolene; Figure 1.5j) or bicyclic (e.g. β-caryophyllene; Figure 1.4c).



(a) α-Farnesene b) β-Pinene (c) Limonene (d) α+β-Thujone

(e) Pulegone (f) Linalool (g) Nerolidol (h) α-Bisabolol

(i) α-Myrcene (j) β-Bisabolene (k) Gossypol

Figure 1.5: Various chemical structures of essential oil constituents (a - j) and a secondary metabolite (k = gossypol) showing some degree of cyclization (adapted from Nakatsu *et al.*, 2000).

1.5.2 Terpenoids

These are oxygen-containing analogues of the terpenes and are subdivided according to the number of carbon atoms in the same manner as are terpenes and can be linear (e.g. nerolidol; Figure 1.5g), monocyclic (e.g. pulegone; Figure 1.5e) or bicyclic (α+β thujone; Figure 1.5d). The skeleton of terpenoids may differ from just adding isoprene units by the loss or shift of a fragment, generally a methyl group is replaced or shifted by an O_2 in various functional groups (Nakatsu *et al.*, 2000). These oxygenated compounds belong to a number of different chemical groups including the alcohols (nerolidol), aldehydes (citronellal), esters (linalyl acetate), ketones (fenchone), oxides (1,8-cineole) and phenols (eugenol). Terpenoids are usually very expensive, because like most secondary metabolites they are synthesized by plants in relatively small amounts (Lawrence, 2000). In addition, due to their complex

structures, most terpenoids cannot be obtained in a profitable way through chemical synthesis and as are normally isolated by steam distillation (Lahlou, 2004b).

1.5.2.1 Alcohols

Alcohols are not water soluble and are less prone to oxidation and for this reason they evaporate quite slowly (Nakatsu *et al.*, 2000). They can further be subdivided into monoterpene alcohols (e.g. linalool; Figure 1.5f) and sesquiterpene alcohols (e.g. α-bisabolol; Figure 1.5h). Other examples are nerol, geraniol, citronellol and patchoulol.

1.5.2.2 Aldehydes

The majority of the lemon scented oils fall into this group (Onawumni *et al.*, 1984), and include citral, geranial, neral and citronellal.

1.5.2.3 Esters

Esters sometimes have a trademark fruity aroma and are formed when acids react with alcohols (Cowan, 1996). Linalyl acetate, neryl acetate and geranyl acetate are common examples.

1.5.2.4 Ketones

The ketones consist of some of the most toxic elements of essential oils (Zhou *et al.*, 2004). Some of these include α+β-thujone (Figure 1.5d) and pulegone (Figure 1.5e).

1.5.2.5 Oxides

Oils containing oxides are generally camphoraceous in nature (Nakatsu *et al.*, 2000). This group contains essential oil constituents like asarone and ascaridol and some non-toxic oxides like 1,8-cineole (eucalyptol), bisabolol oxide and linalool oxide.

1.5.2.6 Phenols

Phenols found in essential oils normally have a carbon side chain and are more hydrophilic and evaporate more quickly than oils that do not contain phenols (Nakatsu *et al.*, 2000). Examples of phenols are eugenol, thymol, carvacrol, gaiacol, chavicol and australol.

1.6 Medicinal applications and adverse effects of essential oils

In humans, essential oils exhibit a wide spectrum of pharmacological activities such as infection control, wound healing, pain relief, nausea, inflammation and anxiety

(Onawunmi *et al*., 1984; Halcon, 2002; Kalemba and Kunicka, 2003; Shin and Kang, 2003). Traditional medicines containing essential oils have been scientifically proven to be effective in treating various ailments like malaria and others of microbial origin (Campbell *et al*., 1997; Cragg *et al*., 1997; Lopes *et al*., 1999; Nakatsu *et al*., 2000; Goulart *et al*., 2004). Nakatsu *et al*. (2000) reported that although each essential oil is different in composition, there is considerable overlap in their actions. For example, essential oils of *Lippia javanica*, *Tetradenia riparia* and *Virola surinamensis* have reported antimalarial activities yet they have different essential oil profiles (Lukwa, 1994; Campbell *et al*., 1997; Lopes *et al*., 1999).

The biological activities of the essential oils have been attributed to the composition or specific essential oil constituent, for example:

a) Sesquiterpenes in German chamomile (*Matricaria recutita*) have anti-inflammatory properties and are also useful in the treatment of asthma (Nakatsu *et al*., 2000);
b) Alcohols in tea tree (*Melaleuca alternifolia*) or true lavender (*Lavandula angustifolia*) or baboonwood (*Virola surinamensis*) have antimicrobial and antimalarial properties (Cowan, 1996; Lopes *et al*., 1999);
c) Aldehydes in lemonsgrass (*Cymbopogon citratus*) have anti-inflammatory properties (Onawumni *et al*., 1984; Shale *et al*., 1999);
d) Esters in clary sage (*Salvia sclarea*) have anticholinesterase properties (Savelev *et al*., 2003);
e) Ketones in sweet fennel (*Foeniculum vulgare*) aid in wound healing and dissolving mucus and fats (Nakatsu *et al*., 2000).
f) Oxides in ‘lanyana’ (*Artemisia afra*) have effective expectorant properties and are used to treat bronchitis and common colds (Subramanyam *et al*., 1996);
g) Phenols in thyme (*Thymus vulgaris*) and clove (*Eugenia caryophyllata*), have antimicrobial properties and can be used as food preservatives (Nakatsu *et al*., 2000).

However, Zhou *et al*. (2004) reported that while some of the traditional medicines with their essential oils may be unequivocally helpful, others may be dangerous because ultimately they do more harm than good. For example, pennyroyals (*Mentha pulegium*) and their essential oil constituents used as flavourants and in fragrances have been reported for their hepatic, renal, and central nervous system toxicities (Zhou *et al*., 2004). Whilst some esters such as geranyl acetate and neryl acetate are skin sensitizers and their dermal application may cause skin irritation (Halcon, 2002). Ketones like thujone and pulegone are toxic and have been associated with epileptic seizures, convulsions, abortive effects, and mental confusion

(Zhou *et al*., 2004). Some oxides such as asarone and ascaridol may cause convulsions (Nakatsu *et al*., 2000). Another example of an essential oil containing oxides is peppermint oil. In high oral doses it has produced severe toxic reactions, and its major component, menthol, has been associated with apnoea and severe jaundice in babies and patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency, and hepatotoxicity (Halcon, 2002; Zhou *et al*., 2004).

1.7 Route of administration

When applying essential oils for any medical reasons, the route of administration is selected based on pathophysiology, desired outcome, safety and toxicity data, professional practice parameters, and cultural preferences (Cowan, 1996; Halcon, 2002). For example, although inhalation is the best route of administration for treating respiratory symptoms and for affecting mood or cognition, topical application is likely to work best for burns, wound care and most dermatological conditions. For instance, essential oils of 'lanyana' (*Artemisia afra*) can be inhaled or used as a chest rub (topically) to treat common colds and bronchial disorders (Viljoen *et al*., 2006). Whilst essential oils of lavender (*Lavandula angustifolia*) may be applied topically to treat burns and wound infections (Nakatsu *et al*., 2000).

Some essential oils, such as *Eucalyptus globulus*, can be toxic even when a teaspoon is ingested. Thus, the patient or provider should choose another route of administration or another species of *Eucalyptus* that has similar therapeutic effects, but presents less danger of toxicity (Cowan, 1996; Halcon, 2002). Internal application of essential oils by suppository or oral ingestion is very common in places such as France, where essential oils are prescribed by physicians. While in the United Kingdom and the United States topical preparations and inhalation are common, where they are employed by nurses and other health care professionals (Halcon, 2002). In Africa, oral ingestion, topical application and inhalation of essential oils are common routes of administration and are commonly used by traditional healers.

1.8 The commercial importance of essential oils

Essential oil terpenes such as α-pinene, *d*-limonene and turpentine are used as a component of semi-aqueous cleaning solutions or by themselves (Mahmoud and Corteau, 2002; Nakatsu *et al*., 2000). Terpenes are volatile organic compounds, thus flammable. Regardless, in the industrial world they are used as solvents for rosin fluxes, fingerprints, heavy petroleum greases, and oils (Constantin *et al*., 1991). In addition, they can be used with immersion and

ultrasonic systems, often working well at room temperature (Constantin *et al*., 1991). Terpenes may produce explosive mists when sprayed, thus they should only be used in spray applications with proper safety precautions, such as inert gas blanketing (Halcon, 2002). Moreover, they need to be incinerated at an approved facility and not be discarded via drainage system (Constantin *et al*., 1991).

Overall essential oils are pertinent for pharmaceutical, cosmetic and food research and development, and are widely viewed as templates for structure optimization programs with the goal of creating new drugs (Cragg *et al*., 1997). Essential oils forming part of the bioactive molecules of medicinal plants have been in existence for much longer than any drug company (De Smet, 1997). Because most essential oil constituents like terpenoids are biologically active, they are employed for medical purposes. For instance, the antimalarial drug artemisinin (from *Artemisia annua*) and the anticancer drug paclitaxel (Taxol®) are of a terpene nature with an established medical application (Cowan, 1996; Cragg *et al*., 1997; De Smet, 1997).

With respect to the pharmacokinetic profile of essential oils, it is believed that they are absorbed and metabolized according to their chemical composition, dose and route of administration, as is the case with other substances (Halcon, 2002). However, this cannot be assumed. To avert this serious dilemma such that essential oils can be clinically used, scientific evidence confirming their safety and efficacy including their pharmacokinetics (i.e. their bioavailabilty, distribution rate and dosage regimen) are necessary (Cragg *et al*., 1997). This requires phytotherapeutic and pharmacological studies to confirm their use as medical drugs or lead molecules for novel drug development.

1.9 Public interest in the use of essential oils

The use of essential oils forms the basis of a fascinating plant-based traditional medicine system that has been utilized in countries such as China, France and India, but predominantly in the developing countries of the African continent where finance to buy classical synthetic drugs is a major challenge (Nakatsu *et al*., 2000). In addition, one of the issues driving the interest in essential oils is the increased public use of complementary therapies. As a result, health sciences education and training in complementary therapies have taken a lead to encourage medical personnel to be aware of the risk and benefits of essential oils and to also educate their patients regarding these issues (Shale *et al*., 1999).

In general, public interest in the therapeutic uses of essential oils has grown much faster than the available scientific research to back it up. There is, however, a growing body of published laboratory and human studies on specific essential oils that are employed for selected health outcomes. This information can aid practitioners in advising patients and can provide direction for future clinical research (Halcon, 2002). Among South African medicinal plants 'lanyana' (*Artemisia afra*) is a common example that is used extensively as an antibacterial and an antifungal and is also used as a component of fragrances in toiletries, cosmetics and perfumes (Subramanyam *et al*., 1996).

1.10 Essential oils in the twentieth century

The volatile compounds from plants have played an important part in the medical field throughout the twentieth century (Cragg *et al*., 1997; De Smet, 1997). Peppermint oil is a good example of an essential oil that has a history of continuous wide use and holds promise for medicine, but also warrants caution because of its potential side effects (De Smet, 1997; Shale *et al*., 1999). Peppermint in a variety of forms can be found in toothpaste and countless other products and foods (Halcon, 2002). Although, numerous studies on essential oils have been undertaken by the food, cosmetics, and tobacco industries, access to the information by the healthcare community has been limited (Halcon, 2002; Mahmoud and Corteau, 2002; Nakatsu *et al*., 2000). Essential oils of thyme and clove are also ubiquitous as food and cosmetic additives, drawing on their well known preservative (antimicrobial) properties (Nakatsu *et al*., 2000).

1.11 Single versus combination therapy

From the historical perspective, people traditionally using medicines believed that complex diseases could be treated with a 'single magic herb'. A perfect example is that of *Melaleuca alternifolia* (tea tree) and its essential oils, which has been widely used over the past 30 years (Carson and Riley, 1995; Cox *et al*., 2001). Numerous studies have reported the efficacy of *M. alternifolia* essential oil against a variety of pathogenic microorganisms including many *Staphylococcus aureus* isolates (including methicillin-resistant *S. aureus*), as well as other bacteria and fungi (Carson and Riley, 1995; Cox *et al*., 2001). However, it is also important to note that many plants are referred to as "tea tree," but that the above applies only to *M. alternifolia* (Carson and Riley, 1995).

Traditionally in South Africa, aromatic plants used in combination include the following few examples. The Cape snowbush (*Eriocephalus africanus*) can be blended with sandalwood

(*Santalum album*) or bergamot (*Citrus bergamia*) and the mixtures applied topically or via inhalation for the treatment of depression, colds and flu, menstrual problems, muscular aches and pain (Constantin *et al*., 1991). Another example is the combination of buchu (*Agathosma betulina*) with lavender (*Lavandula angustifolia*), when applied topically or via inhalation are useful in the treatment of burns, wound infections and as an anxiolytic/sedative (Subramanyam *et al*., 1996).

It is unequivocally incorrect and unwise to rely on monotherapy, as a possible drawback is the tendency of the organisms to develop resistance to the antimicrobial and antimalarial drug (Cox *et al*., 2001). If medical science has to rely on a single therapeutic compound to eradicate disease, it would mean a serious catastrophe to human health as microbial resistance is already increasing at an unprecedented rate (Cassella *et al*., 2002). It is widely accepted that agents should be used in combination to avoid treatment failure (Bell, 2005). Thus, the discovery of new and effective chemical entities is urgently required. Essential oil constituents are possible candidates to be used in combination and together with standard antimicrobials to combat this dilemma.

Cassella *et al*. (2002) reported that it is an accepted premise by practising aromatherapists, that essential oils act better when used in combination in order to optimise their efficacy. The interaction of two or more drugs is said to be synergistic if the potency of the combination is higher than the expected activity of the individual drug activity. However, if the potency of the combination is lower, then the end result is defined as an antagonistic interaction (Bell, 2005). It has been claimed that the most dominating constituents in the essential oil are the ones eliciting the activity attributed to the whole oil (Nakatsu *et al*., 2000). Despite this fact, constituents in very small amounts are often found to be as useful as the principal constituent (Cassella *et al*., 2002; Nakatsu *et al*., 2000). For example, when the antibacterial activity of the whole oil of thyme (*Thymus vulgaris*) was compared to its principal constituents (borneol, carvacrol, camphene, thymol, *p*-cymene and α-pinene) the whole oil was more active than these individual constituents (Nakatsu *et al*., 2000; Viljoen *et al*., 2006). This suggests that minor essential oil constituents played a significant role in the biological activity of this essential oil.

Moreover, synergistic and antagonistic effects could also be achieved by using the essential oils in combination, but also by combining the essential oils with other standard drugs. However, there are few published studies investigating such interactions (Cassella *et al*.,

2002), which are vital since essential oils have great potential as adjuvants in the symptomatic management or treatment of a wide range of health problems (Cowan, 1996; De Smet, 1997).

1.12 Future of essential oils

Essential oils applied at the correct concentrations and appropriately administered have resulted in few known complications (Gollin, 1999). As such, physicians who want to include the use of essential oils in their therapies must review literature, participate in established courses, or partner with a qualified and experienced individual (Gollin, 1999). Halcon (2002) mentioned that conservatism is essential to protect patients, but it is also vital to recognize that exposure to essential oils is neither new nor rare. The increasing public and professional interest in essential oils is prompting an urgent need for laboratory and clinical research to expand and clarify the evidence base as well as for additional research on safety and toxicity. The World Health Organization (WHO) Guidelines for the Assessment of Herbal Medicines (WHO, 2003) allows variation in the usual clinical trial path in the case of therapeutic substances that have a long history of apparent safe usage. Some essential oils including those of *M. alternifolia* may fit this criterion (Cox *et al*., 2001). Aromatic plants will continue to be used in South Africa as long as the majority of people keep on consulting traditional healers. Thus, the layperson as well as the medical and traditional healthcare workers need to be better informed in order to optimize safety and therapeutic applications of essential oil preparations (Nchinda, 1998).

While the reported biological properties are quite varied, there are numerous studies that report on the efficacy of essential oils against bacteria, fungi and parasites (e.g. *Plasmodium falciparum*) and their use as anti-oxidants and acetylcholinesterase inhibitors (Burfiled and Reekie, 2005; Goulart *et al.*, 2004; Nakatsu *et al.*, 2000; Atindehou *et al*., 2004; Pauli and Schilcher, 2004). However, very little information is known about the contribution of the individual essential oil constituents to the overall activity of the crude essential oils and their combined interaction with each other and standard drugs. Thus, this project was undertaken to contribute and enhance this versatile and growing reservoir of scientific knowledge on essential oils.

1.13 Study objectives

1. To assess the biological activity of twenty specific essential oil constituents from seven structural chemical groups. Activities included:
   - 1.1 Antibacterial and antifungal
   - 1.2 Antimalarial
   - 1.3 Anti-oxidant
   - 1.4 Anticholinesterase
2. To determine the toxicity profile of the essential oil constituents.
3. To determine whether the combination of selected essential oil constituents interact in a synergistic, additive, indifferent or antagonistic manner with each other, as well as with standard antimalarial and antimicrobial agents.

CHAPTER TWO

SELECTION OF ESSENTIAL OIL CONSTITUENTS

2.1 Introduction

Essential oils are complex mixtures comprising many single compounds, with each of these constituents contributing to the beneficial or adverse effects of the oils (Dormans and Deans, 2000). Many essential oils have been applied for centuries in local healing rites, however, their composition and biological activity are poorly recorded (Nakatsu *et al*., 2000; Zhou *et al*., 2004). The analysis of essential oils is generally performed using gas chromatography (GC; quantitative analysis) and gas chromatography-mass spectroscopy (GC-MS; qualitative analysis) (Lahlou, 2004b).

Identification of the main components is carried out by the comparison of both the GC retention times and mass spectroscopic (MS) data against those of the reference standards. Lahlou (2004b) reported that, analytical conditions and procedures used should carefully be described and these include:

- Apparatus of oil analysis (make and model number of the equipment);
- Column type and dimensions;
- Carrier gas flow rate;
- The temperature programming conditions including injector temperature, detector and column temperatures, in addition to mass spectra (electronic impact).

Many essential oils are isolated, analysed and their main components are identified, characterized and then published without any biological testing whatsoever (Campbell *et al*., 1997; Nakatsu *et al*., 2000). Their useful biological activities can remain unknown for years (Carson and Riley, 1995; Cowan, 1996). Therefore, there is an urgent need to increase the knowledge base on the potential uses and benefits of essential oils and the specific constituents. Guided by the fact that essential oil constituents vary quantitatively between various plant species (Halcon, 2002), twenty essential oil constituents (EOC´s) were selected based on the reported biological activity.

2.2 Methodology

2.2.1 Materials used

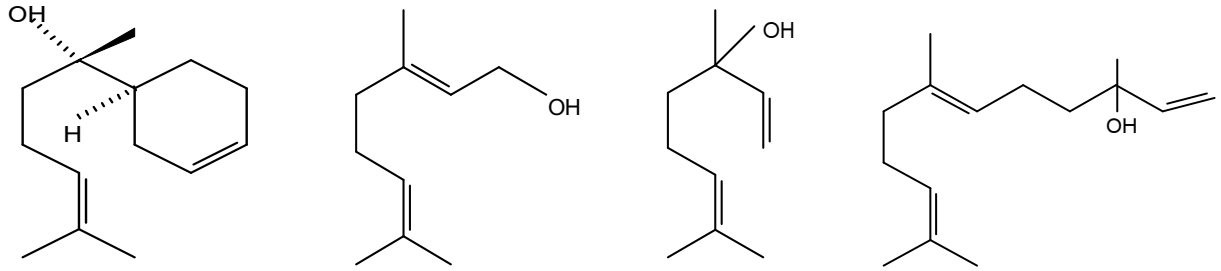
Twenty EOC´s were purchased from Sigma-Aldrich (USA) and Fluka (Switzerland). These EOC´s included both terpene hydrocarbons and oxygenated terpenes (Figure 2.1 and Table 2.1).

2.2.2 Gas chromatographic analysis of essential oil constituents

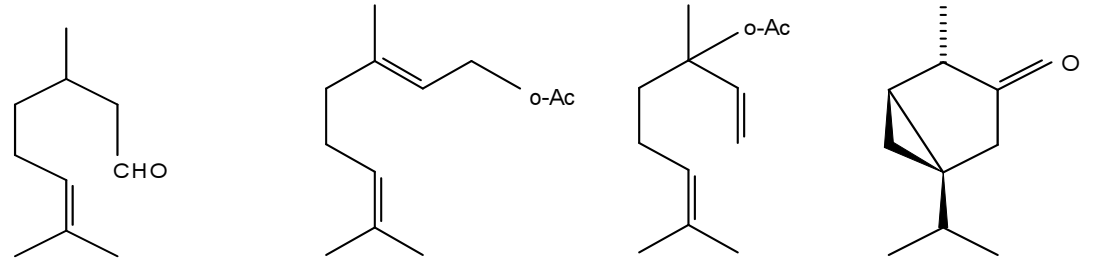
Analysis of all standards was performed on a Shimadzu 17A gas chromatograph using the following conditions; Column: J&W-DB1 (60m x 0.25mm id., 0.25μm film thickness); Temperatures: injection port 230°C, column 60°C for 1min, 5°C/min to 180°C, 180°C for 2min, (total = 25min). Helium was used as a carrier gas.

2.2.3 The chromatogram

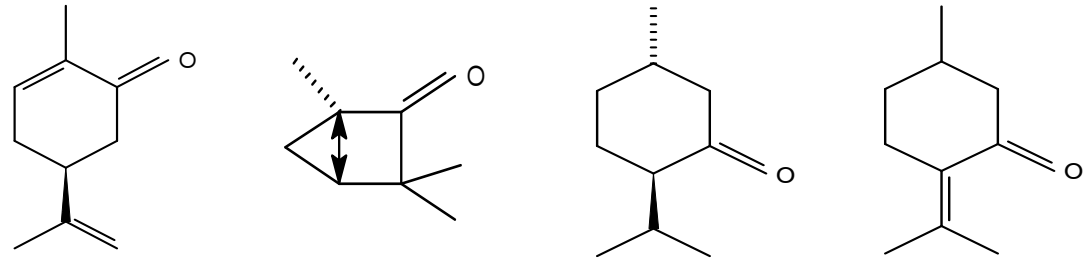
The chromatogram is observed as a series of peaks where each peak represents a chemical compound. The x-axis represents the time scale and the time at which the peak is recorded is called the retention time (R_t). The peak height and peak area is an indication of the quantity of the compound in the mixture. The peak area is integrated as a percent of the total. The purity of the purchased EOC´s was verified by GC to those supplied by Sigma-Aldrich and Fluka (Figure 2.1 and Table 2.2). The specific chirality was not confirmed and the suppliers specifications were considered correct.



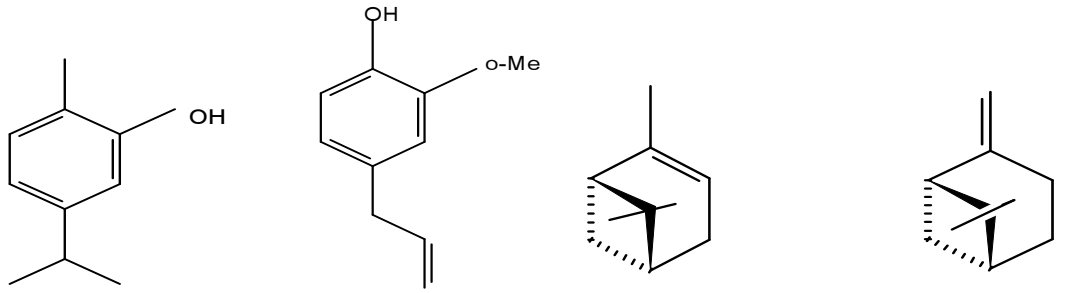
(a) (-)-α-Bisabolol (b) *trans*-Geraniol (c) (±)-Linalool (d) *E*- and *Z*-(±)-Nerolidol



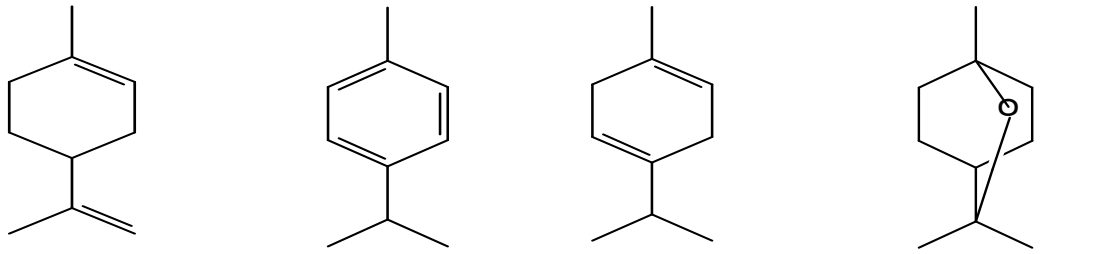
(e) (-)-Citronellal (f) Geranyl acetate (g) Linalyl acetate (h) (±)-α+ β-Thujone



(i) (+)-Carvone (j) (-)-Fenchone (k) (-)-Menthone (l) (-)-Pulegone



(m) Carvacrol (n) Eugenol (o) (+)-α-Pinene (p) (+)-β-Pinene



(q) γ-Terpinene (r) (R)-(+)-Limonene (s) *p*-Cymene (t) 1,8-Cineole

Figure 2.1: The chemical structures of 20 essential oil constituents from seven different structural groups (Nakatsu *et al*., 2000).

Table 2.1: Properties of 20 selected essential oil constituents (values and synonyms have been obtained from the cited references as well as from the supplied compounds, Sigma-Aldrich or Fluka, 2006 and Merck, 2001).

Essential oil constituents	Chemical structure	Empirical formula	Molecular weight (g/mol)	Density (g/l)	Boiling point (°C/mmHg)	Synonyms	References
Alcohols							
(-)-α-Bisabolol	Figure 2.1 a	$C_{15}H_{26}O$	222.37	0.929	157-158	6-Methyl-2-(4-methyl-3-cyclohexene-1-yl)-5-hepten-2-ol	Mahmoud and Croteau (2002)
trans-Geraniol	Figure 2.1 b	$C_{10}H_{18}O$	154.25	0.889	229-230	*trans*-3,7-Dimethyl-2,6-octadien-1-ol; (±)-3,7-Dimethyl-1,6-octadien-3-ol	Nakatsu *et al*. (2000)
(±)-Linalool	Figure 2.1 c	$C_{10}H_{18}O$	154.25	0.870	194-197	(±)-3,7-Dimethyl-3-hydroxy-1.6-octadien-1-ol	Lukwa (1994)
E- and *Z*-(±)Nerolidol	Figure 2.1 d	$C_{15}H_{26}O$	222.37	0.875	114	3,7,11-Trimethyl-1,6,10-dodecatrien-3-ol	Nakatsu *et al*. (2000)
Aldehyde							
(-)-Citronellal	Figure 2.1 e	$C_{10}H_{18}O$	154.25	0.851	207	(*S*)-3,7-Dimethyl-6-octenal; Hydroxy-citronellal	Nakatsu *et al*. (2000)
Esters							
Geranyl acetate	Figure 2.1 f	$C_{12}H_{20}O_2$	196.29	0.916	200-201	*trans*-3,7-Dimethyl-2,6-octadien-1-yl acetate; Geraniol acetate	Onawumni *et al.* (1984)
Linalyl acetate	Figure 2.1 g	$C_{12}H_{20}O_2$	196.29	0.895	115-116	3,7-Dimethyl-1,6-octadien-3-yl acetate	Onawumni *et al.* (1984)

Table 2.1 continued: Properties of 20 selected essential oil constituents.

Essential oil constituents	Chemical structure	Empirical formula	Molecular weight (g/mol)	Density (g/l)	Boiling point (°C/mmHg)	Synonyms	References
Ketones							
(*cis*+*trans*)-(±)α+β-Thujone	Figure 2.1 h	$C_{10}H_{16}O$	152.23	0.920	100	1-Isopropyl-4-methylbicyclo [3.1.0] hexan-3-one	Lahlou (2004b)
(+)-Carvone	Figure 2.1 i	$C_{10}H_{14}O$	150.22	0.960	228-230	*p*-Mentha-6,8-diene-2-one; (*S*)-5-Isopropenyl-2-methyl-2-cyclohexenone	Campbell *et al.* (1997)
(-)-Fenchone	Figure 2.1 j	$C_{10}H_{16}O$	152.24	0.945	191-195	(1R)-1,3,3-Trimethylbicyclo[2.2.1] heptan-2-one; 2-Norbornanone; (−)-1,3,3-Trimethyl-2-norbornanone; (-)-1,3,3-Trimethylnorcamphor	Nakatsu *et al.* (2000)
(-)-Menthone	Figure 2.1 k	$C_{10}H_{18}O$	154.25	0.896	85-88	ρ-Menthan-3-one; 2-Isopropyl-5-methylcyclohexanone	Zhou *et al.* (2004)
(-)-Pulegone	Figure 2.1 l	$C_{10}H_{16}O$	152.24	0.936	223-224	(*S*)-2-Isopropylidene-5-methylcyclo-hexanone; ρ-Menth-4-(8)-en-3-one	Zhou *et al.* (2004)
Phenols							
Carvacrol	Figure 2.1 m	$C_{10}H_{14}O$	150.22	0.976	236-237	5-Isopropyl-2-methylphenol; ρ-Cymen-2-ol	Nakatsu *et al.* (2000)
Eugenol	Figure 2.1 n	$C_{10}H_{12}O_2$	164.21	1.067	254	2-Methoxy-4-(2-propenyl) phenol ; 4-Allylguaiacol	Nakatsu *et al.* (2000)

Table 2.1 continued: Properties of 20 selected essential oil constituents.

Essential oil constituents	Chemical structure	Empirical formula	Molecular weight (g/mol)	Density (g/l)	Boiling point (°C/mmHg)	Synonyms	References
Terpene Hydrocarbons							
(+)-α-Pinene	Figure 2.1 o	$C_{10}H_{16}$	136.24	0.858	155-156	(1*R*,5*R*)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene; (1*R*,5*R*)-2-Pinene; Pseudopinene;	Cowan (1996)
(+)-β-Pinene	Figure 2.1 p	$C_{10}H_{16}$	136.24	0.872	164-165	(1R,5R)-6,6-Dimethyl-2-methylenebicyclo [3.1.1]heptane; (1R,5R)-2(10)-Pinene; Nopinene	Dormans and Deans (2000)
γ-Terpinene	Figure 2.1 q	$C_{10}H_{16}$	136.24	0.848	183-186	1-Isopropyl-4-methyl-1,4-cyclohexadiene; ρ-Menth-1-en-8-ol	Halcon (2002)
(R)-(+)-Limonene	Figure 2.1 r	$C_{10}H_{16}$	136.24	0.840	176-177	(*R*)-4-Isopropenyl-1-methyl-1-cyclohexene; (+)-*p*-Mentha-1,8-diene; (+)-Carvene; Dipentene	Lahlou (2004b)
P-Cymene	Figure 2.1 s	$C_{10}H_{14}$	134.22	0.860	176-178	1-Isopropyl-4-methylbenzene; 4-Isopropyltoluene; ρ-Cymol	Buchbauer (1993)
Oxide							
1,8-Cineole	Figure 2.1 t	$C_{10}H_{18}O$	154.25	0.921	176-177	1,3,3-Trimethyl-2-oxabicyclo[2.2.2] octane; Eucalyptol	Campbell *et al.* (1997)

Table 2.2: Verification of percentage purity of 20 selected essential oil constituents.

Essential oil constituents	Determined Purity (%)	Reported Purity (%)
Alcohols		
(-)-α-Bisabolol	90.32	~97.0
trans-Geraniol	95.65	98.0
(±)-Linalool	97.00	97.0
E- and *Z*-(±)-Nerolidol	94.43	98.0
Aldehyde		
(-)-Citronellal	100.00	≥98.0
Esters		
Geranyl acetate	98.73	98.0
Linalyl acetate	96.81	97.0
Ketones		
(±)-α+β-Thujone	98.73	~98.0
(+)-Carvone	96.81	≥99.0
(-)-Fenchone	96.81	~98.0
(-)-Menthone	80.85	≥97.0
(-)-Pulegone	98.62	~99.0
Phenols		
Carvacrol	95.48	~98.0
Eugenol	96.32	~97.0
Terpene Hydrocarbons		
(+)-α-Pinene	95.48	~99.0
(+)-β-Pinene	96.32	≥97.0
γ-Terpinene	94.71	98.0
(R)-(+)-Limonene	92.98	97.0
P-Cymene	93.19	≥99.5
Oxide		
1,8-Cineole	93.13	≥99.7

CHAPTER THREE
ANTIMALARIAL ACTIVITY

3.1 Definition of malaria

Malaria is a common public health catastrophe affecting millions of people throughout the tropics and subtropics (Nchinda, 1998). People thought that this life threatening disease came from fetid marshes, hence the name ´*mal aria*` (bad air) (Hayward *et al*., 2000). Later in 1880, scientists discovered the real cause, a one-cell protozoal parasite named *Plasmodium* (Hayward *et al*., 2000). It was found to be transmitted from person to person through the bite of a female *Anopheles* mosquito, which depends on a blood meal to mature her eggs (Nchinda, 1998). Less common modes of transmission include: inoculation of infected blood, use of contaminated needles and from an infected mother to her infant during pregnancy (Nchinda, 1998).

3.1.1 Malaria burden

Today malaria causes more than 300 million acute illnesses and at least one million deaths annually in the world (Atindehou *et al*., 2004; Benoit-Vical *et al*., 1999; WHO, 2005). It continues to be a major obstacle in the social and economic development of developing countries. In the last decade, the prevalence of malaria has been escalating at an unprecedented and uncontrolled rate, especially in Africa (WHO, 2005). Cases in Africa account for 90% of malaria cases in the world, and the disease reduces the working capacity of those infected (Nchinda, 1998). It affects the overall quality of life and undermines efforts at sustainable development. Young adults and adolescents are now dying of severe forms of the disease, where in Africa alone it is estimated that more than a million children die of malaria each year (Boyom *et al.*, 2003). Between 1994 and 1996, malaria epidemics in 14 countries of Sub-Saharan Africa resulted in an unacceptably high number of deaths, in many areas previously free of the disease (Nchinda, 1998). In South Africa alone about 13399 malaria cases and 89 fatal cases were reported in 2004, while in 2005, 4539 malaria cases and 35 deaths were reported (Department of Health, 2005).

3.1.2 Malaria distribution

Many of the tropical developing countries in Africa, America and Asia continue to experience the occurrence of malaria (WHO, 2005). In South Africa, malaria occurs more commonly in low altitude areas below 1000m in north eastern KwaZulu-Natal, Limpopo and Mpumalanga.

Other limited areas in which malaria may occasionally occur are the north west and northern Cape Provinces along the Molopo and Orange rivers. Malaria is a distinctly seasonally based problem with the highest risk occuring during the wet summer months (October to May) (Department of Health, 2005). Malaria cases have also been reported in Gauteng with 366 cases and five deaths being reported since the beginning of 2006. These cases were due to people returning from holiday in countries outside the borders of South Africa. But cases reported in Johannesburg have been attributed to mosquitoes being imported from malaria areas by being transported in planes, motor vehicles and trains (Department of Health, 2005).

3.1.3 Signs and symptoms of a malaria infection

Human malaria is an infectious disease caused by a protozoan of the genus *Plasmodium* and the subspecies *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. In Sub-Saharan Africa over 90% of human malaria infections are due to *P. falciparum* and is accountable for severe morbidity and mortality. The other three species cause a milder illness, with *P. ovale* and *P. vivax* infections sometimes recurring if treatment is not appropriate. Infections are characterized by a high degree of parasitaemia causing death through complications such as renal failure, severe haemolysis and anaemia, pulmonary oedema and a variety of serious neurological disorders (Noedl *et al.*, 2003). The symptoms of a malaria infection develop after 10 – 14 days following a mosquito bite. These may initially resemble a “flu-like” illness with one or more of the following; fever, rigors, headache, sweating, fatigue, mylagia, diarrhoea, loss of appetite, nausea, vomiting and a cough. The most severe manifestations are cerebral malaria (mainly in children and persons without previous immunity) and anaemia (mainly in children and pregnant women) (Noedl *et al.*, 2003). Persons repeatedly exposed to the disease acquire a considerable degree of clinical immunity, but is unstable and disappears after a year away from the endemic-disease environment (Nchinda, 1998).

3.1.4 Diagnosis

The diagnosis of malaria should be an early and serious consideration for any patient with fever who has travelled to or lives in a malaria area, even if chemoprophylaxis has been taken (Hayward *et al.*, 2000; Noedl *et al.*, 2003). An examination of blood for parasites can be performed by a rapid malaria test (dipstick method), a blood smear or flow cytometry (Boyom *et al.*, 2003). In recent years a number of new techniques based on the ´dip-stick` format have become available for diagnostic purposes. Hayward *et al.* (2000) reported that these methods detect plasmodial histidine rich protein-2 (HRP-2) in *P. falciparum* or parasite-specific lactate

dehydrogenase (pLDH), which can be used to differentiate between *P. falciparum* and *P. vivax* infections (Boyom *et al.*, 2003).

3.1.5 Life cycle of *Plasmodium*

The life cycle of the *Plasmodium* malaria parasite involves two hosts (Figure 3.1), namely: human (host stage) and the female *Anopheles* mosquito (vector stage). During a blood meal, a malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host ❶. Sporozoites infect the liver cells ❷and mature into schizonts ❸, which rupture and release merozoites ❹. (Of note, in *P. vivax* and *P. ovale* a dormant stage [hypnozoites] can persist in the liver and cause relapses by invading the bloodstream weeks, or even years later.) After this initial replication in the liver (exo-erythrocytic schizogony Ⓐ), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony Ⓑ). Merozoites infect the red blood cells ❺. The ring stage trophozoites mature into schizonts, which rupture releasing merozoites ❻. Some parasites differentiate into sexual erythrocytic stages (gametocytes) ❼. Blood stage parasites are responsible for the clinical manifestations of the disease.

The gametocytes, male (microgametocytes) and female (macrogametocytes), are ingested by an *Anopheles* mosquito during a blood meal ❽. The parasites' multiplication in the mosquito is known as the sporogonic cycle Ⓒ. While in the mosquito's stomach, the microgametes fuse with the macrogametes generating zygotes ❾. The zygotes in turn become motile and elongate into ookinetes ❿which invade the midgut wall of the mosquito where they develop into oocysts ⓫. The oocysts grow, rupture and release sporozoites ⓬, which make their way to the mosquito's salivary glands. Inoculation of the sporozoites ❶ into a new human host perpetuates the malaria life cycle.

3.1.5.1 Human stage

The life cycle within the human host occurs in both erythrocytes (intra-erythrocytic) and in the hepatic cells (extra-erythrocytic) (Nchinda, 1998). The mosquito infected with *Plasmodium* injects the sporozoites through the skin of human host, which travel within seconds to the liver via the bloodstream (Figure 3.1).

In the hepatocytes, sporozoites develop into exo-erythrocytic schizonts and undergo nuclear division to mature into approximately 32000 daughter merozoites that are released into the blood stream. Within seconds the merozoites penetrate the erythrocytes, and initiate the asexual intra-erythrocytic life cycle. The merozoites are small in size, approximately 1µm in

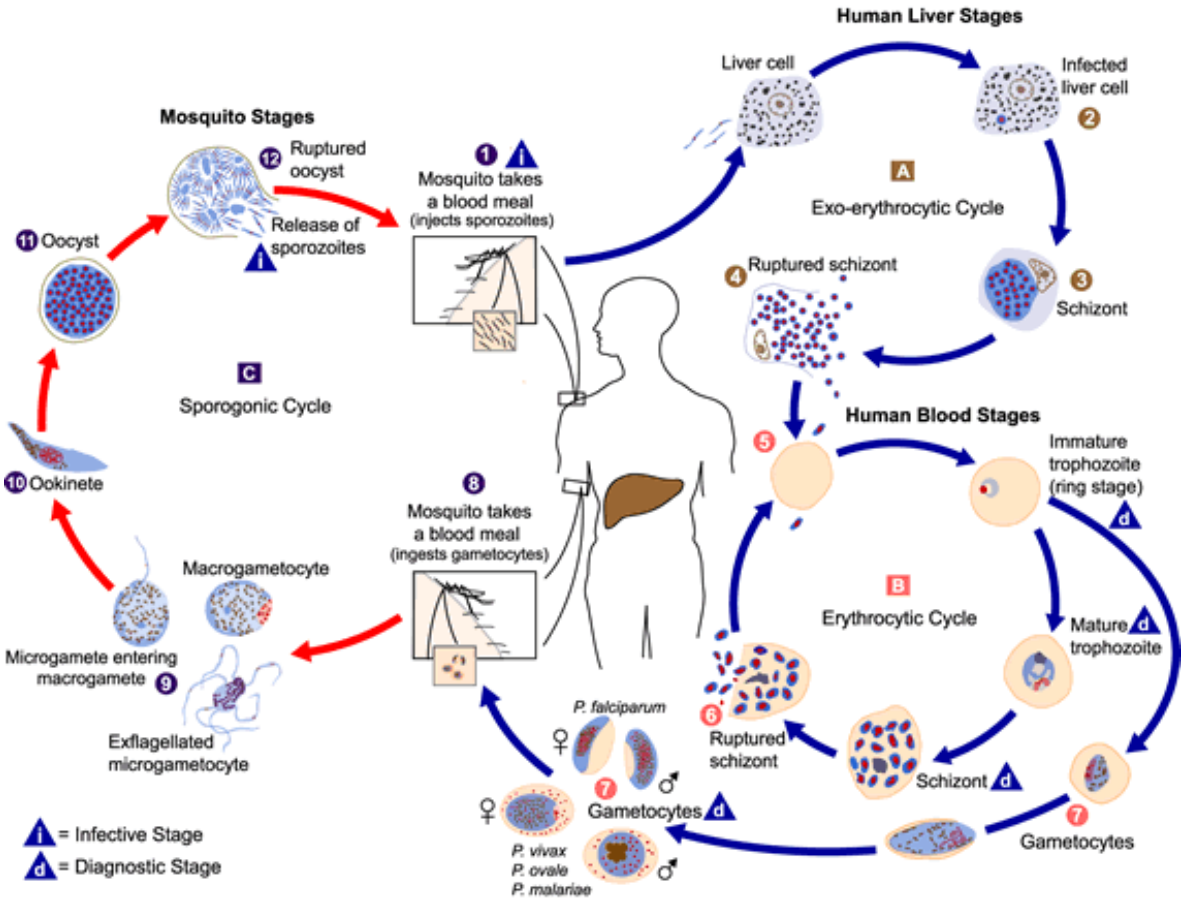


Figure 3.1: The various stages of the life cycle of the malaria parasites (http://www.cdc.gov/malaria/biology/lifecycle.htm).

diameter and each consists of a single nucleus in the cytoplasm (Noedl *et al.*, 2003). A vacuole forms in the parasite as it transforms into the ring stage. After about 12 - 24h the yellow-brown crystalline pigment appears in a food vacuole. The malaria pigment or haemozoin is the inert end product of the digested red blood cell haemoglobin, which was endocytosed as a source of amino acids essential for the parasites (Noedl *et al.*, 2003). The parasite appears as a solid body and occupies a variable portion in the host cell, and is now known as a trophozoite (Figure 3.1).

When nuclear division starts to occur within the trophozoite, the parasite is referred to as a schizont. The nucleus starts to divide very quickly in *P. falciparum* as compared to other species. Nuclear division continues until the schizont matures with the formation of 16 - 32 daughter merozoites (Boyom *et al.*, 2003). The erythrocyte-containing schizont ruptures releasing the merozoites into the plasma, such that they reinvade new erythrocytes, thereby initiating the next cycle of asexual development (Figure 3.1). Some of these merozoites

differentiate into the sexual forms of the parasite: the macrogametocytes (female) and the microgametocytes (male) (Boyom *et al*., 2003). The male gametocyte is more easily distinguishable than the female gametocyte by its larger nucleus and the pale cytoplasm.

3.1.5.2 Vector stage

This next phase that occurs in the female *Anopheles* mosquito is referred to as sporogony (i.e. spore formation). The blood is ingested into the midgut of the insect, where the macrogametocyte rapidly escapes the erythrocyte to form a macrogamete. Microgametogenesis is a slower process, but after about ten minutes, the nucleus divides into eight flagellated microgametes that are actively motile and break free from the parent body and move quickly to fertilize the female macrogamete (Noedl *et al*., 2003). The two nuclei fuse and a zygote is formed, which elongates into a slow motile ookinete (Nchinda, 1998).

The ookinete penetrates the epithelial layer of the midgut and comes to rest on the external surface, where it develops into an oocyte. The oocyte is enclosed within a thin cyst wall, and contains a single nucleus, pigment granules and small residual structures of the ookinete. The oocyte expands and its nucleus divides repeatedly until maturity is reached. From a single oocyte, nuclear division gives rise to approximately 10000 sporozoites (Noedl *et al*., 2003). The mature oocyte ruptures and the sporozoites travel in the haemocoelomic fluid from the midgut and accumulate in the acrinal cells of the salivary glands of the mosquito. Thus, when the mosquito bites the human host, the sporozoites pass with the saliva into the host, and the life cycle repeats itself.

3.1.6 Parasite resistance to classical antimalarial drugs

Parasite populations are known to adapt in an environment where a sub-therapeutic concentration of an antimalarial agent is introduced and thereby initiating drug resistance (Noedl *et al*., 2003). Malaria parasites are capable of passing their genetic information to future generations to ensure the succession of drug-resistant malaria parasites (Goulart *et al*., 2004). Drug resistance in malaria has therefore also been defined as the ability of the parasite strain to survive and/or multiply, despite the administration and absorption of the drug given in doses higher than those usually recommended, but within the limits of tolerance of the subjects (Noedl *et al*., 2003).

A factor contributing to the development of resistance is drug failure, which involves pharmacokinetic issues such as absorption, bioavailability (distribution and metabolism),

elimination and dosage regimen. If sufficient amounts (i.e. the low doses) of the active metabolites of a drug cannot reach the target site, the drug will not be able to kill and clear the parasite from the patient's body (Noedl *et al*., 2003). But these sub-therapeutic doses allow for the more resistant strains to survive, whilst the sensitive ones are killed. This contributes to the ever increasing number of cases of severe malaria and even deaths. Treatment failure, poor patient compliance and inappropriate drug regimens have all contributed to the development of multi-drug resistant parasites.

Advances in molecular techniques, have allowed several markers to be identified and have been investigated for their association with antimalarial drug resistance (Hayward *et al*., 2000). Resistance has developed more rapidly against the folate antagonists, which include pyrimethamine, sulfadoxine, proguanil and dapsone. The synergistic interaction between pyrimethamine and sulfadoxine inhibiting the consecutive dihydrofolate reductase and dihydropteroate synthetase enzymes, respectively, prevents the synthesis of folinic acid and subsequent DNA/RNA synthesis (Gregson and Plowe, 2005). This combination has up until the early 2000´s been the first line of treatment in uncomplicated cases, but the development and spread of resistance to this effective combination have necessitated alternative therapeutic agents (Noedl *et al*., 2003). The association between pyrimethamine resistance and point mutations on the dihydrofolate reductase (*dhfr*) gene, as well as sulfadoxine resistance and point mutations on dihydropteroate synthetase (*dhps*) gene, have been well documented (Hayward *et al*., 2000; Gregson and Plowe, 2005).

The first cases of *P. falciparum* resistance to chloroquine were recorded in 1959 in Thailand, and from then on there was rapid worldwide spread of chloroquine-resistant *P. falciparum* malaria (Hayward *et al*., 2000). With the first case in Africa being reported in 1979 in Kenya (WHO, 2003). However, despite the extensive spread of resistance in *P. falciparum*, chloroquine is still widely used to treat *P. vivax*, as sporadic resistance cases have only recently been reported. Currently in South Africa, the prophylactic combination of chloroquine and proguanil is less favoured over mefloquine, doxycycline and the new combination of atovaqoune and proguanil (Department of Health, 2005). But monotherapy of chloroquine is still used in Mozambique to maintain asymptomatic parasitaemias in patients to induce semi-immunity in the infected patients.

The resistance of the parasite to chloroquine ranges from a minimal loss of effect, detected only by delayed recrudescence (RI), to a high level of resistance, at which the drug has no

suppressive effect (RIII) (WHO, 2005). These varying levels of resistance are attributed to various genetic changes (Hayward *et al*., 2000; WHO, 2005).

3.1.7 Antimalarial drug development

Several attempts have been made to synthesize a vaccine or monoclonal antibodies to alter immune response to a malaria infection (Noedl *et al*. 2003; Nchinda, 1998). However, no therapeutic agent has as yet been successful throughout the continent. Thus, until the release of a successful vaccine, therapeutic management includes preventative non-pharmacological measures as well as antimalarial drugs.

In the last 30 years very few antimalarial drugs have been developed (Ridley, 2002), and it is now common practice to use these drugs in combination in attempts to decrease the rate of resistance from developing.

Looking at the history of antimalarial drug development, it could be noted that the majority were plant-derived. One of the most important lead compounds against malaria is quinine, isolated from *Cinchona* bark and was used as the template for the synthesis of chloroquine and mefloquine (Schwikkard and van Heerden, 2002). More recently, artemisinin isolated from the Chinese plant *Artemisia annua*, has successfully been used to synthesize a series of derivatives to which chloroquine-resistant *P. falciparum* are susceptible.

With the realisation that medicinal plants could yield more lead compounds or structures modelled on these molecules, there has been a renewed interest in phytomedicine and traditional remedies (Anthony *et al*., 2005). According to the South African Health Ministry, an estimated 70% of the population uses traditional medicines on a regular basis (Scott *et al*., 2004). For example, most of the South Africans treat various illnesses including malaria and its associated symptoms by relying on the use of herbal plant remedies (Watt and Breyer-Brandwijk, 1962). Approximately 500 plant species are currently used as traditional medicines in South Africa. A range of these medicinal plants have been scientifically validated for their antimalarial properties and have shown potential to be as effective as Western medicine (Prozesky *et al*., 2001; Clarkson *et al*., 2004; Kamatou *et al*., 2005; Waako *et al*., 2005).

When evaluating the pharmacological effects of plants, most studies have concentrated on the solvent extracts (aqueous or alcoholic), with little work carried out on the purified essential

oils. The essential oils could be as or more effective in treating or preventing a parasitic disease (Anthony *et al*., 2005; Burfield and Reekie, 2005). Several studies have shown the antimalarial activity of essential oils isolated from plants such as *Salvia* (Kamatou *et al*., 2005), *Helichrysum* (van Vuuren *et al*., 2006), *Cochlospermum* (Benoit-Vical *et al*., 1999), *Lippia* (Valentin *et al*., 1995), *Virola* (Lopes *et al*., 1999) and *Tetradenia* (Campbell *et al*., 1997).

Although these studies have determined the composition of the essential oil, there has been little or no work done with regards to which of the major and/or minor constituents are responsible for the inhibitory activity. Thus, it was the aim of this study to investigate the antimalarial effects of 20 essential oil constituents (EOC´s) that would be present in varying quantities in these active plant essential oils. Since the essential oil is composed of numerous EOC's, it is likely that an interaction will exist between the EOC´s. To examine this possibility, selected EOC´s with varying activity profiles were combined and their pharmacological interaction recorded.

3.2 Methodology

3.2.1 Culture maintenance

The chloroquine-resistant strain of *Plasmodium falciparum* (FCR-3) was continuously maintained *in vitro* according to the method of Jansen and Trager (1976) and the departmental protocol. The parasites were maintained in culture in human red blood cells (RBC´s) to which complete culture medium (Section 3.2.1.1) was added. Parasitised red blood cells (pRBC´s) were maintained at a parasitaemia of 5-10% and haematocrit of 5%. The stock culture was flushed with a gas mixture of 5% CO_2, 3% O_2 and 92% N_2, sealed and incubated at 37ºC. There was a fast growth rate of the stock culture as seen by the doubling of parasitaemia every 42 - 48h. In order to ensure optimal growth of the parasites, the culture medium was replaced daily, and the culture was diluted with freshly washed red blood cells every second day when the parasites were in the trophozoite-schizont stage.

3.2.1.1 Preparation of complete culture medium

The culture medium was prepared in autoclaved Milli-Q® water with: 10.4g/l RPMI-1640 (Highveld Biologicals, South Africa), 5.9g/l HEPES (N-2-hydroxyethyl-piperazine-N-2-ethane-sulfonic acid) buffer (Merck), 4.0g/l D-glucose (Merck), 44mg/l hypoxanthine (Sigma-Aldrich) and 50mg/l gentamicin sulphate (Sigma-Aldrich). The culture medium was sterilized through a Sterivex-GS® 0.22µm filter and stored at 4ºC. Before addition to the

cultures, the complete hypoxanthine culture medium was prepared by adding 10% (v/v) human plasma (Section 3.2.1.2) and 4.2ml of sterile sodium bicarbonate [$NaHCO_3$; 5% (w/v)] (Merck), to adjust the pH of the complete culture medium to approximately 7.4.

3.2.1.2 Plasma preparation

Human plasma was obtained from the South African Blood Transfusion Service and heat inactivated at 56ºC in a water bath for 2h. The inactivated plasma was centrifuged at 3000rpm for 10min. The supernatant was aliquoted into 50ml sterile tubes and stored at -20ºC until required.

3.2.1.3 Red blood cell preparation

The whole blood was drawn and stored in a test tube containing citrate phosphate dextrose adenosine-1 (CPDA) (to avoid coagulation) at 4ºC for 3 weeks. The whole blood was washed three times by centrifugation at 2000rpm for 5min in phosphate buffer saline (PBS, pH 7.4) solution. Care was taken to remove the buffy coat. The PBS solution consisted of 136.89mM NaCl (Merck), 4.02mM KCl (Merck), 4.10mM $Na_2HPO_4.2H_2O$ (Merck) and 1.47mM KH_2PO_4 (Merck), which was sterilized by autoclaving the solution at 120ºC for 20min. To avoid dehydration the washed red blood cells were suspended in experimental medium and stored at 4ºC. The experimental medium was prepared as the hypoxanthine containing culture medium (Section 3.2.1.1), but did not contain gentamicin and hypoxanthine.

3.2.1.4 Assessment of parasite growth using light microscopy

To ensure optimal growth conditions, the culture was examined microscopically each day. A thin smear was made by taking a small amount of blood from the bottom of the culture flask and depositing it on a clean slide. The smear was air dried before being fixed with 100% methanol. Thereafter, the dried slide was immersed in diluted Giemsa stain for approximately 20min. The diluted stain consisted of neat Giemsa stain (BDH) diluted in a Giemsa buffer in a 1:10 (v/v) ratio. The Giemsa buffer consisted 26mM KH_2PO_4 and 40mM $Na_2HPO_4.12H_2O$. The slide was then washed with water, dried and examined under oil immersion at 1000x magnification. From the slide, the morphology of the parasites was determined and the percentage parasitaemia in at least 10 fields was calculated using Equation 3.1.

Equation 3.1:

$$\% \text{ Parasitaemia} = \frac{\text{Total number of parasitised red blood cells} \times 100}{\text{Total number of uninfected + parasitised red blood cells}}$$

3.2.2 Synchronization of the culture

In order to obtain a culture consisting predominately of ring-infected RBC´s the methodology described by Lambros and Vanderberg (1979) was used. The pelleted RBC´s were treated with 10 volumes of 5% (w/v) D-sorbitol (Merck) for 10 - 20min at room temperature. As a result of an osmotic action, D-sorbitol has the ability to lyse the mature trophozoite and schizont stages but does not affect the ring-infected and uninfected RBC´s. The culture was then centrifuged at 2000rpm for 5min and the resultant pellet resuspended in complete hypoxanthine culture medium (Section 3.2.1.1), gassed and incubated at 37ºC.

3.2.3 Tritiated hypoxanthine incorporation assay

To determine the effects of the EOC´s on parasite growth, the basic method of tritiated hypoxanthine uptake assay according to Desjardins *et al*. (1972) was used. Hypoxanthine is required by the parasite for DNA synthesis. The parasites cannot synthesise hypoxanthine *de nova* and thus relies on the human host to supply the nucleotide precursor. The uptake of radio-labelled tritiated hypoxanthine was used as a marker of the sensitivity of parasites to the potentially novel antimalarial agents. The experiment was repeated thrice to ensure that there was consistency among all runs.

3.2.3.1 Preparation of uninfected red blood cells

Uninfected control RBC´s were washed three times by centrifugation at 2000rpm for 5min in PBS buffer (Section 3.2.1.3), before being suspended at a 1% haematocrit in complete experimental medium. The complete experimental medium consisted of experimental medium (Section 3.2.1.3) with 10% human plasma and 4.2ml of 5% (w/v) $NaHCO_3$ (Section 3.2.1.1).

3.2.3.2 Preparation of the parasites

Ten fields of the Giemsa stained blood smear were counted to obtain the average percentage parasitaemia. The stock culture was centrifuged for 5min at 2000rpm. The supernatant was discarded and parasite pellet resuspended in PBS and centrifuged again. To remove any trace amounts of hypoxanthine this was done three times. Ten fields of the Giemsa stained blood smear were counted to obtain the average percentage parasitaemia (Section 3.2.1.4). The parasitaemia was then adjusted to 0.5% with the addition of washed uninfected RBC´s and the haematocrit to 1% with the addition of complete experimental media.

3.2.3.3 Preparation of the drug and essential oil constituents

The effect of quinine and EOC´s on the resistant strain of *P. falciparum* was tested. Stock solutions were freshly prepared before each experiment and the highest possible grades of the drug and EOC´s were purchased from Sigma-Aldrich and Fluka.

3.2.3.3.1 Preparation of the classical antimalarial drug

Quinine sulphate (M_r = 324.43g/mol) (Sigma-Aldrich) served as the reference antimalarial agent. The stock solution of quinine was prepared with a dilution factor of 10 taken into account. This stock solution was prepared in autoclaved Milli-Q® water, to the concentration of 0.1mM. The dilutions were prepared in plastic eppendorf tubes using incomplete experimental medium.

3.2.3.3.2 Preparation of essential oil constituents

The stock solution of 10% (v/v) of each EOC was prepared by diluting 10µl of neat pure EOC in 90µl of 100% DMSO (dimethyl sulfoxide) (Merck). Notably the density of each EOC was taken into account when the stock solution was prepared. All further EOC dilutions were made up in plastic eppendorfs using DMSO. The dilution factor resulting from addition to wells in plates was taken into account and determined to be 125x by dividing the amount of oil added (2µl) plus 23µl of experimental medium into the total volume of the wells (250µl).

3.2.3.4 Preparation of microtitre plates

Two microlitres of EOC dilution plus 23µl of incomplete experimental medium were added in triplicate from A1 – G12 (Figure 3.2). Thereafter, 200µl of the parasite suspension (Section 3.2.3.2) was added to all the wells except the first four wells of row H. These wells served as the uninfected RBC´s control, where 200µl of 1% (v/v) uninfected RBC suspension (Section 3.2.3.1) was added. The last eight wells served as the drug-free parasitised RBC´s control. To ensure that the total volume in each well was 250µl, 25µl of experimental medium was added to all wells of row H.

3.2.3.5 Incubation in a micro-aerobic environment

To create a micro-aerobic environment suitable for optimal parasite growth, the candle jar method described by Jansen and Trager (1976) was used. After preparing the microtitre plates, they were placed in an airtight humified glass desiccator with candles. These candles were lit before the cover was fitted and when the flame died the gas content in the candle jar

was similar to that used to maintain the cultures, namely 3% O_2, 5% CO_2 and 92% N_2. The candle jar was then incubated for 24h at 37ºC.

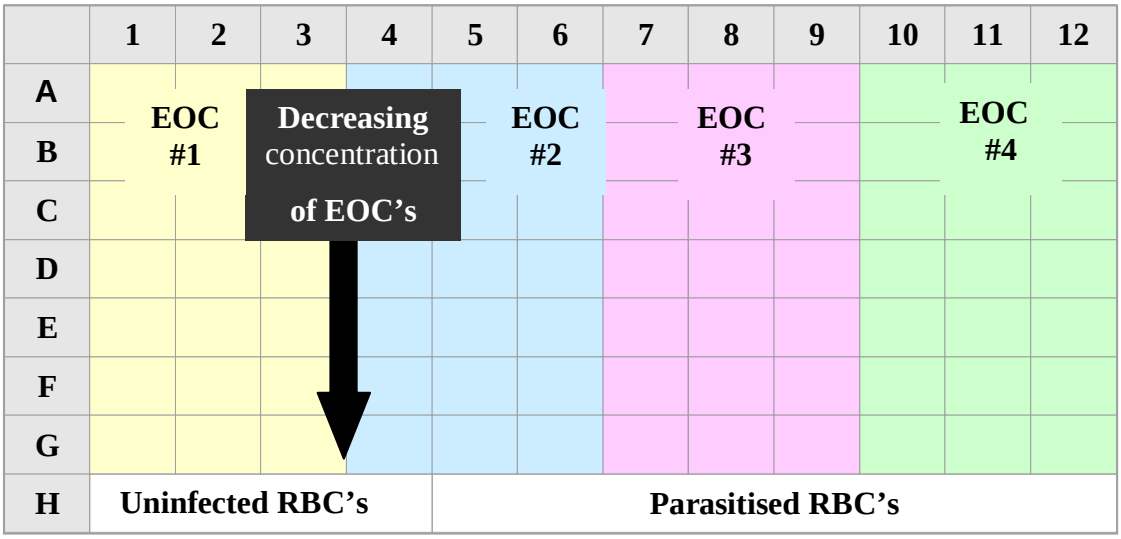


Figure 3.2: A schematic representation of the 96-well microtitre plate.

3.2.3.6 Preparation of the isotope and labelling of parasites

Tritiated labelled hypoxanthine (5mCi/ml) (Amersham Pharmacia Biotech, UK) was stored in 50% ethanol/water at -20ºC. Ten microlitres of [^{3}H]-hypoxanthine was taken from the stock solution and the ethanol was evaporated off with sterile filtered CO_2 (0.22µm filter). Thereafter, 2.7ml experimental medium was added to the isotope and 25µl of this mixture (0.5µCi) was added to each well. The plates were returned to the candle jar as before for a further 24h. This incubation period is hereafter referred to as the "single parasitic erythrocytic life cycle" as the typical life cycle of *P. falciparum* takes 42 - 48h to complete (Jansen and Trager, 1976).

3.2.3.7 Harvesting and scintillation counting

After 48h of incubation, a semi-automated cell harvester (Titertek® cell harvester, Flow Laboratory, Norway) was used to aspirate the parasite suspension from each well and deposit the parasitic DNA onto a glass fibre (GFB) filtermat. Thereafter, the filtermat was allowed to dry overnight. The filtermat along with 10ml Wallac Betaplate Scint® scintillation fluid was enclosed in a sample bag and heat sealed. Thereafter, the filtermat was correctly aligned in a cassette and placed in a Wallac Betaplate® liquid scintillation counter and each sample counted for one minute. The results from the scintillation counter were expressed in counts per minute (CPM). Equation 3.2 was used to express the CPM values as a percentage [^{3}H]-

hypoxanthine incorporated into the parasite DNA, with the uninfected RBC and parasitised controls taken into account.

Equation 3.2:

$$\% [^3H]\text{-Hypoxanthine incorporation} = \frac{\text{CPM (EOC)} - \text{CPM (mean uninfected RBC control)} \times 100}{\text{CPM (mean parasitised RBC control)} - \text{CPM (mean uninfected RBC control)}}$$

The concentration that inhibited 50% of [^{3}H]-Hypoxanthine incorporation or parasite growth (IC_{50} values) was obtained from the log sigmoid dose response curve generated by the Enzfitter® software (Biosoft, UK).

3.2.4 The combinations between the standard antimalarial and essential oil constituents

To evaluate the interactions between the EOC´s, the above described tritiated hypoxanthine incorporation assay (Section 3.2.3) was modified to accommodate testing selected EOC´s in combination. These combinations were repeated in triplicate to confirm consistency. Based on the antimalarial activity of the individual EOC´s, eight combinations were selected as seen in Table 3.1, and one of the most active EOC (*E*- and *Z*-(±)-nerolidol) was also combined with quinine.

Table 3.1: Essential oil constituents used in the combination study (n = 3).

EOC_1	**EOC_2**
Linalyl acetate	(+)-α-Pinene
Linalyl acetate	Carvacrol
p-Cymene	*E*- and *Z*-(±)-Nerolidol
E- and *Z*-(±)-Nerolidol	(-)-Pulegone
(-)-Pulegone	*p*-Cymene
(+)-α-Pinene	Carvacrol
Carvacrol	*p*-Cymene
p-Cymene	γ-Terpinene

3.2.4.1 Essential oil constituent and drug preparation

Eight fixed ratios of the EOC's depicted in Table 3.1 were prepared such that as the concentration of one EOC increased, the concentration of the other decreased. Both EOC's were also prepared in the absence of the other EOC to serve as a positive control. The eight

dilutions for each individual EOC were prepared in DMSO as described above (Section 3.2.3.3.2).

The dilutions were then combined in a 1:1 (v/v) ratio. A further dilution factor of 2 was taken into account, so that the final dilution factor increased to 250x for the EOC's and 20x for quinine. In the wells of row A, 2µl of the combined EOC's and 23µl of experimental medium were plated out in triplicate. From row B to F, a 1:10 serial dilution was prepared in experimental medium, while a 1:100 dilution was prepared in row G. After serially diluting the combined EOC's, 65µl of the content from row B – F was discarded and 175µl discarded from row G. To the remaining 25µl EOC's, 200µl parasitised suspension was plated out as described in Section 3.2.3.3 and incubated for 24h (Section 3.2.3.5), before [^{3}H]-hypoxanthine was added for a further 24h (Section 3.2.3.6) and then the DNA harvested and filtermats counted (Section 3.2.3.7).

3.2.5 Data analysis

The data was initially analysed as for the individual EOC´s (section 3.2.3). From the eight log sigmoidal dose response curves, eight IC_{50} values were calculated, and in turn the IC_{50} values for the other EOC's were calculated using direct proportion. The drug ratio values (or otherwise known as fractional inhibitory concentration (FIC) values) were calculated by dividing the IC_{50} values of each EOC's combination by the IC_{50} values of the individual EOC.

3.2.5.1 Isobologram construction

The drug ratio values (IC_{50} value of combined EOC's/IC_{50} value of individual EOC) were then used to construct an isobologram, from which the nature of the interaction between the two EOC´s could be determined as seen in Figure 3.3 (Berenbaum, 1989; Meadows *et al*., 2002). Each experimental run was done in triplicate to confirm consistency. In order to observe the interaction between EOC´s and classical antimalarial agents used in the treatment of malaria, one of the most active EOC´s was combined with quinine. Quinine was prepared as described in Section 3.2.3.3.1. It must be noted that there may be some points on the isobolograms overlying on each other and cannot be clearly seen sometimes. This can be the case irrespective that six fixed ratios of the two EOC´s were combined.

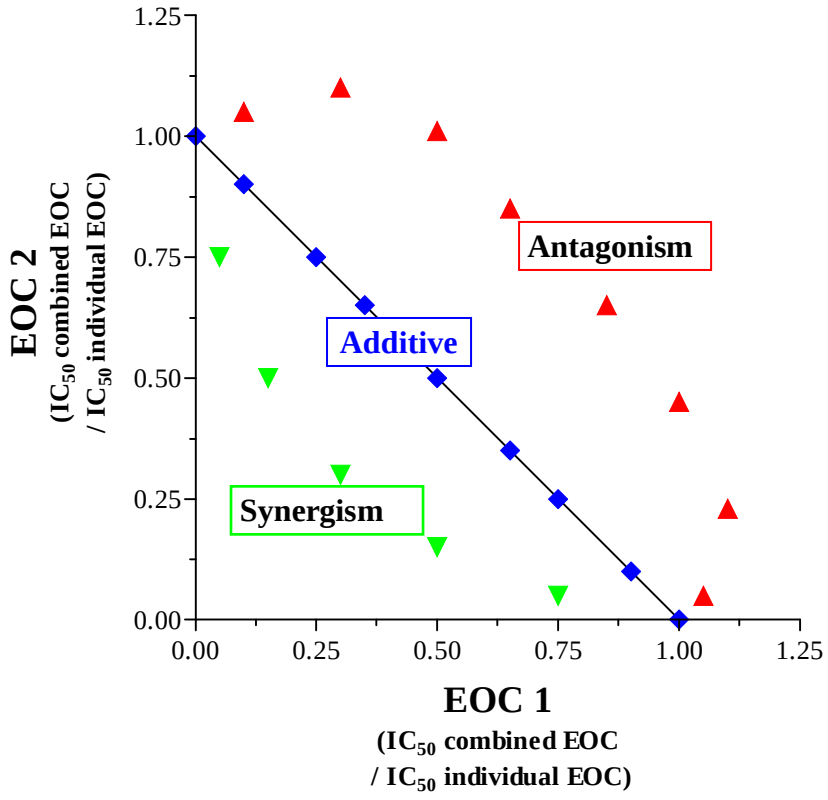


Figure 3.3: An isobologram showing possible interactions of the essential oil constituents (Berenbaum, 1989).

3.2.5.2 Fractional inhibitory concentration determination

To verify the interaction read from the isobolograms the sum of fractional inhibitory concentrations (∑FIC or FIC_{AB}) was calculated for each combination using Equation 3.3. Notably, the sum of the FIC values <0.5 is synergistic and 0.5-1.0 is additive, >1.0 is indifferent while that >2.0 is antagonistic (Berenbaum, 1989).

Equation 3.3:

$$FIC_{AB} = \frac{IC_{50A} \text{ in combination}}{IC_{50A} \text{ alone}} + \frac{IC_{50B} \text{ in combination}}{IC_{50B} \text{ alone}}$$

Where A and B are the two selected EOC's used in combination.

3.2.6 Statistics

The data were presented as mean ± s.d. of the triplicate values. To test for correlation between variables, linear regression analysis was performed and squared correlation coefficient (r^2) was calculated; with a p value of <0.05 being regarded as significant.

3.3 Results

3.3.1 The antimalarial activity of individual essential oil constituents

The twenty EOC´s were found to inhibit the growth of *P. falciparum* to varying degrees with IC_{50} values ranging between 0.99 to 1528.8µM (Figure 3.4 and 3.5). *E*- and *Z*-(±)-Nerolidol, (-)-pulegone, (+)-α-pinene and linalyl acetate were approximately two to three times more active than the plant-derived compound, quinine (Figure 3.5).

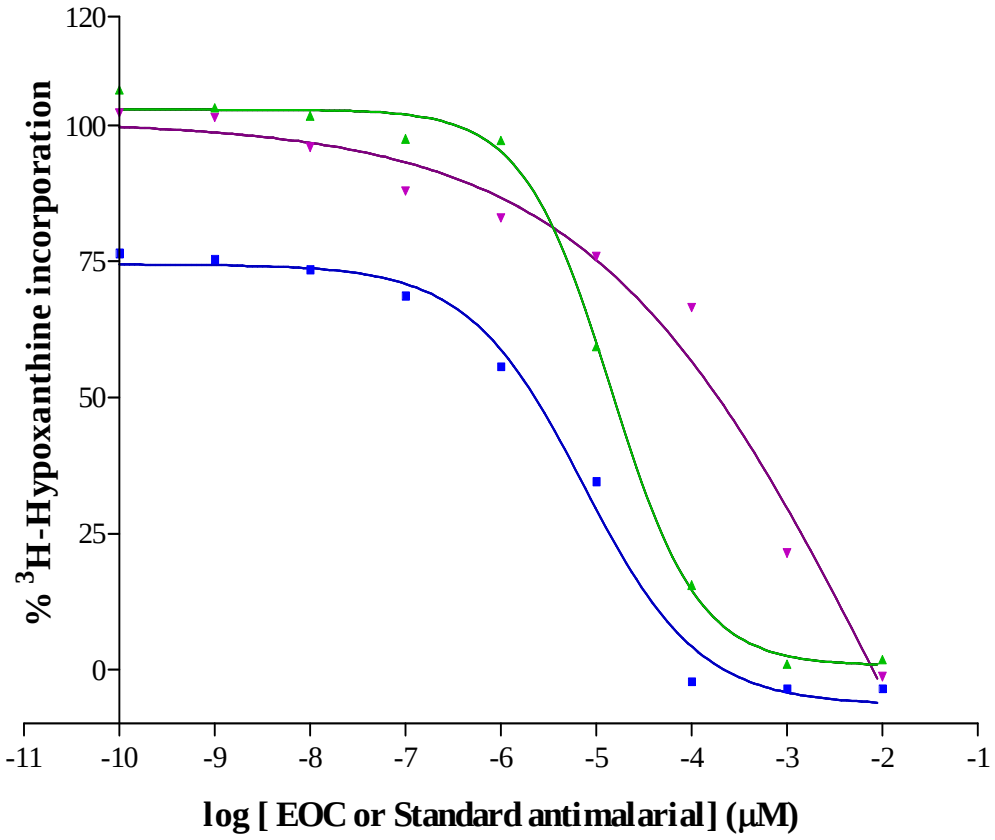


Figure 3.4: The log sigmoidal dose response curves of quinine (▲), *p*-cymene (▼) and *E*- and *Z*-(±)-nerolidol (■).

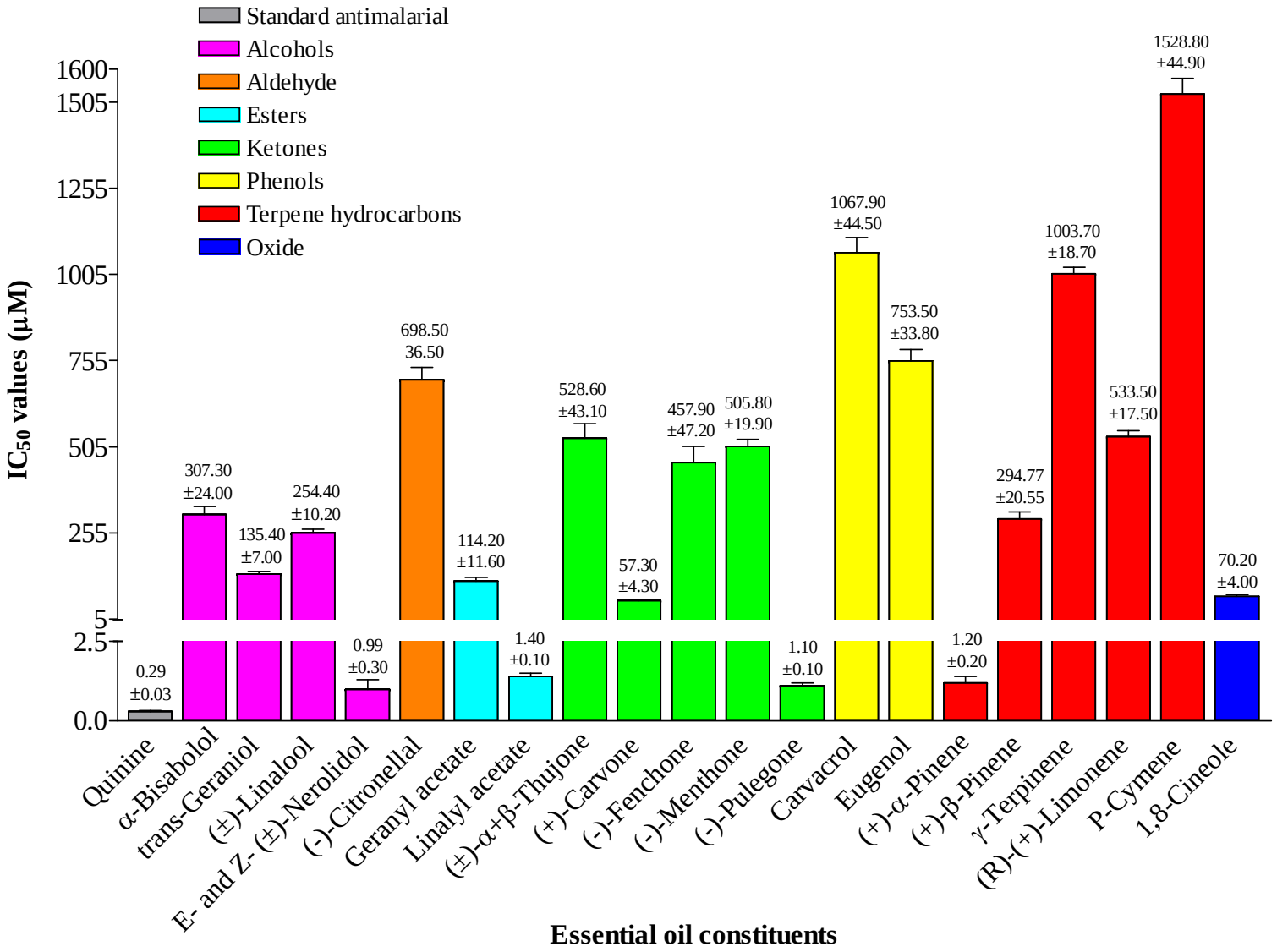


Figure 3.5: The antimalarial activity of the 20 essential oil constituents and quinine.

3.3.2 The relationship between essential oil constituent density and antimalarial activity

There was no correlation (r^2 = 0.008) between the antimalarial activity and the densities or lipophilicity of the 20 EOC's (Figure 3.6).

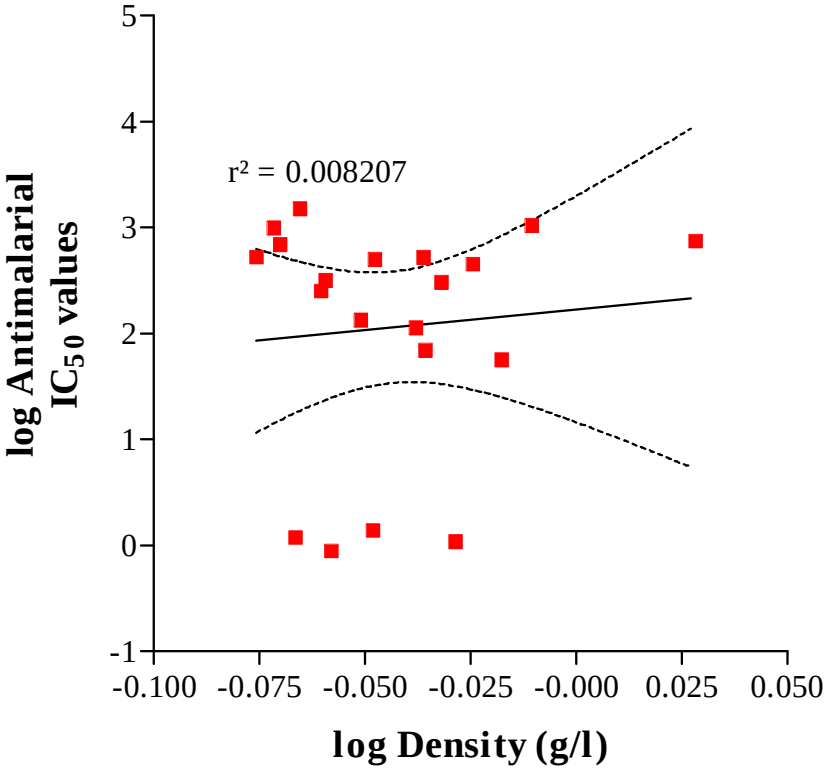


Figure 3.6: The linear relationship between the density of the essential oil constituents and their antimalarial activity.

3.3.3 The antimalarial activity of the combined essential oil constituents

After investigating the antimalarial activity of individual EOC´s, various combinations depending on spectrum of activity of EOC´s were performed (Table 3.2). Representative isobolograms showing the synergistic (between *p*-cymene and *E*- and *Z*-(±)-nerolidol or γ-terpinene) and antagonistic (between *E*- and *Z*-(±)-nerolidol and (-)-pulegone) interactions are illustrated in Figures 3.7 and 3.8, respectively.

Table 3.2: The combined interaction between two essential oil constituents (n = 3).

EOC_1	EOC_2	∑FIC value	Interaction
Linalyl acetate	(+)-α-Pinene	0.91	Additive
Linalyl acetate	Carvacrol	0.14	Synergistic
p-Cymene	*E*- and *Z*-(±)-Nerolidol	0.09	Synergistic
E- and *Z*-(±)-Nerolidol	(-)-Pulegone	2.03	Antagonistic
(-)-Pulegone	*p*-Cymene	11.30	Antagonistic
(+)-α-Pinene	Carvacrol	0.22	Synergistic
Carvacrol	*p*-Cymene	0.02	Synergistic
p-Cymene	γ-Terpinene	0.37	Synergistic

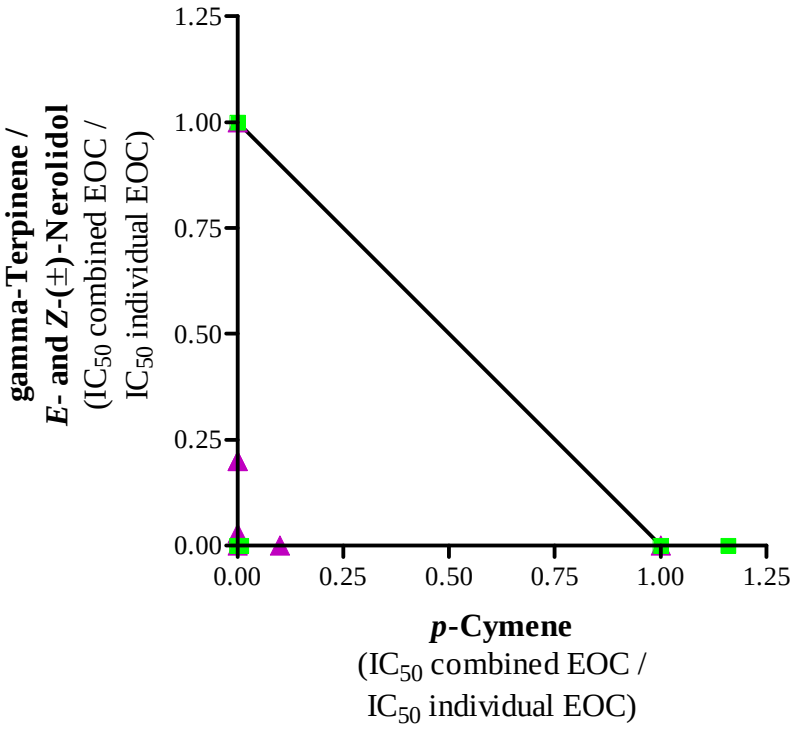


Figure 3.7: *p*-Cymene interacted in a synergistic manner when combined with γ-terpinene. (■) and *E*- and *Z*-(±)-nerolidol (▲).

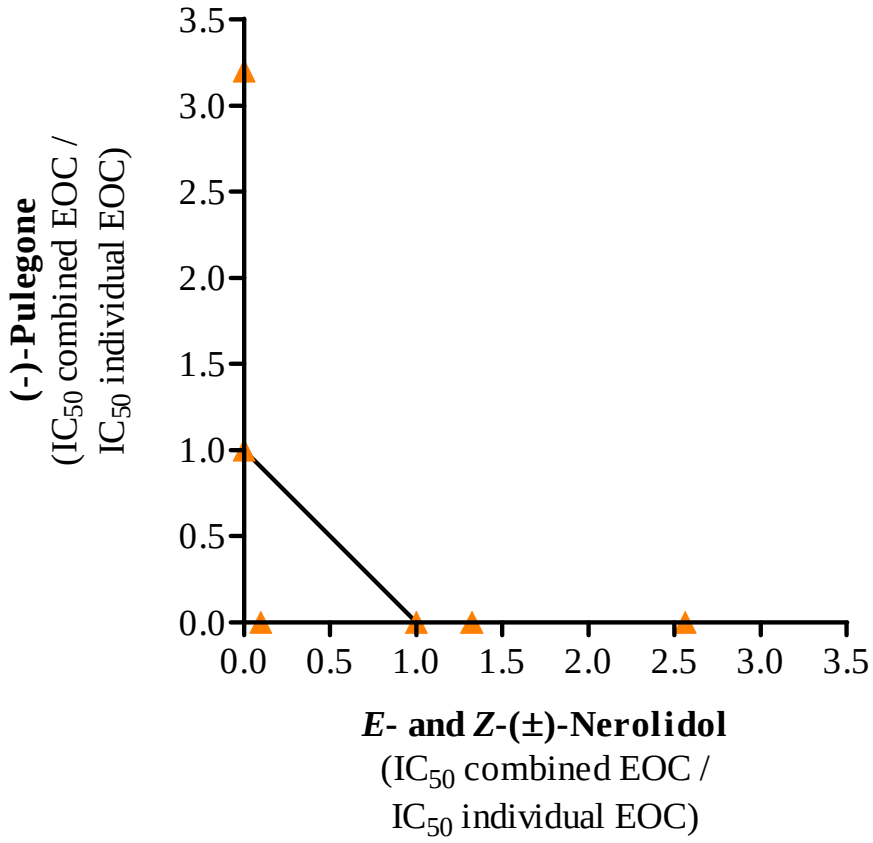


Figure 3.8: An antagonistic interaction between *E*- and *Z*-(±)-nerolidol and (-)-pulegone.

3.3.4 The antimalarial activity of quinine and the most active essential oil constituent

A synergistic interaction (∑FIC = 0.01) was observed when the most active EOC, namely *E*- and *Z*-(±)-nerolidol, was combined with the standard antimalarial agent, quinine.

3.4 Discussion

3.4.1 The antimalarial activity of individual essential oil constituents

Of the twenty EOC´s tested, *E*- and *Z*-(±)-nerolidol was found to be the most active (IC_{50} = 0.2µg/ml = 0.9µM) (Figure 3.5). The antimalarial activity of the EOC's did not correlate with their density or lipophilicity (Figure 3.6), which indicated that all the EOC's crossed the various red blood cell and parasite membranes equally well in order to reach their respective targets. But once across the membranes, their ability to interfere or bind with their respective target differed greatly.

E- and *Z*-(±)-Nerolidol is also one of the EOC´s in the essential oils of numerous plant species that have been reported to possess antimalarial activity. *E*- and *Z*-(±)-Nerolidol found in the oil of *Virola surinamensis* (Myristiceae) has previously been reported to have antimalarial

activity where 100, 10 and 1μg/ml inhibited the development of young trophozoites to the schizont stage (without haemozoin formation) (Lopes *et al*., 1999). In trying to determine the mechanism of action of *E*- and *Z*-(±)-nerolidol, Lopes *et al.* (1999) found that 100μM had no effect on overall protein synthesis (as indicated by L-[^{35}S]–methionine incorporation), but did inhibit the incorporation of D-[U-^{14}C]-glucose especially when young trophozoites were differentiating into mature trophozoites (22 - 48h culture).

From this it was proposed that *E*- and *Z*-(±)-nerolidol interferes with malaria growth by inhibiting glycoprotein biosynthesis (Lopes *et al*., 1999). Possibly by inhibiting specific pathways in the biosynthesis of isoprenoids that play an important role as components of structural cholesterol and in ubiquinones (Figure 3.9) (Goulart *et al*., 2004). It has been shown that *E*- and *Z*-(±)-nerolidol inhibits the biosynthesis of coenzyme Q and other intermediates of the isoprenoid pathway (dolichol) in all intra-erythrocytic stages (De Macedo *et al*., 2002). This could be achieved by *E*- and *Z*-(±)-nerolidol competing with natural substrates in an enzyme-substrate reaction (Goulart *et al*., 2004).

Terpenes such as *E*- and *Z*-(±)-nerolidol have a chemical structure similar to that of some intermediates of the isoprenoid pathway such as farnesyl pyrophophate (FPP, C_{15}) and geranylgeranyl pyrophosphate (GGPP, C_{20}) (Figure 3.9) (Holstein and Hohl, 2003). As such, *E*- and *Z*-(±)-nerolidol could inhibit the biosynthesis of the polyprenyl pyrophosphate side and chain attached to ubiquinone (De Macedo *et al*., 2002). Alternatively *E*- and *Z*-(±)-nerolidol could inhibit the biosynthesis of both dolichol and the isoprenic chain of coenzyme Q. De Macedo *et al*. (2002) proposed that *E*- and *Z*-(±)-nerolidol interfered with the mechanism of elongation involved in the biosynthesis of dolichol and the isoprenic chain attached to the benzoquinone ring of coenzyme Q.

In addition, *E*- and *Z*-(±)-nerolidol as well as the monoterpene (R)-(+)-limonene, have been shown to inhibit the isoprenylation of proteins, resulting in the interference with mitochondrial metabolic processes like pyrimidine biosynthesis (De Macedo *et al*., 2002). The difference in the mechanism of action of these two constituents is probably due to the difference in chemical structure, where the hydroxyl group and linear structure of *E*- and *Z*-(±)-nerolidol could allow better interaction with the target enzyme compared to the six-member ring backbone of (R)-(+)-limonene (Figure 2.1, d and s) (De Macedo *et al*., 2002).

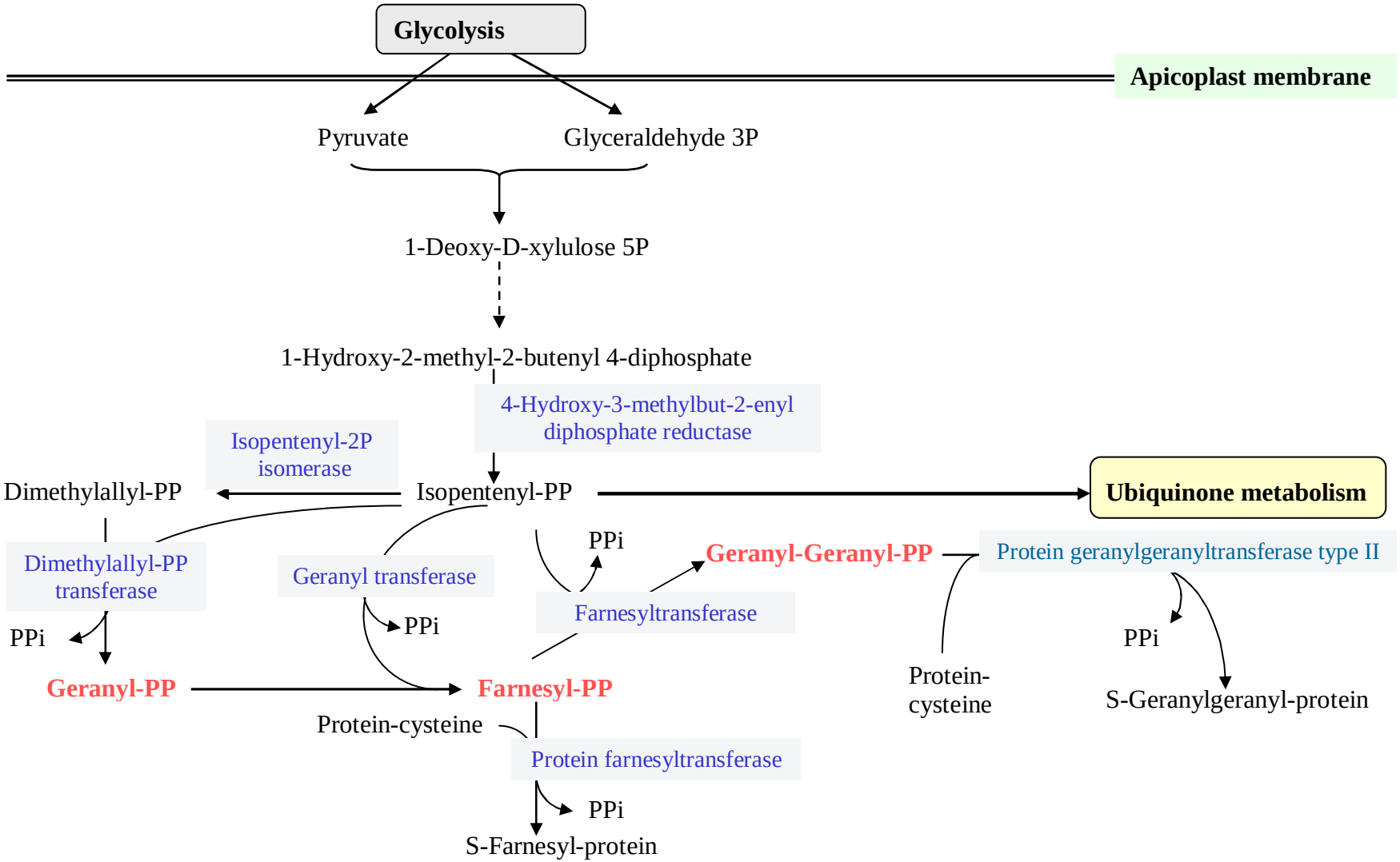


Figure 3.9: The biosynthetic pathway of isoprenoids in *Plasmodium falciparum* (http://sites.huji.ac.il/malaria/maps/isoprenoidmetpath.html).

The antimalarial activities obtained in this study (0.9µM) are similar to those reported by Goulart *et al*. (2004) (0.8µM), even though a different *P. falciparum* strain was used. Regardless of this potent activity, the *E*- and *Z*-(±)-nerolidol-rich oil (45.2%) from *Lippia multiflora* only displayed moderate antimalarial activity (47.6µg/ml) even in the presence of a high concentration of (±)-linalool (20.2%) (Valentin *et al*., 1995). Thus, the interaction between major and minor constituents dictates the overall activity of the oil and needs to be taken into account.

Both (R)-(+)-limonene and (±)-linalool were found to inhibit the isoprenoid biosynthesis pathway at a much earlier step compared to farnesol. It is possible that these monoterpenes ((R)-(+)-limonene and (±)-linalool) inhibit the biosynthesis of dolichol and ubiquinones by interfering with the condensation between isopentenyl-PP and dimethylallyl-PP (Figure 3.6) (Goulart *et al*., 2004).

In addition (R)-(+)-limonene was found to induce some inhibition of Ras-like protein isoprenylation in the ring and schizont stages (Moura *et al*., 2001). Holstein and Hohl (2003) noted that the (+)- and (-)-isomers of menthol displayed varying inhibitory activity on isoprenylation, where (-)-menthol was active compared to the inactive (+)-isomer. It was also suggested that alcohols with an open chain such as (±)-linalool and *trans*-geraniol are inactive or have low inhibitory activity on isoprenylation (Holstein and Hohl, 2003).

The antimalarial activity of *E*- and *Z*-(±)-nerolidol, (±)-linalool and R-(+)-limonene are comparable, even though a 3D-7 chloroquine-sensitive strain was used compared to FCR-3 chloroquine-resistant strain in this study. Goulart *et al*. (2004) reported values of 0.8µM, 0.28mM and 1.22mM respectively, and this study reported values of 0.9µM, 0.3mM and 0.5mM, respectively (Figure 3.5). The differences in antimalarial activity of these terpenes could be attributed to their structural differences. While the (R)- and (S)-enantiomers displayed similar activity to downregulate Ras-related proteins, overall it was found that the order of activity was related to the presence of an oxygenated substituent at the C7 position; where alcohol followed by aldehydes and acids were consequently more active (Holstein and Hohl, 2003). Whilst the deoxygenated form of (R)-(+)-limonene was found to be inactive (Holstein and Hohl, 2003).

Even though (±)-linalool is similar in structure to *E*- and *Z*-(±)-nerolidol and contains a hydroxyl group, the shorter chain greatly reduces the antimalarial activity of (±)-linalool and

trans-geraniol (Figure 2.1, b, c, and d). In contrast, the presence of an acetate group increases (250 fold) the activity of linalyl acetate compared to (±)-linalool (Figure 2.1, c and g).

One of the proposed mechanisms by which the malaria parasite induces drug resistance is the presence of a mutant *pfcrt* protein transporter that is present in the membrane of the food vacuole (Figure 3.10). It is in this food vacuole that chloroquine accumulates and inhibits the formation of the inert haemozoin.

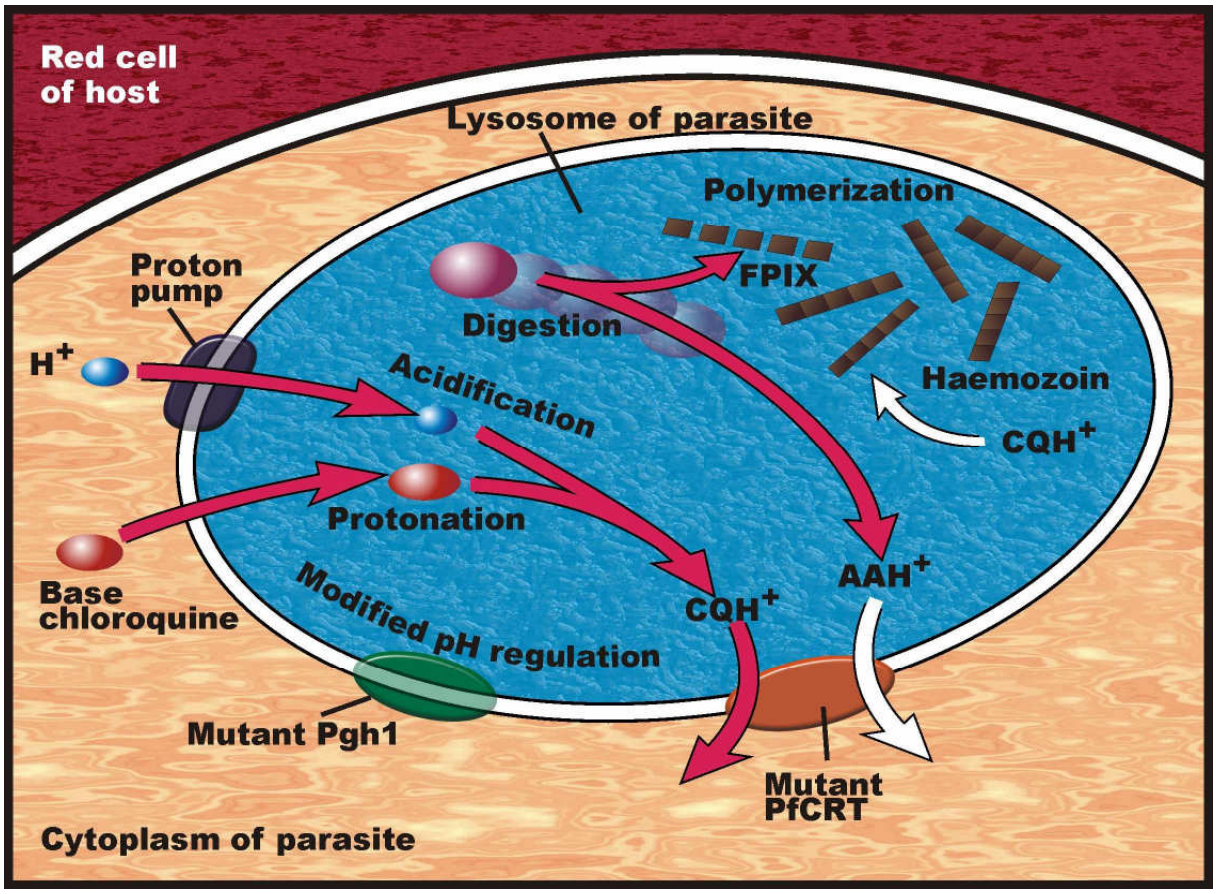


Figure 3.10: The effect of chloroquine (CQ) on haem (FP9) detoxification in the lysosome of a chloroquine-resistant malaria parasite (Warhurst, 2001). Where PfCRT = *Plasmodium falciparum* chloroquine-resistant transport; Pgh1 = p-glycoprotein homologue 1 and AA = amino acid.

The chloroquine-haem complex is toxic to the parasite, resulting in lipid peroxidation and parasite death (Warhurst, 2001). The *pfcrt* pump transports the accumulated chloroquine out of the food vacuole, thereby decreasing its concentration and the possibility of interacting with the haem units and results in the death of the parasite. This process closely resembles the mechanisms by which cancer cells become multidrug resistant.

Yoshiba *et al*. (2005) reported that the essential oil of *Zanthoxyli fructus* inhibited the activity of the p-glycoprotein transport pump, resulting in the accumulation of anticancer drug in the cells and subsequent cell death. The chief components of this essential oil were the acyclic monoterpenoids (geraniol, linalool, citronellal and citronellol) and the monocyclic monoterpenoids (limonene and 1,8-cineole). It was found that the acyclic monoterpenoids, (R)-(+)-citronellal and (S)-(-)-β-citonellol were potent inhibitors of p-glycoprotein compared to the other EOC´s.

However, in this study (-)-citronellal was relatively inactive as an antimalarial agent (IC_{50} = 698.5±36.5µM) (Figure 3.5). This aldehyde compound like drugs such as verapamil (a calcium channel blocker) have the ability to block the action of p-glycoproteins in cancer cells (Yoshiba *et al*., 2005). In addition, it can block transport pumps (*pfcrt*) in the malaria parasite (Warhurst, 2001), even if it is relatively ineffective in directly inhibiting parasite growth. These drugs including verapamil are termed "reversal agents" which, when combined with an antimalarial agent such as chloroquine reverse the resistance in the strain, resulting in the parasite being more sensitive to the inhibitory effects of chloroquine. To verify this hypothesis, chloroquine should be combined with (-)-citronellal to determine if the interaction is synergistic. If so, then (-)-citronellal could act as a "reversal agent" and could be used as adjunct therapy in the treatment of malaria.

The oils of *Tetradenia riparia* (Lamiaceae) contain the ketone fenchone (13.6%) and have also been reported to have moderate antimalarial activity (IC_{50} = 50.3µg/ml) (Campbell *et al*., 1997). However, this activity is possibly due to interactions with other constituents present in the oil, as (-)-fenchone alone has poor antimalarial activity (Figure 3.5).

It is interesting to observe that the two structural isomers, (+)-α-pinene and (+)-β-pinene (Figure 2.1, o and p, respectively) displayed contrasting activity with (+)-α-pinene being 250 times more active than (+)-β-pinene against *P. falciparum*, although the two compounds

displayed identical toxicity profiles (Figure 5.4). This could indicate that the conformation of (+)-α-pinene is crucial for its interaction and binding to its plasmodial target.

3.4.2 The antimalarial activity of the combined essential oil constituents

When two EOC´s were combined and assessed for their antiplasmodial activity the overall interaction between the various combinations were found to be synergistic, although to varying degrees (Table 3.2). The combination between the least active and most active EOC´s, *p*-cymene (1528.8μM) and *E*- and *Z*-(±)-nerolidol (0.99μM) displayed a pronounced synergistic interaction (Figure 3.7). *E*- and *Z*-(±)-Nerolidol with its reported mechanism of action (Lopes *et al*., 1999) potentiated the antimalarial activity of *p*-cymene which had minimal activity when used individually (or vice versa) (Figure 3.5). Goulart *et al*. (2004) reported that most terpenes inhibit the isoprenoid biosynthetic pathway in *P. falciparum*. It is likely that the interaction of *E*- and *Z*-(±)-nerolidol with *p*-cymene might assist *p*-cymene to reach the site of action in the parasites (or vice versa), or they may have acted on the complementary targets.

The volatile oils from *V. surinamensis* also showed the predominance of *p*-cymene (42.0%), α-pinene (11.7%), and β-pinene (5.2%) and nerolidol (3.8%) (Lopes *et al*., 1999). From the results of this study, it can be seen that *p*-cymene and β-pinene (which constitute 47% of the whole oil) were both relatively poor inhibitors of parasite growth (Figure 3.6). However, 100μg/ml of this oil inhibited 100% parasite growth over 48h of treatment. Thus, the remaining two major constituents, namely α-pinene and nerolidol contributed to the effective antimalarial activity of this essential oil. This illustrates the importance of considering the combined effect of EOC´s even though the active constituents only represent 15.5% of the essential oil.

In this present study the two least active EOC´s, namely carvacrol (1067.9μM) and *p*-cymene interacted synergistically (Table 3.2). *p*-Cymene is the biological precursor of carvacrol, but lacks a phenolic hydroxyl group (Figure 2.1, m and r). While carvacrol is structurally similar to (R)-(+)-limonene but has one less hydroxyl group and appeared to act well in combination with *p*-cymene. Limonene has been found to inhibit the isoprenylation of proteins, resulting in interference with mitochondrial metabolic processes like pyrimidine biosynthesis (De Macedo *et al*., 2002).

When the least active EOC´s, γ-terpinene (1003.7μM) and *p*-cymene (1528.8μM) were combined a synergistic interaction was observed. Whilst when the most active EOC´s, *E*- and *Z*-(±)-nerolidol and (-)-pulegone were combined an antagonistic interaction occured (Figure 3.8). Likewise the combinations of the two most active EOC's [linalyl acetate (1.4μM) and (+)-α-pinene (1.2μM)] showed an additive interaction (Table 3.2). While that of the most and the least active EOC's ((-)-pulegone and *p*-cymene) showed an antagonistic interaction (Table 3.2). It may be that these two EOC's competed for the same site in an essential biochemical process in *P. falciparum*. Moreover, this study shows that the interactions that exist when two EOC´s are combined, vary depending on the concentration and ratio of the agents used. Boyom *et al*. (2003) stated that in the case of malaria, the physical properties of essential oil constituents namely its low density (~0.94g/ml) and lipophilicity which facilitates rapid diffusion across cell membranes, might enhance targeting of the active components into the intracellular malaria parasite. But the final interaction between the two EOC´s depends on their respective cellular targets.

3.4.3 The combined effect of quinine and *E*- and *Z*-(±)-nerolidol

The combination of a classical antimalarial drug, namely quinine with *E*- and *Z*-(±)-nerolidol displayed a synergistic interaction (∑FIC value = 0.01). The ability of these two agents to target separate cellular components may have contributed to their enhanced interaction; with *E*- and *Z*-(±)-nerolidol able to inhibit isoprenoid biosynthesis (Goulart *et al*., 2004) and quinine able to inhibit haemozoin formation (Warhurst, 2001). Quinine is also proposed to inhibit malaria growth by decreasing the plasmodial DNA strand separation and transcription, thereby inhibiting protozoal protein synthesis (Noedl *et al*., 2003). Thus, if a patient does combine a volatile oil (for example through inhalation) with the more classical agents to treat malaria, the patient will not be compromised by this combination.

In the absence of vaccines, people rely on chemotherapeutic agents to prevent and treat an infection. These results indicate that plant-derived or chemically synthesised EOC´s have the potential to be used in adjunct therapy with antimalarial agents. In addition, they could be used as new templates for drug design.

CHAPTER FOUR
ANTI-OXIDANT AND ANTICHOLINESTERASE ACTIVITIES

4.1 Introduction

The study of anti-oxidants and anticholinesterases is producing a medical revolution that promises a new age in health and disease management. In the past few years, there has been renewed interest in studying the anti-oxidant activity of essential oils from aromatic plants due to their health promoting benefits (Choi *et al*., 2000; Nakatsu *et al*., 2000). There have been numerous reports on the anti-oxidant properties of essential oils (Sanchez-Moreno, 2002), however, there is little or no research conducted on the anticholinesterase activity of essential oils (Graβmann *et al*., 2000). Natural anti-oxidants found in plants play a major role in the prevention of complex pathological processes including cardiovascular diseases, cerebrovascular diseases and carcinogenesis (Sanchez-Moreno, 2002). In addition, both anti-oxidants and anticholinesterases play a vital role in minimising the progression of neurodegenerative and chronic diseases like Alzheimer's disease (Mi-Yeon *et al*., 2000).

In many pathological cases, reactive oxygen species (ROS) play an important role; where ROS are generated by several types of inflammatory cells, for example: neutrophils and macrophages in order to defend the host from invading microorganisms (Choi *et al*., 2000). A continuous or over production of ROS and resultant inflammatory reactions may lead to tissue damage. In the central nervous system of patients with Alzheimer's disease, not only do free radicals such as ROS damage the mitochondrial and nuclear DNA, but the development of senile plaques and neurofibrillary tangles, all contribute to the pathology of Alzheimer's disease (Mi-Yeon *et al*., 2000).

It is also pertinent to note that apart from being therapeutically used in Alzheimer's disease, anticholinesterases are used as pesticides and to treat other medical conditions. These anticholinesterases are able to inhibit cholinesterase reversibly or irreversibly (Ringman and Cummings, 1999). Generally, the reversible anticholinesterases, such as edrophonium, pyridostigmine, neostigmine, distigmine and physostigmine are used as medicines; whilst the irreversible inhibitors such as parathion and malathion are used as pesticides. Although ecothiophate, which is an irreversible inihibitor is used to treat glaucoma. It is important to know that all pesticides are lipophilic and are readily absorbed through the skin and cross the blood brain barrier causing central cholinergic effects, in addition to the full spectrum of

peripheral nicotinic and muscarinic effects (Pappano, 2004). These include markedly impaired vision, copious lacrimation, nasal discharge and salivation, accompanied by slurred speech, wheezing, impaired respiration, gastro-intestinal cramping, diarrhoea, urination, profuse sweating, vomiting, dehydration, bradycardia and hypotension. These complications are treated with atropine (an anticholinergic agent) accompanied by an acetylcholinesterase enzyme regenerator, obidoxime (Pappano, 2004).

When studying any disease profile or in any situation where there is possible tissue damage, one has to bear in mind that free radicals may be involved. Along with the knowledge that essential oils possess anti-oxidant properties and *Salvia* essential oils have been used in the treatment of Alzheimer's disease (Savelev *et al*., 2003), the anti-oxidant and anticholinesterase activities of the individual essential oil constituents were investigated.

4.1.1 Free radicals

Anti-oxidants play a vital role in preventing disease and strengthening the immune system, particularly when one is suffering from a chronic disease. Their main function is to protect the body against free radicals (also called oxidants), by neutralising or scavenging the oxidants before they can damage cells and tissues (Sanchez-Moreno, 2002).

What are free radicals? A free radical can be any molecule containing one or more unpaired electron(s) and is capable of independent existence (Sanchez-Moreno, 2002). Each atom has two elements: a nucleus, consisting of protons and neutrons, and an electron that revolves around the core (Peiwu *et al*., 1999). Free radicals are highly reactive, with the result that they have a very limited lifespan. They are, however, not only produced within the body but also from substances in the environment, such as tobacco smoke, exhaust fumes, compounds in food, pollutants in drinking water and alcohol that may enter our bodies (Graβmann *et al*., 2000).

Extensive research has shown that the body produces more free radicals under certain conditions, which in turn places greater pressure on the immune system (Choi *et al*., 2000). For example, prolonged or uncontrolled stress over a long period can significantly increase free radical production (Graβmann *et al*., 2000). Sports participants also produce more free radicals on account of increased catabolic metabolism (Graβmann *et al*., 2000).

Free radicals are extremely destructive and if the body is exposed to high levels over an extended period of time, oxidative stress is the resultant effect. Oxidative stress occurs when the body′s supply of anti-oxidants is insufficient and is unable to cope with the excess free radicals (Sanchez-Moreno, 2002). Oxidative stress is the major cause of endogenous damage to DNA, proteins, lipids and other macromolecules.

During oxidative stress ROS like superoxide, hydrogen peroxide (H_2O_2) and hydroxyl (OH)-type radicals are generated by inflammatory cells (e.g. macrophages and neutrophils) to defend the host from invading microorganisms (Peiwu *et al.*, 1999). Phagocytes release superoxide, which at physiological pH undergoes dismutation to form H_2O_2 and oxygen (O_2). While neutrophils produce nitric oxide (NO) which react with superoxide to form peroxynitrite (Peiwu *et al.*, 1999).

Myeloperoxidase (MPO) an enzyme contained in the neutrophils, oxidizes halides to their corresponding hypophalous acids using H_2O_2 as an oxidant. These hypophalous acids in return react with H_2O_2 and form single reactive oxygen species. Other ROS formed include OH-radical produced by iron-catalyzed decomposition of H_2O_2 (Figure 4.1).

$$O_2 + e^- + H^+ \rightarrow HO_2^{\cdot} \text{ (hydroperoxyl radical)}$$
$$HO_2^{\cdot} \rightarrow H^+ + O_2^- \text{ (superoxide radical)}$$
$$O_2^- + 2H^+ + e^- \rightarrow H_2O_2 \text{ (hydrogen peroxide non radical)}$$
$$^{\cdot}OH + e^- \rightarrow H_2O$$

Figure 4.1: A stepwise reduction of an oxygen molecule resulting in different free radicals (Peiwu *et al.*, 1999).

Human health is unwillingly vulnerable to disease and infections and is under attack from free radicals every hour of the day. Free radicals weaken the body′s immune system and are implicated in most chronic diseases, depending on which cells, tissue and organs are attacked.

If the pancreas is attacked, free radical damage may contribute to the development and progression of diabetes; they may cause or worsen cardiovascular disease by adversely affecting blood vessels; they may aid in the process of aging; while in the brain and nerves they may cause neurodegenerative diseases like Alzheimer′s disease (Sanchez-Moreno, 2002). All forms of chronic diseases such as osteoarthritis and rheumatoid arthritis, cancer,

emphysema and HIV/AIDS result in an increased production of free radicals, which further potentiate and prolong the damage caused by the disease. Thus, an increased intake of anti-oxidants may prevent cellular damage by free radicals.

4.2 Anti-oxidant activity

Humans have their own natural anti-oxidant defenses in the form of enzyme systems. However, we rely greatly on external anti-oxidants such as vitamin C and E, α-tocopherol, β-carotene, glutathione and other flavonoids in our diet (Nakatsu *et al*., 2000). Non-enzymatic anti-oxidants also play a role in resistance against oxidative stress. They protect normal human body function from free radical damage by preventing the formation of excess free radicals, scavenging free radicals and repairing damaged molecules (Sanchez-Moreno, 2002). Vitamin E is considered the “master” anti-oxidant as it is a very efficient neutralizer of the free radical damage, especially to the lipid structure of our cell membranes (Choi *et al*., 2000).

Furthermore, anti-oxidants complement each other’s activity, for instance, L-ascorbic acid (vitamin C) helps to regenerate vitamin E. As a water-soluble anti-oxidant, L-ascorbic acid is in a unique position to "scavenge" aqueous peroxyl radicals before these destructive substances have a chance to damage the lipids. It works along with vitamin E, a fat-soluble anti-oxidant, and the enzyme glutathione peroxidase to stop free radical chain reactions. Anti-oxidants have been shown to be beneficial in preventing or delaying the progression of a number of disease states such as cancer, Alzheimer’s disease, and macular degeneration (Sramek and Cutler, 2000). A study in China including 29584 adults showed that supplementation with α-tocopherol, β-carotene and selenium reduced stomach cancer by 21% and oesophageal cancer by 42% (Sanchez-Moreno, 2002). An observational study of 991522 participants from 1982 to 1988 showed that regular long-term use of vitamin E reduced the risk of death from bladder cancer by 40% (Sanchez-Moreno, 2002).

The evidence for anti-oxidants is strong enough for the Food and Drug Administration (FDA) to allow a limited claim of anti-oxidants in the prevention of cancer (Graβmann *et al*., 2000). The Age-Related Eye Disease Study (AREDS) underlined the benefits of anti-oxidant intake in people who suffer from age-related macular degeneration (AMD), a leading cause of blindness in the elderly (Sramek and Cutler, 2000). In this interventional trial, patients with intermediate or advanced stage AMD who received anti-oxidant supplementation (vitamin C, vitamin E, β-carotene and zinc) had a 28% reduced risk of further progression of this disease

(Sramek and Cutler, 2000). Furthermore, anti-oxidants showed benefits in immunity and reduced the risk of infections in elderly patients in nursing homes.

A healthy diet rich in vegetables and fruits is the first step toward obtaining a well-balanced anti-oxidant regimen. However, the beneficial doses used in many studies are higher than one can obtain from diet alone, since essential nutrients are lost in the harvesting, processing and cooking of food even before it is consumed (Nakatsu *et al*., 2000). So it is advisable that consumers choose a supplement that has a wide spectrum of anti-oxidants in their most natural and bioavailable form (Peiwu *et al*., 1999). The anti-oxidant dose should be sufficient to aid in the defense against free radicals (above a multivitamin), but not mega-doses that may be harmful (Nakatsu *et al*., 2000).

There has been much attention on using anti-oxidants to inhibit lipid peroxidation or at least afford protection from damage by free radicals. Essential oils from *Citrus paradisi* and *Cymbopogon citratus* have been reported to play a vital role as anti-oxidants (Peiwu *et al*., 1999). Some phenols such as carvacrol and eugenol have been reported for their anti-oxidant activity, but there is limited information on the anti-oxidant activity of the individual EOC's present in these essential oils (Nakatsu *et al*., 2000). Thus, twenty essential oils were selected from seven chemical groups and their individual anti-oxidant activity was evaluated in comparison to L-ascorbic acid (vitamin C).

4.2.1 Methodology

4.2.1.1 2,2-Diphenyl-1-picrylhydrazyl assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay is used to measure the ability of anti-oxidants to scavenge the stable free radical of 2,2-diphenyl-1-picrylhydrazyl. This stable free radical is dark violet in colour and is decolourised when an anti-oxidant compound donates an electron. This degree of decolourisation is stoichiometric with respect to the number of electrons seized by DPPH.

A number of assays have been introduced to measure the total anti-oxidant activity of pure compounds. In this study, the DPPH assay was selected and used to assess the *in vitro* anti-oxidant activity of essential oil constituents according to Vardar-Ünlü *et al*. (2003). This assay is considered a valid and easy assay to evaluate the scavenging activity of the anti-oxidants as the radical compound is stable and does not have to be generated as in other free radical scavenging assays.

4.2.1.2 2,2-Diphenyl-1-picrylhydrazyl preparation

A 96.2µM solution of DPPH (Fluka) was prepared in methanol. The solution was stable for a week, but needed to be kept in the dark and stored at 4ºC.

4.2.1.3 Preparation of essential oil constituents

Approximately 32mg of EOC was weighed out and dissolved in 500µl DMSO to prepare a stock solution of 10000ppm (= 650mM), which is equivalent to 1%, since 1ppm equals 0.0001%. This solution was then vortexed before 50µl was added to 950µl of DMSO to prepare various concentrations.

4.2.1.4 Preparation of microtitre plates

Of the dilutions, 50µl (= 3250µM) was added to each well of row B to row G (Figure 4.2). Whilst, 50µl DMSO was added to the wells in rows A to H to serve as the untreated controls. HPLC grade methanol (200µl) was added to columns 2, 4, 6, 8, 10 and 12 to serve as colour control while an equal volume of DPPH solution was plated out in columns 1, 3, 5, 7, 9 and 11. As a result the concentration was diluted to 100ppm (= 650µM). Furthermore, seven dilutions were prepared by 1:10 serial dilutions in plastic eppendorfs using DMSO from the stock solutions and each plated out into its relevant plate. Each plate was set up as mentioned above.

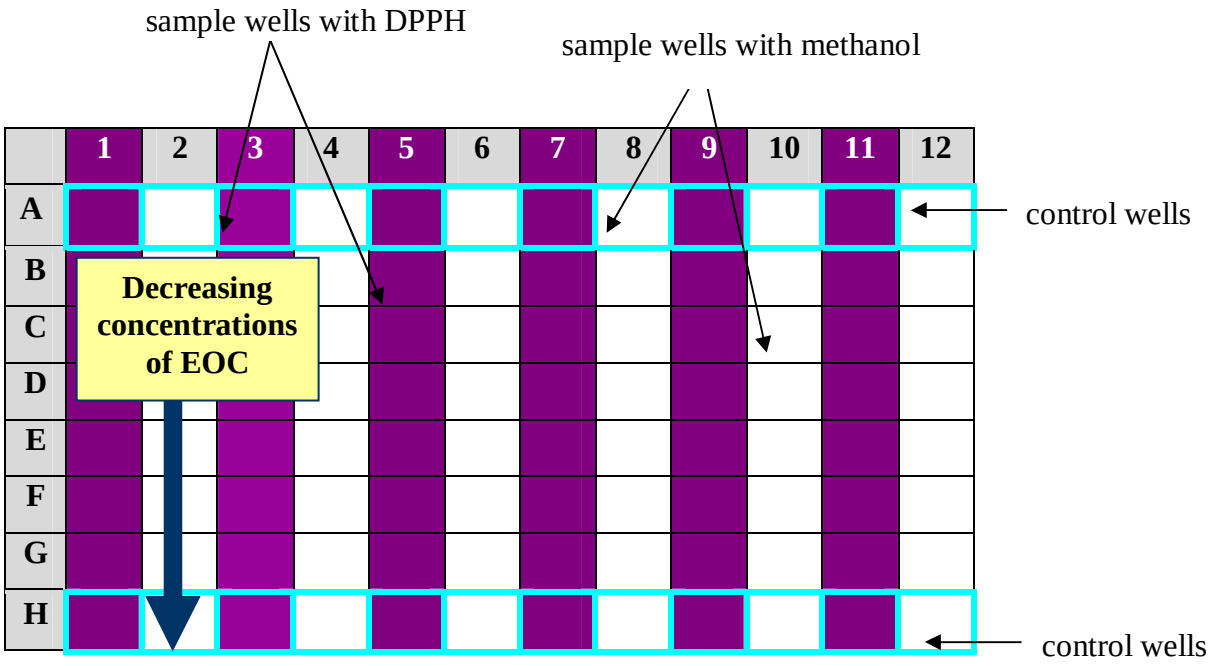


Figure 4.2: The schematic representation of the setup of the microtitre plate used in the 2,2-diphenyl-1-picrylhydrazyl assay.

4.2.1.5 Reading of the plate and data analysis

The test plate was shaken for 2min in a UV-VIS spectrophotometer and thereafter the absorbance was measured at a wavelength of 540nm. The standard plate was then rinsed with methanol and discarded together with its contents. The test plate was left to stand for 30min in an incubator at 37°C. After which, the absorbance was read at 540nm using Genesis® software (Biosoft, UK). Taking the appropriate controls into account, the percentage decolourisation of each well was determined. The experiment was repeated at least twice. The IC_{50} values were calculated using the Enzfitter® version 1.05 software. L-Ascorbic acid (vitamin C) was used as the positive control.

4.2.2 Results

The anti-oxidant activity of the 20 essential oil constituents was very poor. The phenol, eugenol was the only constituent that possessed comparable anti-oxidant activity to L-ascorbic acid (Table 4.1)

Table 4.1: The anti-oxidant activity (IC_{50} values) of essential oil constituents.

Essential oil constituent	Anti-oxidant activity (µM)
Alcohols	
(-)-α-Bisabolol	>449.7
trans-Geraniol	>648.3
(±)-Linalool	>648.3
E- and *Z*-(±)-Nerolidol	>449.7
Aldehyde	
(-)-Citronellal	>648.3
Esters	
Geranyl acetate	>509.5
Linalyl acetate	>509.5
Phenols	
Carvacrol	>665.7
Eugenol	46.6±0.12
Oxide	
1,8-Cineole	>648.3

Essential oil constituent	Anti-oxidant activity (µM)
Ketones	
(±)-α+β-Thujone	>656.9
(+)-Carvone	>665.7
(-)-Fenchone	>656.9
(-)-Menthone	>648.3
(-)-Pulegone	>656.9
Terpene Hydrocarbons	
(+)-α-Pinene	>734.0
(+)-β-Pinene	>734.0
γ-Terpinene	>734.0
(R)-(+)-Limonene	>734.0
p-Cymene	>745.1
Control	
L-Ascorbic acid	17.03±0.06

4.2.3 Discussion

4.2.3.1 The anti-oxidant activity of essential oil constituents

The essential oils from clove (Nakatsu *et al*., 2000) and citrus (Choi *et al*., 2000) possess potent anti-oxidant activity. However other studies have indicated that not all essential oils have anti-oxidant activity (e.g. *Helichrysum* species) (Lourens *et al*., 2004). In this study, eugenol, the major constituent of the essential oil of *Eugenia caryophyllata* (clove) and *Myristica fragrance* (nutmeg) (Nakatsu *et al*., 2000) is the only constituent that possessed comparable anti-oxidant activity to L-ascorbic acid, being approximately three fold less active (Table 4.1). The therapeutic potential of *Ocimum sanctum* as a neuroprotective agent in cerebral ischaemia is attributed to the high content (70%) and very low toxicity (Table 5.1) of eugenol (Yanpallewar *et al*., 2004).

In one study, essential oil from the leaves of Syrian oreganum (*Origanum syriacum*) was found to contain 49.02% monoterpenes, 36.60% oxygenated monoterpenes and 12.59% sesquiterpenes (Peiwu *et al*, 1999). The major components were as follows: γ-terpinene, carvacrol, *p*-cymene and β-caryophyllene. The oil's anti-oxidant activity was compared to that of ascorbic acid, and 2,6-di-tert-butyl-4-methyl phenol (BHT, butylated hydroxytoluene). The results showed that the anti-oxidant activity of the oil was 3 to 4 times lower compared to L-ascorbic acid or BHT (Peiwu *et al*, 1999).

Vardar Ünlü *et al*. (2003) reported that thymol and carvacrol from the essential oil of *Thymus pectinatus*, were individually found to possess weaker anti-oxidant activity than the crude oil itself, indicating that other constituents of the essential oil contribute to the anti-oxidant activity observed. It is also interesting to note that although carvacrol, (±)-linalool, γ-terpinene and *trans*-geraniol have been reported to possess anti-oxidant activity (Choi *et al*., 2000; Nakatsu *et al*., 2000), in this study they had little activity when compared to eugenol and L-ascorbic acid (Table 4.1). The difference in activity between eugenol and carvacrol (both phenols) may possibly be attributed to the presence of a methyl group at the second carbon of eugenol, which is absent in carvacrol (Figure 2.1, m and n).

Epidemiological data suggest that anti-oxidants may have a beneficial effect on many age-related diseases: atherosclerosis, cancer, some neurodegenerative (Alzheimer's disease) and ocular diseases. However, the widespread use of anti-oxidants is hampered by several factors; the lack of prospective and controlled studies; growing evidence that free radicals are not only by-products, but also play an important role in cell signal transduction, apoptosis and

infection control. Despite these, the results of this study show that some essential oil constituents are good anti-oxidants.

4.3 Acetylcholinesterase activity

There are two forms of cholinesterase enzyme found in man, namely acetylcholinesterase (EC 3.1.1.7) and butyrylcholinesterase (or otherwise known as pseudocholinesterase; EC 3.1.1.8). Acetylcholinesterase is an extremely active enzyme present in three isoforms: G1, which is present in the brain; G4, in the brain and the neuromuscular endplate; and G2, in skeletal muscle and blood-forming cells (erythrocytes). With butyrylcholinesterase found in blood plasma and in lower concentrations in the hippocampal and cortical areas known to receive cholinergic innervation (Ellis, 2005). The function for these enzymes is the hydrolysis of acetylcholine to choline and acetic acid. Acetylcholine is the neurotransmitter essential for cholinergic or parasympathetic transmission in the autonomic nervous system (Pappano, 2004).

4.3.1 Cholinesterase inhibitors and alzheimer′s disease

This age-related dementia is associated with a loss of neurons, and alterations to brain tissue, particularly in the hippocampus and basal forebrain. Amyloid plaques and neurofibrillary tangles characterise the condition. The loss of cholinergic fibres (in the basal forebrain nuclei) is proposed to be a key factor in this disease. The development of Alzheimer's disease requires two events to occur. The first one is the age-related abnormal production of free radicals, which damage mitochondrial and nuclear DNA in the brain. The second event is a deficiency of the neurotransmitter acetylcholine in the synapses of cerebral cortex (Savelev *et al*., 2003).

Probable Alzheimer's disease is characterized by the presence of neuritic amyloid plaques and neurofibrillary tangles in the brain, pathologic changes associated with cholinergic denervation. Acetylcholinesterase and butyrylcholinesterase are both associated with amyloid plaques. Although it is unknown whether acetylcholinesterase plays a role in accelerating the neurotoxicity of amyloid plaques, there is some evidence that butyrylcholinesterase may accelerate maturation of benign plaques into plaques associated with neuronal degeneration and Alzheimer's disease. Long-term inhibition of cholinesterase activity may result in the activation of normal amyloid precursor protein processing in patients with Alzheimer's disease, as opposed to abnormal amyloid precursor protein processing, which leads to senile

plaque formation and causes many of the cognitive impairments in Alzheimer's disease (Sramek and Cutler, 2000).

Anticholinesterase drugs currently form the basis of modern drugs available for the symptomatic treatment for patients with Alzheimer's disease, and have a modest efficacy with predictable parasympathomimetic side effects. Drugs such as donepezil and the plant-derived isolates like galanthamine, physostigmine and rivastigmine are effective inhibitors of acetylcholinesterase. Rivastigmine preferentially inhibits the G1 molecular form of acetylcholinesterase and is the only cholinesterase inhibitor to have exhibited preferential selectivity for any of the three isoforms of acetylcholinesterase (Ellis, 2005). Selective inhibition of acetylcholinesterase occurs with galanthamine and donepezil, whereas rivastigmine inhibits both acetylcholinesterase and butyrylcholinesterase.

Galanthamine is a tertiary alkaloid acetylcholinesterase inhibitor that has been approved in several countries for the symptomatic treatment of senile dementia of the Alzheimer's type. Derived from bulbs of the common snowdrop and several Amaryllidaceae plants, (-)-galanthamine has long been used in anaesthetics to reverse neuromuscular paralysis induced by turbocurarine-like muscle relaxants and more recently, has been shown to attenuate drug- and lesion-induced cognitive deficits in animal models of learning and memory (Miyazawa and Yamafuji, 2005).

Galanthamine also stimulates pre- and postsynaptic nicotinic receptors, although the clinical significance of this finding is yet unclear. Numerous variants and analogues of galanthamine have also been developed, with varying potency in inhibiting acetylcholinesterase activity. Galanthamine is readily absorbed after oral administration, with a T_{max} of 52min and plasma elimination T½ of 5.7h (Miyazawa and Yamafuji, 2005). The efficacy of galanthamine administered to Alzheimer's disease patients has been well demonstrated by large-scale clinical trials. Typical of anticholinesterases, the most common adverse events associated with galanthamine are nausea and vomiting.

A growing body of evidence indicates that both acetylcholinesterase and butyrylcholinesterase play important roles in cholinergic transmission. For this reason, both cholinesterases are considered legitimate targets when managing Alzheimer's disease. The enhanced cholinergic function following treatment with cholinesterase inhibitors provides the symptomatic improvements observed in patients with probable Alzheimer's disease.

Although a number of other therapeutic approaches have been investigated in order to enhance cholinergic function and cognition in patients with Alzheimer's disease, cholinesterase inhibition is the only strategy that has thus far proven to have beneficial effects. In light of this and the reported traditional use of the essential oil from *Salvia lavandulaefolia* as having anticholinesterase properties (Savelev *et al*., 2003), further research is warranted in determining which of the essential oil constituents are able to inhibit acetylcholinesterase.

4.3.2 Methodology

A simple and rapid qualitative thin layer chromatography (TLC) bioautographic assay (Marston *et al*., 2002) was used to screen the selected essential oil constituents for the anticholinesterase activity. Acetylcholinesterase (EC 3.1.1.7) and the reference compound, galanthamine were purchased from Sigma-Aldrich (USA). Tris-HCl; bovine serum albumin and 1-napthyl acetate were obtained from Merck, and Fast Blue B salt was from Fluka (Switzerland).

4.3.2.1 Preparation of essential oil constituents

One microlitre of an essential oil constituent was diluted in seven microlitres of methanol and 1µl was applied to the TLC plates.

4.3.2.2 Preparation of acetylcholinesterase

Acetylcholinesterase (1000U) was dissolved in 150ml of 0.05M Tris-HCl buffer (pH 7.8). Bovine serum albumin (150mg) was added to the solution in order to stabilise the enzyme during the bioassay.

4.3.2.3 Bioautography

TLC plates were eluted with an appropriate solvent (acetone) in order to wash them, and were thoroughly dried just before use. The mobile phase used contained a ratio of 93:7 (v/v) of toluene and ethyl acetate, respectively. After migration of the EOC sample, the TLC plate was dried with a hair dryer for complete removal of acetone. The plate was then sprayed with the enzyme stock solution and thoroughly dried again. For incubation of the enzyme, the plate was laid flat on plastic plugs in a plastic tank containing little water, such that the water did not come into direct contact with the plate, but the atmosphere was kept humid. The cover was placed on the tank and incubation was performed at 37˚C for 20min.

The enzyme had satisfactory stability under these conditions. For detection of the enzyme, solutions of 1-naphthyl acetate (250mg) in ethanol (100ml) and Fast Blue B salt (400mg) in distilled water (160ml) were prepared immediately before use to prevent decomposition. After incubation of the TLC plate, 10ml of the naphthyl acetate solution and 40ml of the Fast Blue B salt solution were mixed and sprayed onto the plate to give a purple colouration after 1 - 2min. The relative to the front (Rf) values were determined.

Galanthamine was used as the positive control, as this alkaloid initially isolated from *Galanthus nivalis*, the snowdrop, has for many years been used as an acetylcholinesterase inhibitor (Pappano, 2004).

4.3.3 Results

Based on the interpretation of the results, the anticholinesterase activity of each tested compound was expressed as: ++++ = very active; +++ = moderately active; ++ = slightly active; - = not active, relative to the intensity of the white spots of the positive control, galanthamine (Figure 4.3 and Table 4.2). Eugenol, 1,8-cineole, (±)-α+β-thujone, *trans*-geraniol, (+)-α-pinene and geranyl acetate were the most active comparable to the standard anticholinesterase compound, galanthamine; while the other compounds had little ((-)-menthone) or no activity ((-)-fenchone).

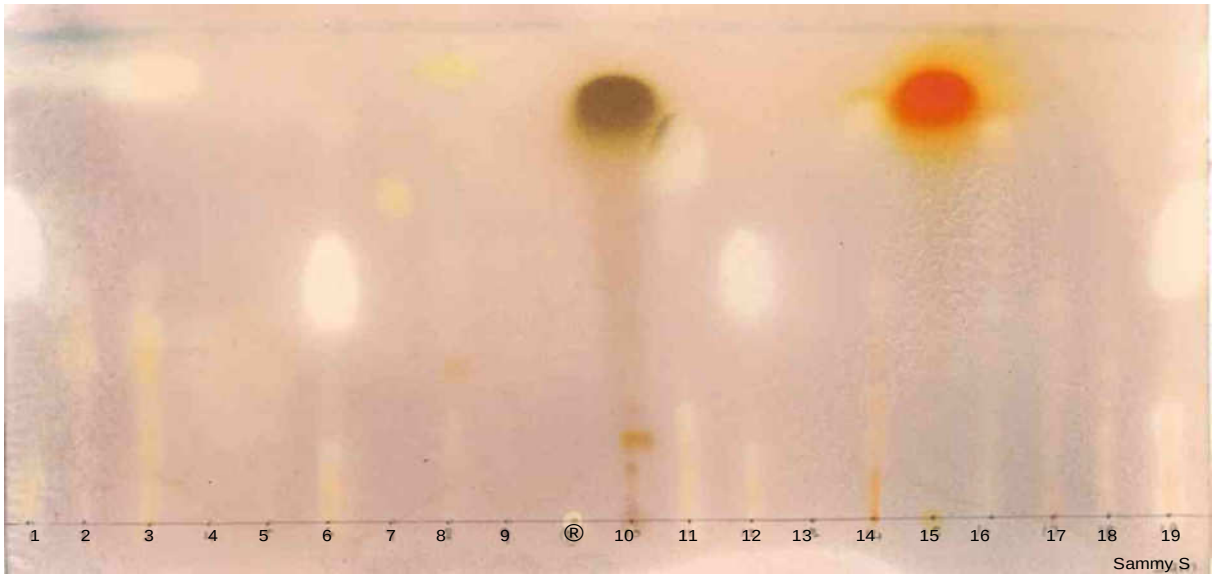


Figure 4.3: A thin layer chromatographic plate showing the anticholinesterase activity of 19 essential oil constituents. The essential oil constituents and the reference compound with their corresponding numbers are depicted in Table 4.2

4.3.4 Discussion

Overall the acetylcholinesterase enzyme was found to be generally susceptible to the action of the essential oil constituents (Table 4.2). Although this was the general trend for the tested constituents, eugenol, 1,8-cineole, (±)-α+β-thujone, *trans*-geraniol, (+)-α-pinene and geranyl acetate were comparable to galanthamine exhibiting promising anticholinesterase activity (Figure 4.3). The oxide, 1,8-cineole has previously been reported to possess anticholinesterase activity (Mills *et al*., 2004; Savelev *et al*., 2003), which is in agreement with the present study. Although, Miyazawa and Yamafuji (2005) reported that essential oil constituents with the pinane skeleton, like (+)-α-pinene and not (+)-β-pinene, showed a strong inhibitory effect on acetylcholinesterase, as is the case with the α-isomer, α-pinene in the present study (Table 4.2).

Lahlou (2004a) also reported that some monoterpene derivatives such as linalyl acetate, geranyl acetate, eugenol and carvacrol inhibit acetylcholinesterase, which is congruent with the results obtained in this present study (Table 4.2). The action of acetylcholinesterase in the neuromuscular junction ensures muscle contraction, thus if the action of acetylcholinesterase is inhibited there will be an increased accumulation of acetylcholine in the synaptic cleft which will induce further muscle contraction or could antagonise the effects of pancuronium (a neuromuscular blocker). However, this is not the only mechanism by which EOC's are proposed to cause muscle contraction. Lahlou (2004a) reported that EOC's like eugenol are able to induce muscle contraction (or blockade of excitation-contraction coupling) by releasing calcium ions from the sarcoplasmic reticulum. Moreover, the spasmolytic effect of (±)-linalool was thought to be mediated through cyclic adenosine monophosphate (cAMP).

Miyazawa and Yamafuji (2005) also reported that overall the hydrocarbon compounds showed potent inhibition of acetylcholinesterase as compared with alcohols and ketones. However, in our study most EOC's like (R)-(+)-limonene, carvacrol, linalyl acetate and *E*- and *Z*-(±)-nerolidol showed equipotent activity (Table 4.2 and Figure 4.3), irrespective of the class each falls under. Whilst the alcohol, *trans*-geraniol showed moderate anticholinesterase activity. The degree of saturation, including the position of the C=C double bond, affected the inhibitory activity of acetylcholinesterase and is also related to the strength or potency of inhibition of acetylcholinesterase (Miyazawa and Yamafuji, 2005).

Table 4.2: The anticholinesterase activity of 19 essential oil constituents. Where: ++++ = very active; +++ = moderately active; ++ = slightly active; - = not active.

Essential oil constituents	Corresponding number in TLC plate (Figure 4.3)	Anticholinesterase activity
Alcohols		
(-)-α-Bisabolol	13	-
trans-Geraniol	11	++++
(±)-Linalool	5	-
E- and *Z*-(±)-Nerolidol	16	+++
Aldehyde		
(+)-β-Citronellal	4	-
Esters		
Linalyl acetate	7	+++
Geranyl acetate	19	++++
Ketones		
(±)-α+β-Thujone	6	++++
(+)-Carvone	8	++
(-)-Fenchone	15	-
(-)-Menthone	18	++
(-)-Pulegone	10	-
Phenols		
Carvacrol	2	+++
Eugenol	1	++++
Terpene hydrocarbons		
(+)-α-Pinene	12	++++
(+)-β-Pinene	9	-
(R)-(+)-Limonene	14	+++
p-Cymene	17	++
Oxide		
1,8-Cineole	3	++++
Control		
Galanthamine	®	++++

EOC's like (-)-α-bisabolol, (-)-fenchone, (-)-pulegone, and (-)-citronellal, (±)-linalool and (+)-β-pinene did not show any anticholinesterase activity. However, (-)-α-bisabolol from the *Salvia* species has been reported to possess anticholinestrase activity (Perry *et al*., 2003). The difference between the activities of the EOC's from study to study could be attributed to the existence of infraspecific chemical differences in the essential oils of some aromatic plants (or chemovarieties). It is important to pinpoint that the test method applied in this study may not be the best when testing the anticholinesterase activity and might have contributed to the poor activity of most EOC's. Regardless, it is relevant to mention that acetylcholinesterase inhibitors including most EOC's mentioned above, have a broader spectrum of activity and thus possess possible therapeutic potential other than just for Alzheimer's disease. These include:

1. Anthelmintic infections: drugs such as pyrantel pamoate used in the treatment of pinworm (*Enterobius vermicularis*), ascariasis (*Ascaris lumbricoides*), hookworm (*Ancylostoma duodenale* and *Necator americanus*) and *Trichostrongylus orientalis* infections are known to be depolarising neuromuscular blockers, but also inhibit the activity of helmintic acetylcholinesterase enzyme, resulting in paralysis and subsequent expulsion of the helminth (Rosenthal and Goldsmith, 2004).

2. Insecticidal agents: organophosphates (parathion) and organocarbamates are irreversible pesiticides and used to control infestations (Pappano, 2004).

3. Bilharzia infections: the agent metrifonate used in the treatment of *Schistosoma haematobium* (bilharzia) and as an insecticide, is an organophosphate cholinesterase inhibitor; where the active metabolite, 2,2-dimethyldichlorovinyl phosphate irreversibly inhibits acetylcholinesterase enzyme. The pharmacokinetics of this metabolite is favourable for long-term treatment of Alzheimer's disease with the half-life of cholinesterase inhibition being approximately 26 days; even though the plasma half-life of the drug is 2-3h. This drug is currently under investigation to determine its safety and efficacy in the treatment of the cognitive symptoms of Alzheimer's disease (Ringman and Cummings, 1999).

4. Antagonists to neuromuscular blockers: acetylcholinesterase inhibitors, such as physostigmine and neostigmine are regularly used to reverse the effects of neuromuscular blockers, such as pancuronium. Thus, if the safety profile of the essential oil constituents (e.g. geranyl acetate, *trans*-geraniol, (±)-α+β-thujone, eugenol, (+)-α-pinene and 1,8-cineole) will be acceptable then further investigations may be warranted in using the EOC´s in this scenario.

5). Ectoparacidal agents: organophosphates (dichlorvos, diazinon) and organocarbamates (carbaryl, propoxur) are regularly used in the veterinary treatment of ectoparasites such as

ticks, lice and fleas. The organophosphates irreversibly phosphorylate acetylcholinesterase, while the organocarbamates reversibly inhibit enzyme activity such that the increased endogenous acetylcholine causes muscle fasciculations with subsequent paralysis and death. Dichlorvos is effective in the treatment of roundworm in pigs and horses (McKellar and Jackson, 2004).

Although the inhibition of acetylcholinesterase activity may be beneficial in many diseases and clinical situations, it should also be remembered that this enzyme is necessary for the metabolism and breakdown of numerous drugs such as the ester-type local anaesthetic (procaine) and neuromuscular blockers (succinylcholine, mivacurium). The consequence being increased plasma concentrations of the drugs with increased risk of adverse drug effects. These types of interactions should be taken into account when further investigating the potential of using the EOC´s.

CHAPTER FIVE
CYTOTOXICITY PROFILE

5.1 Introduction

Toxicology is concerned with the deleterious effects of chemical agents on all living systems. However, in the biomedical area toxicology is primarily concerned with unfavourable effects in humans resulting from exposure to drugs and other chemicals, including plant extracts and essential oils (Plaa, 2004). As with all drugs, plant essential oils can also exhibit unfavourable or deleterious effects on mammalian cells such as: 1) topical effects including the skin, mucous membranes, eye irritation, phototoxicity, skin sensitization and photosensitization; 2) systemic effects including mutagenicity, carcinogenicity, embryotoxicity and reproductive toxicity (Yoo *et al.*, 2005). Furthermore, these adverse effects can involve specific organs like the liver, heart, kidney and brain. The cytotoxicity of an essential oil may be changed by the presence of other compounds, where the compounds may potentiate each other´s activity or chemically modify each other or alter their respective bioavailability and overwhelm their therapeutic range. For example, Tea Tree oil consists of many chemical constituents that are also prominent in pine oil and because of their similar properties, simultaneous administration could potentiate the expected adverse effects if their doses are not adjusted (Nakatsu *et al*., 2000). Therefore, essential oil usage by people using other drugs may lead to complications of essential oil-drug interactions, because some drugs have a narrow therapeutic range (e.g. an anticoagulant warfarin), thus these can compromise the pharmacokinetic and pharmacodynamic profiles of therapeutic agents. Several clinical cases have been documented on the interaction and treatment failure between herbal remedies and prescribed medications (Constable, 2006). The combined use of several herbal preparations (garlic, feverfew and ginseng) can increase the risk of bleeding, as all three preparations inhibit platelet aggregation (Constable, 2006).

Overdosing or incorrect usage of essential oils may result in serious side effects (Zhou *et al*., 2004), since most essential oils should not be used in their pure (neat) form, but diluted with carrier oils before used topically and on mucous membranes. Infants, children, the elderly, pregnant and lactating women and epileptics are vulnerable, therefore, particular care should be taken when using essential oils (Lawrence, 2000). The general rule of thumb is to avoid prolonged use of oils; so it is advisable to take breaks from their usage (Lawrence, 2000). This is due to the tendency of building up a residue in the body which may result in

deleterious effects (Zhou *et al*., 2004). There is well established evidence that the formation of reactive metabolites of drugs is associated with drug toxicity (Zhou *et al.*, 2004). Likewise, there is data suggesting that the formation of reactive metabolites through bioactivation has a significant role in herbal toxicity and carcinogenicity. Reports revealed that these resultant reactive metabolites covalently bind to cellular proteins and DNA, leading to toxicity through multiple mechanisms such as direct cytotoxicity, oncogene activation, and hypersensitivity reactions (Zhou *et al*., 2004). For example, with aristolochic acids present in *Aristolochia* species, the nitro group is reduced by hepatic cytochrome P450 (CYP1A1/2) or peroxidases in extrahepatic tissues, this results in a reactive cyclic nitrenium ion. The nitrenium ion possibly reacts with DNA and proteins, resulting in activation of H-ras oncogene, gene mutation and ultimately carcinogenesis (Zhou *et al*., 2004). Other examples are pulegone present in essential oils from many mint species; and teucrin A, a diterpenoid found in germander (*Chamaedrys*) used as add on therapy to slimming tablets (Lawrence, 2000; Yoo *et al*., 2005). An excessive metabolism of pulegone may generate *p*-cresol which depletes glutathione (Zhou *et al*., 2004). The furan ring of the diterpenoids in germander is oxidized by CYP3A4 to a reactive epoxide which reacts with CYP3A and epoxide hydrolase (Yoo *et al*., 2005). Zhou *et al*. (2004) reported that hepatic cytochrome P450 enzymes (CYPs) are known to metabolize more than 95% of therapeutic drugs and activate a number of procarcinogens as well. Therefore, mechanism-based inhibition of CYPs may explicitly explain some reported herb-drug interactions and chemopreventive/chemotherapeutic herbal and essential oil activities (Zhou *et al*., 2004).

Toxic reactions vary qualitatively depending on the duration of the exposure, with a single exposure occurring over a day or two, presenting acute exposure which is reversible and tends not to be severe. However, multiple exposures continuing over a longer period of time represent a chronic exposure, which result in severe adverse effects (Plaa, 2004). Overexposure to essential oils, topically or via inhalation can result in nausea, dizziness, headache or a light-headed feeling (Yoo *et al*., 2005); but more serious topical and systemic effects as mentioned below, have also been reported.

5.1.1 Topical effects

- Allergic reactions are common with essential oils and include possible symptoms like nausea, dizziness, sweating, palpitations, stomach pains and irritation to mucous membranes. If these problems are experienced, it is advisable to discontinue the use of essential oils as the reactions are reversible.

- Skin irritation is an inflammatory reaction to certain essential oils and the severity depends upon the concentration and duration of exposure to the oil. It may be partially attributed to the terpene content in the oils, but it is more likely to be caused by the high phenol content in certain oils (Yoo *et al.*, 2005).
- Dermatitis is possible even when a diluted essential oil is applied to the skin, sometimes even the vapours can cause a sensitization reaction. The effects can present as a rash, blotches and itching, or blistering. Allergies involve the interaction of the immune system forming antibodies to render the antigen/essential oil harmless. However, investigations demonstrated that dermatitis is an immunological response not an antigen-antibody reaction (Yoo *et al*., 2005). By far, only a few skin sensitizing agents have been identified, namely: basil, cinnamon, lemon, lemongrass, melissa, peppermint, thyme and tea tree oils (Nakatsu *et al*., 2000).
- Phototoxic reactions resulting in photosensitization, redness and trauma to the skin when exposed to sunlight have been reported to occur with bergamot, lemon, lime, orange and other citrus oil (Nakatsu *et al*., 2000).
- Essential oils with thujone or apiol are abortifacient and have been proposed to cause blood accumulation in the pelvic-uterus area and liver damage (Zhou *et al*., 2004). These oils include hyssop, mugwort, parsley, pennyroyal, sage and thuja. Thus, pregnant women should avoid these essential oils and also others such as basil, camphor, clary sage, cedarwood, juniper, marjoram, myrrh, rue, tansy and wormwood (Yoo *et al*., 2005).
- Overdosage of oils containing thujone and apiol can also have narcotic, analgesic or paralyzing effects (Zhou *et al*., 2004).
- Essential oils that are excreted via the renal system such as juniper and sandalwood are rendered nephrotoxic and their use must be discontinued after four weeks (Yoo *et al*., 2005).

5.1.2 Systemic effects

Hypertensive patients must be cautious when using essential oil of hyssop, rosemary, sage and thyme (Zhou *et al*., 2004), while those patients with epileptic seizures must be careful when using oils from fennel, hyssop, rosemary, sage or wormwood (Halcon, 2002).

Toxicological cases caused by volatile oils such as eucalyptus, sassafras, turpentine, wintergreen, chenopodium, citronella oil, pennyroyals and peppermint oils have also been reported (Halcon, 2002). In infants these aforementioned oils may cause respiratory depression or even death, when applied directly into the nose or close to the nose onto the

skin or mucous membranes (Halcon, 2002). These problems arise due to misidentification, drug-herb interactions and lack of standardization. The mechanism of herbal or essential oil toxicity remains elusive and the following systemic problems may also arise from abuse of essential oils.

5.1.2.1 Hepatotoxicity

The liver has various functions and helps with carbohydrate and lipid metabolism, secretion of bile as well as the synthesis of proteins, yet most people perceive the liver as only a detoxification organ for blood. Metabolism of essential oil constituents is via human hepatic cytochrome P450 enzymes to non-toxic metabolites, but the formation of toxic metabolites is also possible (Zhou *et al*., 2004). The monooxygenases oxidizing metabolism of these essential oil constituents require a haemoprotein P450, a flavoprotein NADPH-P450 reductase and phospholipids (Ueng *et al*., 2005).

In the human liver, CYP1A2, CYP2A6, CYP2C9, CYP2D6, CYP2E1, and CYP3A4 are the major forms of the cytochrome P450 enzymes, which constitute about 13%, 4%, 18%, 2%, 7%, and 29% of total P450 contents, respectively (Ueng *et al*., 2005). Hepatotoxicity is unlikely to occur following administration of the essential oil to the skin; but it is more likely to occur following oral ingestion. No essential oil should be taken internally unless so prescribed by a licensed medical practitioner. Essential oils which are hepatotoxic when swallowed are aniseed, buchu, basil, clove and cinnamon bark (Ueng *et al*., 2005).

5.1.2.2 Carcinogenicity

Some essential oils are carcinogenic and have been banned (Table 5.1) in aromatherapy and cannot be applied for internal usage. The oils which still raise doubts are calamus oil (contains asarone), and those containing ample amounts of safrole like sassafras found in yellow and brown camphor (Nakatsu *et al*., 2000). Other carcinogenic compounds in essential oils include methyl chavicol found in basil oil. Notably, this statement does not make basil dangerous, but care should be taken to select the basil oil containing ample amounts of linalool and lowest methyl chavicol content (Ueng *et al*., 2005). It must however be pointed out that studies showed that these oils can be carcinogenic when applied daily in high concentration for many months (Lawrence, 2000; Ueng *et al*., 2005).

Table 5.1: IFRA banned aromatic raw materials (modified from http://www.users.globalnet.co.uk/~nodice/new/magazine/magsafetylecture.htm).

NAME	BOTANICAL NAME	ACTION
Cade crude oil	*Juniperus oxycedrus*	Carcinogenic
Calamus oil	*Acorus calamus*	Carcinogenic
Costus root	*Saussurea costus*	Sensitizer
Elecampane oil	*Inula helenium*	Sensitizer
Fig leaf absolute	*Ficus carica*	Sensitizer
Horseradish oil	*Armoracia rusticana*	Toxic and irritant
Mustard oil	*Brassica nigra*	Toxic and irritant
Peru balsam	*Myroxylon var. pereirae*	Sensitizer
Savin oil	*Juniperus Sabina*	Toxic and sensitizer
Verbena oil	*Lippia citriodora*/*Aloysia triphylla*	Sensitizer
Tea absolute	*Thea sinensis*/*Camellia sinensis*	Sensitizer
Sassafras oil	*Sassafras albidum*	Carcinogenic
Stryax gum	*Liquidambar orientalis*	Sensitizer
Wormseed	*Chenopodium ambrosioides*	Toxic
Wormwood oil	*Artemisia absinthium*	Neurotoxin

5.1.2.3 Cardiac problems

Although essential oils and aromatherapy can be conducive to radiant health, caution is necessary when using them with certain existing medical conditions (Ueng *et al*., 2005). This refers to any cardiovascular problem that may be present. Thus, it is best to exclude peppermint oil as part of the treatment regimen or even during an aromatherapy massage (Zhou *et al*., 2004).

Certain aromatic raw material including essential oils are banned (Table 5.1) and others restricted (Table 5.2) by the International Fragrances Research Association (IFRA), because of their particular deleterious effects. Even though those in Table 5.2 are not banned, caution is indispensable with regards to the amount of active ingredients/compounds contained in

them, as the existence of the side effects such as sensitization and phototoxicity is possible when treating patients with various clinical conditions (Ueng *et al*., 2005).

Table 5.2: IFRA restricted aromatic raw materials (modified from http://www.users.globalnet.co.uk/~nodice/new/magazine/magsafetylecture.htm).

NAME	BOTANICAL NAME	ACTION
Angelica root oil	*Angelica archangelica*	Phototoxic
Bergamot oil	*Citrus bergamia*	Phototoxic
Bitter orange oil	*Citrus aurantium*	Phototoxic
Cassia oil	*Cinnamomum cassia*	Sensitizer
Cinnamon bark	*Cinnamomum zeylanicum*	Sensitizer
Cumin oil	*Cuminum cyminum*	Phototoxic
Grapefruit oil	*Citrus paradisi*	Phototoxic
Lemon oil	*Citrus limon*	Phototoxic
Lime oil	*Citrus aurantifolia*	Phototoxic
Tagetes oil and absolute	*Tagetes minuta*	Phototoxic
Oak moss absolute and resinoid	*Evernia prunastri*	Sensitizer
Pinaceae oils	*Pinaceae mugo, P. nigra, P. pinaster, P. sylvatica*	Sensitizer
Rue oil	*Ruta graveolens*	Sensitizer
Verbena absolute	*Lippia citriodora/Aloysia triphylla*	Sensitizer
Tree moss absolute	*(Pseudo) Evernia furfuracea*	Sensitizer

Due to the wide use and easy availability of herbal medicines, there is increasing concern about essential oil toxicity (Constantin *et al.*, 1991). Despite this fact, there is still a great necessity for more scientific evidence on the toxicity of volatile oils. Thus, this study took the opportunity to meet this increasing demand by investigating the toxic profiles of selected essential oil constituents when used individually and in combination.

5.2 Methodology

5.2.1 Principles of the method

The methods to measure the number of viable proliferating cells vary widely, thus a reliable, versatile and quantitative method for detecting the viability is a prerequisite. Tetrazolium compounds are an increasing popular non-radioactive option. Moreover, assays involving tetrazolium salts such as the MTT [3-(4,5-dimethyl-2-thiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay, can easily detect living cells, and the resultant colour formation depends on the degree of functionality of the mitochondria. The main advantages of this assay are: 1) no washing steps; 2) no radioisotope is required; and 3) results can be read with high degree of accuracy, reproducibility and rapidity (Mosmann, 1983).

The MTT assay is reliant on succinate dehydrogenase which is an enzyme localised in the mitochondria of living cells that cleaves the tetrazolium ring of a tetrazolium dye. The yellow, water soluble MTT dye changes to a violet, insoluble product (MTT-formazan) (Mosmann, 1983). This chemical reaction can easily be measured by a spectrophotometer, and the amount of formazan formed is directly proportional to cell density.

5.2.2 Culture maintenance

The transformed human kidney epithelial (Graham) cells were continuously maintained *in vitro* at 37ºC in 5% CO_2 according to the departmental protocol. The cells were maintained with complete culture medium at least thrice weekly and trypsinized (Section 5.2.2.4) once weekly to subculture the cells.

5.2.2.1 Preparation of complete culture medium

The culture medium was prepared in Milli-Q® water with the following ingredients: 9.8g/l HAM F10 supplemented with 1.18g/l $NaHCO_3$. The culture medium was sterilized through a Sterivex-GS 0.22µm filter unit (Highveld Biologicals, South Africa) and stored at 4ºC. Before use, the complete culture medium was prepared by supplementing the culture medium with 5% foetal calf serum (FCS, Section 5.2.2.2) and 0.1ml of sterile 50mg/ml gentamicin. The gentamicin stock was prepared by dissolving 50mg gentamicin sulphate in 1ml PBS (Section 3.2.1.3), filtered through a Sterivex-GS 0.22µm filter unit and stored at 4ºC until required.

5.2.2.2 Preparation of foetal calf serum

FCS (Highveld Biologicals, South Africa) was heat inactivated at 56ºC in a water bath for 1h. The inactivated FCS was centrifuged at 1500rpm for 10min. Thereafter, aliquots of the supernatant were stored at -20ºC until required.

5.2.2.3 Assessment of cell growth

The cells were examined microscopically to assess their growth at least thrice weekly. Following which the spent medium was discarded and 50ml of the fresh complete culture medium was added and the cells were then incubated at 37ºC.

5.2.2.4 Trypsinization

Trypsinization was carried out to adjust the number of adherent proliferating cells in the flask in order to optimize growing conditions. A volume of 4ml of 0.25% trypsin-0.1% Versene-EDTA (Highveld Biologicals, South Africa) was added to the culture flask and allowed to cover the bottom of the flask, left to stand at room temperature for 5 - 10min, thereafter the flask was gently agitated to loosen the cells. Once the cells were in suspension, 6ml complete culture medium was added to neutralise the trypsin. The FCS in the culture medium has the ability to inactivate trypsin. Thereafter, the cells were gently agitated to ensure a single cell suspension. Thereafter, 9ml of this suspension was either discarded or used for experimental purposes. To the remaining cells, 50ml complete culture medium (Section 5.2.2.1) was added and the cells were then incubated at 37°C in 5% CO_2 such as to readhere and propagate.

5.2.3 MTT cytotoxicity assay

To investigate the toxicity profile of the EOC´s, the MTT assay was performed on the human kidney (Graham) epithelial cells according to the method of Mosmann (1983). Various dilutions of twenty essential oil constituents were incubated under optimal conditions with the cells for 48h to determine their effect on the proliferation of the cells.

5.2.3.1 Preparation of the cells

From Section 5.2.2.4, the cell suspension was centrifuged at 1500rpm for 5min and the supernatant discarded. A volume of 20ml experimental medium (i.e. the complete culture medium (Section 5.2.2.1) but without gentamicin) was then added to the cells in a 50ml centrifuge tube. The cells were once again gently agitated to ensure a single cell suspension. The cells were then stained with 0.2% trypan blue in a 1:1 (v/v) ratio and two grids of cells counted using a haemocytometer at 100x magnification. The trypan blue is taken up into

compromised or dead cell, whilst viable cells exclude the dye. At least, 95% cellular viability was ensured before using the cells in the experiment. The number of cells/ml was determined using Equation 5.1 before the cell suspension was adjusted with experimental medium to 0.25 million cells/ml.

Equation 5.1:

$$\text{Cells/ml} = \text{number of counted cells/2 x dilution factor (2) x } 10^4$$

5.2.4 Preparation of essential oil constituents and control drugs

Test compounds and a positive control drug, quinine, were prepared in eppendorfs as previously described in Sections 3.2.3.3; 3.2.3.3.1; 3.2.3.3.2; 3.2.4 and 3.2.4.1.

5.2.5 Selection and preparation of essential oil constituents used in combination assays

Based on the IC_{50} values obtained from the individual EOC´s, various EOC combinations were selected; namely: two of the most active EOC´s, one of the most active with one of the least active, as well as two of the least active EOC´s. Eight fixed ratios of the two predetermined EOC´s were then prepared such that the concentration of one EOC increased, the concentration of the second EOC decreased. Both EOC were also prepared in the absence of the other EOC to serve as a positive control. The eight dilutions for each individual EOC were prepared in DMSO as described in Chapter 3 (Section 3.2.3.3.2). Further, at least six dilutions from each of the abovementioned eight dilutions were prepared by 1:10 serial dilution in eppendorfs using DMSO.

The dilutions were then combined in a 1:1 (v/v) ratio. A further dilution factor of 2 was taken into account, so that the final dilution factor increased to 200x for the EOC´s. In the wells of row A, 2µl of the first dilution of the combined EOC's were plated out in triplicate and 18µl RPMI experimental medium (Section 3.2.1.3) was added. In rows B to G 18µl RPMI experimental medium was added before 2µl of previous dilution was transferred from row to row. To row H, only 20µl of 18µl RPMI experimentalmedium was added, while 180µl of the adjusted cell suspension (Section 5.2.3.1) was added to all the wells of the plate (Section 5.2.6), except in the first well of row H. This latter well served as a cell free blank, and contained 180µl of HAM F10 experimental medium and 20µl of RPMI experimental medium. The experimental runs were repeated in triplicate for reasons of consistency.

5.2.6 Preparation of microtitre plates

A volume of 180µl of the adjusted cell suspension was added to all wells except one. This well served as the cell-free blank control, to which 180µl of experimental HAM F10 culture medium containing FCS plus 20µl hypoxanthine-free medium (Section 3.2.1.3) were added. The cells were incubated for 6h under humidified conditions in 5% CO_2 at 37ºC so that they could adhere to the bottom of the wells of the plate well. After this incubation period, 2µl of the individual EOC or combined EOC´s and 18µl of hypoxanthine-free medium were added to all wells, except the last row (row H). A total of 20µl of each dilution of the test EOC's and control, and quinine were added in triplicate to wells of rows A-G. To row H, 20µl of hypoxanthine-free medium was added and served as the drug-free controls. Thereafter, the cells were incubated under humidified conditions in 5% CO_2 at 37ºC. After 44h of incubation, 40µl of 50mg/ml (= 0.05mM) MTT (Section 5.2.7) was added to all wells and the plate was further incubated for 4h.

5.2.7 Addition of MTT and DMSO

A 0.05mM stock solution of MTT (USB™) prepared in PBS (Section 3.2.1.3) was sterile filtered using a Sterivex-GS 0.22µm filter unit and stored at 4ºC. After 48h of incubation, 200µl of the contents from all wells was aspirated and discarded from all wells. Thereafter, 150µl of DMSO was added to all the wells to stop the reaction and dissolve the purple formazan crystals.

5.2.8 Data analysis

The plate was shaken for 4min using a UV-VIS spectrophotometer to ensure complete dissolution of the crystals and the absorbance was read at the test wavelength of 540nm and reference wavelength of 690nm using the Ascent® software (Biosoft, UK) and log sigmoidal dose response curves drawn. The percentage cell viability was calculated with the appropriate controls taken into account according to Equation 5.2.

Equation 5.2:

$$\text{Cellular viability} = \frac{\text{Drug treated absorbance - Mean absorbance of cell-free control}}{\text{Mean absorbance of drug-free control - Mean cell-free control}} \times 100\%$$

Thereafter, the IC_{50} values were determined using the Enzfitter® software (Biosoft, UK). The IC_{50} values from at least three independent experiments were used to calculate the mean and standard deviation for each EOC. Data from the combination experiments were analysed and

isobolograms constructed to determine the type of interaction between the EOC's. Isobolograms were drawn using Graphpad Prism® software (Biosoft, UK) by plotting fractional IC_{50} values for one compound against fractional IC_{50} values for the second compound. The trend of the graphs gave an indication of synergistic, antagonistic or additive interactions (Berenbaum, 1989). Points lying above the straight diagonal line (convex in shape) and those lying below the straight diagonal line (concave in shape) indicated an antagonistic or synergistic interaction, respectively. Points lying on the straight line indicated that the combination of the two compounds had an additive interaction (Figure 3.3). It must be noted that there may be some points on the isobolograms overlying on each other and cannot be clearly seen sometimes. This can be the case irrespective that six fixed ratios of the two EOC´s were combined. The FIC values were calculated as described in Section 3.2.5.2 (Equation 3.3). In addition, the ratio of the safety index between the antimalarial activity and the toxicity profile of the EOC's was calculated using Equation 5.3.

Equation 5.3: Safety index = Toxicity profile/Antimalarial activity

5.2.9 Statistics

All values were expressed as means ± s.d. (standard deviations). To test for correlation between variables, linear regression analysis was performed and squared correlation coefficient (r^2) was calculated, with $p < 0.05$ considered to be significant.

5.3 Results

5.3.1 The toxicity profiles of the individual essential oil constituents

In vitro toxicity profiles of 20 EOC´s including terpene hydrocarbons and oxygenated compounds have been investigated (Figure 5.1). The most toxic EOC was *E*- and *Z*-(±)-nerolidol, with an IC_{50} value of 5.5±1.2µM; whilst the least toxic was eugenol with an IC_{50} value of 1358.4±13.0µM (Figure 5.1). *E*- and *Z*-(±)-Nerolidol was approximately 13 times more toxic than quinine which had an IC_{50} value of 71.2±0.01µM, whereas eugenol was 19 times less toxic than quinine.

5.3.2 The combination studies of the selected essential oil constituents

Based on the results of the toxic activity of the 20 individual EOC´s, the two most toxic (*E*- and *Z*-(±)-nerolidol and (-)-pulegone), two least toxic (*p*-cymene and γ-terpinene) and one toxic ((-)-pulegone) and one least active (*p*-cymene) EOC were selected and tested for their toxicity profiles.

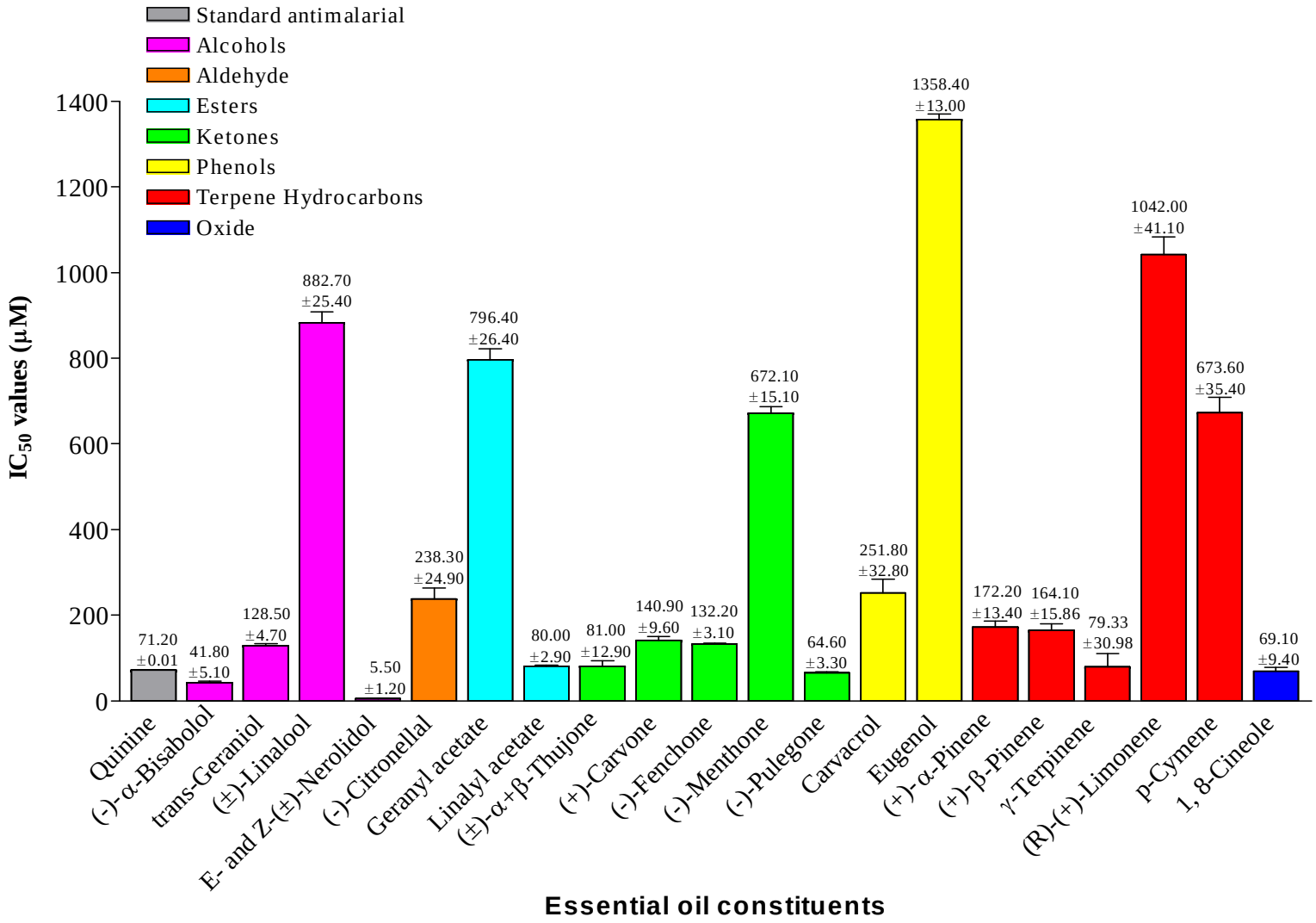


Figure 5.1: The toxicity profile of the individual essential oil constituents.

From the isobolograms of the combinations tested in Table 5.3, it was evident that the toxicity profiles between the combined EOC´s (Figures 5.2 and 5.3) were all potentiated.

Table 5.3: The toxicity profile of the combined essential oil constituents (n = 3).

EOC_1	EOC_2	∑FIC value	Interaction
E- and *Z*-(±)-Nerolidol	(-)-Pulegone	0.076	Synergism
p-Cymene	γ-Terpinene	0.002	Synergism
p-Cymene	(-)-Pulegone	0.306	Synergism

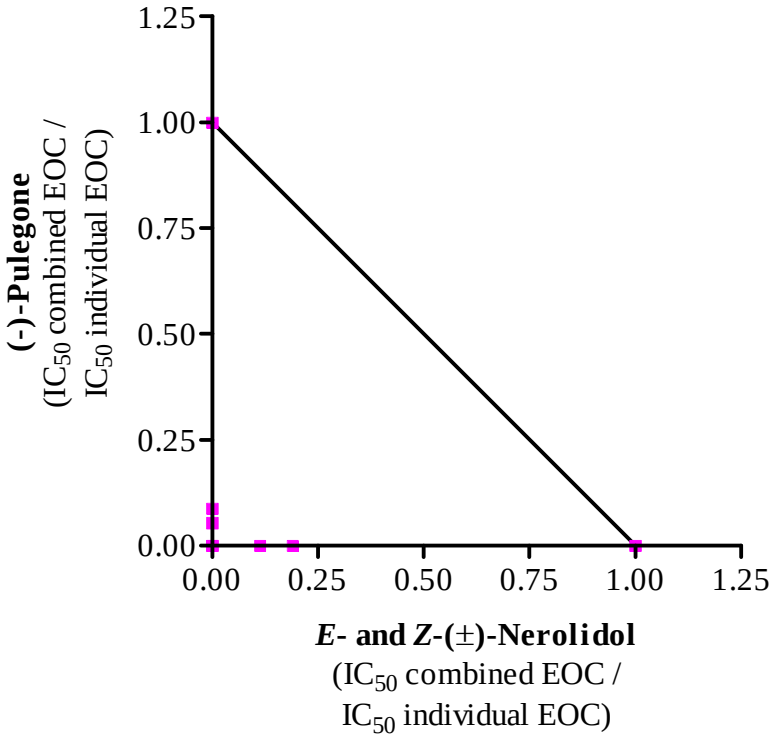


Figure 5.2: The synergistic interaction between *E*- and *Z*-(±)-nerolidol and (-)-pulegone, indicating an increase in their toxicity profile.

5.3.3 The toxicity profile of the essential oil constituent and quinine

Potentiation of the toxicity profile (∑FIC= 0.001) was observed when the most active EOC (*E*- and *Z*-(±)-nerolidol) was combined with the standard antimalarial agent, quinine (Figure 5.4).

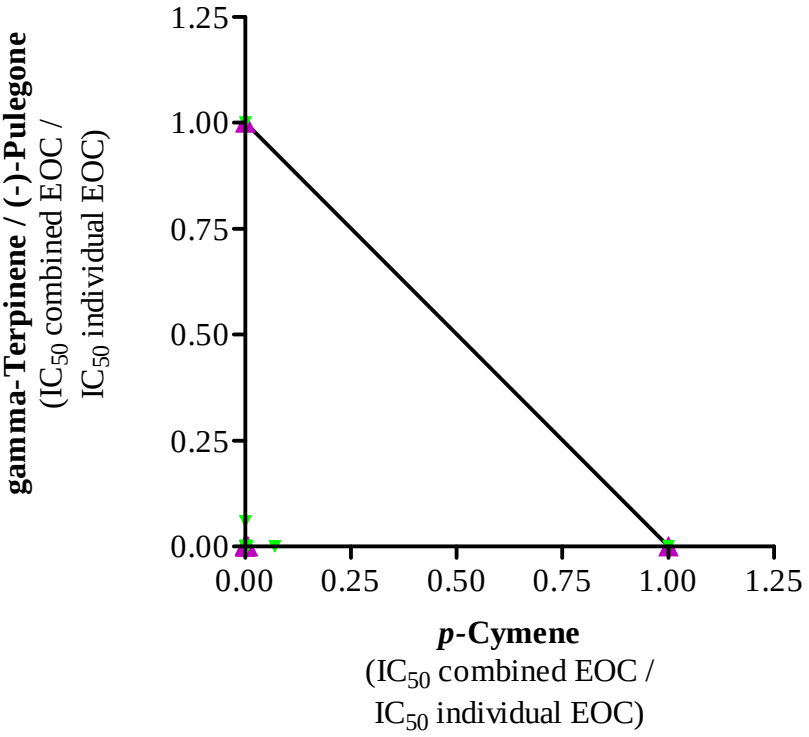


Figure 5.3: *p*-Cymene interacted in a synergistic manner with both γ-terpinene (▲) and (-)-pulegone (▼) to potentiate the toxicity profile of the essential oil constituents.

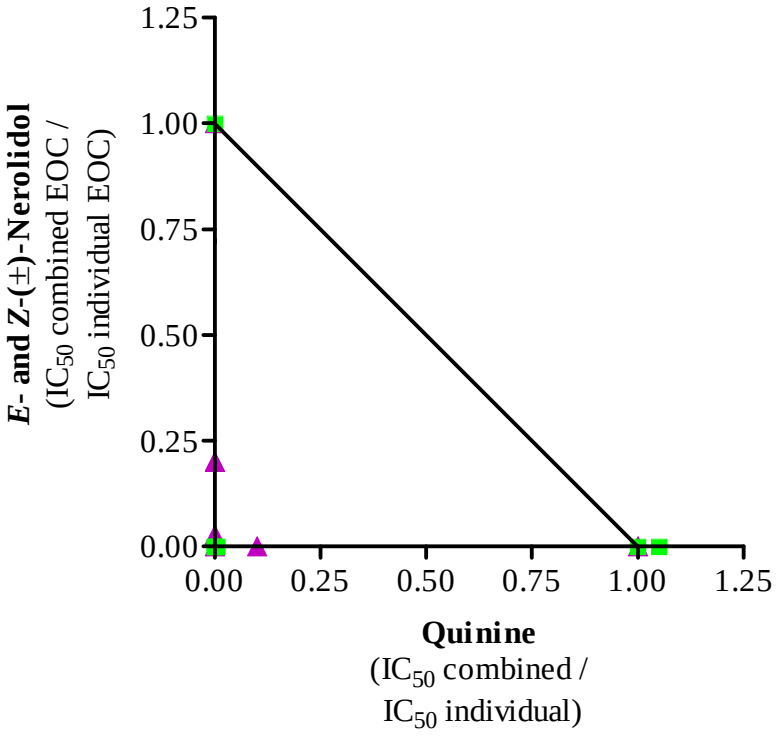


Figure 5.4: The synergistic interaction between quinine and *E*- and *Z*-(±)-nerolidol.

5.3.4. The relationship between essential oil constituent toxicity profiles and density

There was a lack of correlation ($r^2 = 0.045$) observed between the density and the IC_{50} values of the toxicity profile of these 20 EOC´s (Figure 5.5).

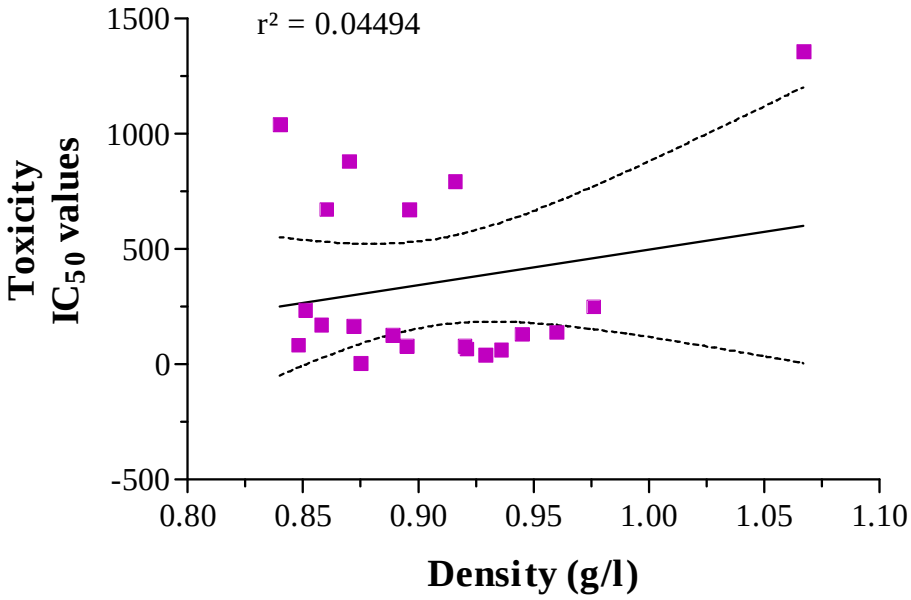


Figure 5.5: The linear relationship between the density of the essential oil constituents and their toxic effects on the human kidney epithelial cells.

5.3.5 The relationship between essential oil constituent toxicity profiles and antimalarial activity

There was no correlation ($r^2 = 0.310$) between the IC_{50} values of the antimalarial activity and the toxicity profile of the 20 EOC´s (Figure 5.6). To determine the selectivity of the EOC for a mammalian cell or the malaria parasite, the safety indices of the 20 EOC's were calculated (Table 5.4). Overall, the EOC's displayed general cytotoxicity rather than selectivity towards the malaria parasites, as shown by the low safety indices of γ-terpinene (0.09) and (-)-α-bisabolol (0.14), in comparison to that of the antimalarial agent, quinine (245.5). The only comparable safety index was that of (+)-α-pinene (143.5), which contrasted the safety index of its (+)-β- isomer (0.52).

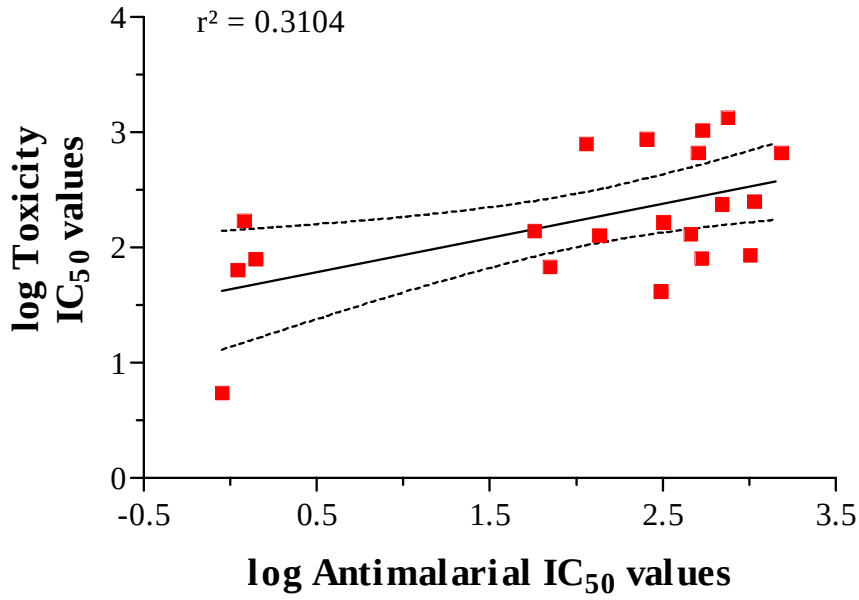


Figure 5.6: The linear relationship between the antimalarial activity of the essential oil constituents and their toxic effects on the human kidney epithelial cells.

5.4 Discussion

5.4.1 The toxicity profiles of the individual essential oil constituents

In spite of the extensive use of EOC´s, not much safety data is available. The results presented here concur with the regulations governing the use of some constituents as food additives (Table 5.4), with eugenol, (R)-(±)-limonene and *p*-cymene being considered safe for public consumption (Buchbauer, 1993; Cox *et al*., 2001).

This study also clearly shows the variability in the toxicity profiles of the constituents with *E*- and *Z*-(±)-nerolidol being approximately 250 times more toxic than eugenol (Figure 5.1). Eugenol is a major constituent of the essential oil isolated from *Eugenia caryophyllata*, which has been widely used as a herbal drug to treat dyspepsia, acute/chronic gastritis and diarrhoea (Lawrence, 2000). The essential oil of this plant has also been reported to have local anaesthetic and analgesic properties, antifungal, anticonvulsant, anticarcinogenic and antimutagenic activities (Yoo *et al.*, 2005). Generally at low concentrations eugenol is known to act as an anti-oxidant and an anti-inflammatory agent, whereas at high concentrations it acts as a pro-oxidant resulting in the enhanced generation of tissue damaging free radicals (Yoo *et al*., 2005). The anti-oxidant property of low concentrations of eugenol was verified in this study (Table 4.1).

Table 5.4: The safety indices of 20 essential oil constituents based on their IC_{50} values as a measure of the toxicity and antimalarial properties.

Essential oil constituent	Toxicity profile (μM)		Antimalarial activity (μM)		Safety index
	Average	s.d.	Average	s.d.	
(-)-α-Bisabolol	41.8	5.1	307.3	0.01	0.14
trans-Geraniol	128.5	4.7	135.4	7.03	0.95
(±)-Linalool	882.7	25.4	254.4	10.2	3.47
E- and *Z*-(±)-Nerolidol	5.5	1.2	0.9	0.3	6.11
(-)-Citronellal	238.3	24.9	698.5	36.5	0.34
Geranyl acetate	796.4	26.4	114.2	11.6	6.97
Linalyl acetate	80.0	2.9	1.4	0.1	57.1
(±)-α +β-Thujone	81.0	12.9	528.6	43.1	0.15
(+)-Carvone	140.9	9.6	57.3	4.3	2.46
(-)-Fenchone	132.2	3.1	457.9	47.2	0.29
(-)-Menthone	672.1	15.1	505.8	19.9	1.33
(-)-Pulegone	64.6	3.3	1.1	0.1	58.7
Carvacrol	251.8	32.8	1067.9	44.5	0.24
Eugenol	1358.4	13.0	753.7	33.8	1.8
(+)-α-Pinene	172.2	13.4	1.2	0.2	143.5
(+)-β-Pinene	166.7	19.6	318.5	35.6	0.52
γ-Terpinene	86.5	11.8	1006.7	18.7	0.09
(R)-(+)-Limonene	1042.0	41.1	533.5	17.5	1.95
p-Cymene	673.6	35.4	1528.8	44.9	0.44
1,8-Cineole	69.1	9.4	70.2	4.0	0.98
Quinine	71.2	0.02	0.3	0.04	245.5

Eugenol is oxidized in an enzymatic or non-enzymic manner via the electron pathway to a phenoxyl radical and subsequently could be converted to eugenol quinomethides, which are involved in cytotoxicity (Lahlou 2004a; Yoo *et al*., 2005). Yoo *et al*. (2005) showed that eugenol is able to induce apoptosis through ROS generation, depleting the intracellular reduced glutathione (GSH) and protein thiols resulting in loss of mitochondrial membrane potential, thus releasing cytochrome C into the cytosol in human leukaemia cells.

Terpenes have a number of common characteristics such as their highly lipophilic properties and most of them readily interact with biomembranes. They can increase the fluidity of the membranes, which leads to uncontrolled efflux of ions and metabolites, resulting in cell leakage and ultimately in cell death (Griffin *et al*., 1999). A conformational change of membrane proteins (e.g. ion channels and transporters) can occur when lipophilic compounds enter biomembranes and accumulate close to the membrane proteins (Griffin *et al*., 1999). If Na^+, K^+ or Ca^{2+} channels are affected, a disturbance of signal transduction could result. The use of essential oils to treat spasms and light pain in the case of dyspeptic or gall disorders could thus be plausibly explained. Since the lipophilic terpenes can cross the blood brain barrier, sedative and analeptic effects ascribed to several complementary drugs could be explained. Dormans and Deans (2000) reported that this ability of terpenes to cross the blood brain barrier may be attributed to their size and densities (Figure 3.6). This membrane activity is rather unspecific, therefore terpenes show antimicrobial, antimalarial, anti-oxidant and cytotoxic activities against a wide range of organisms, ranging from bacteria, fungi, to insects and vertebrates; such that they are used to treat bacterial and parasitic infections and respiratory tract disorders (Halcon, 2002; Nakatsu *et al*., 2000). The lipophilicity of the EOC's enable them to readily cross the membranes, but this is not the sole determinant of their biological or toxic activity as can be seen by a lack of correlation between the toxicity profiles and density (Figure 5.5) or antimalarial activity (Figure 5.6) of the EOC's. Thus, further research is required to determine the precise cellular target(s) of the EOC's.

The monoterpene ketone, pulegone constitutes more than 80% of the terpenes in the oils from many mint species such as the pennyroyals, which is used as a flavourant/fragrant constituent or to induce menstruation (Zhou *et al*., 2004). Many pennyroyals containing pulegone have been reported to cause gastritis, induce abortion, and result in hepatic failure, central nervous system, renal and pulmonary toxicity, and even death (Zhou *et al*., 2004). The toxic profile is confirmed in this study where (-)-pulegone plus *E*- and *Z*-(±)-nerolidol inhibited the growth of human kidney epithelial cells (Figure 5.2). Thus, when examining the efficacy of an essential oil its toxicity profile needs to be considered especially since the composition and ratio of EOC´s are influenced by seasonal and geographical variation (Buchbauer, 1993; Viljoen *et al*., 2005). With all factors considered, the toxicity of the oil may be minimised and the therapeutic benefit optimised.

5.4.2 The toxicity profile of the essential oil constituent and quinine

The combined effects of various EOC´s were assessed for their resultant toxicity properties (Table 5.3; Figures 5.2 to 5.4). Essential oils consist of numerous constituents present in variable concentrations. Thus, to examine the interaction between some of these EOC´s various combinations were assessed to determine how they may contribute to the overall toxicity profile of the plant. All tested EOC combinations potentiated each other´s toxicity profile (Table 5.3). For example, the IC_{50} values of the individual oils *E*- and *Z*-(±)-nerolidol, the most active EOC's, and *p*-cymene, the least active EOC's, were 5.5±1.2µM and 673.6±35.4µM respectively, but when combined the IC_{50} values decreased dramatically. Similarly, for the other combined EOC's, the combined toxic effect was more potent compared to the effects of the individual EOC's.

Thus, although the alcohol, *E*- and *Z*-(±)-nerolidol and the ketone, (-)-pulegone displayed promising antimalarial activity when used individually (Figure 3.5) and interacted antagonistically when used in combination (Table 3.2), they are not ideal candidates to be used in combination therapy due to the increased toxicity profile of the two EOC's when used in combinations (Table 5.3). This possibly indicates that there is a more specific effect on the malaria parasite rather than non-specific inhibitory or toxic effects on viable cells. Notably, even if this is the case, *E*- and *Z*-(±)-nerolidol was the most active antimalarial and also the most toxic. The combination of *E*- and *Z*-(±)-nerolidol and quinine showed promising antimalarial activity (∑FIC = 0.01), but had a high toxicity profile (∑FIC = 0.001) (Figure 5.4).

The combination of two monoterpenes, namely γ-terpinene and *p*-cymene (Figure 5.3) also displayed a very potent toxicity profile (∑FIC = 0.002). Likewise when the ketone, such as (-)-pulegone was combined with the monoterpene, *p*-cymene (Figure 5.3), the combination had a potent toxic profile (∑FIC = 0.306) (Table 5.3). The cytotoxic effects of these EOC´s are confirmed by their low safety indices, ranging from 0.09 to 143.5 (Table 5.4). Thus, based on these observations of the *in vitro* effect of the EOC's on human kidney cells, the route of administration and the concentration used should be carefully monitored to avoid detrimental and even fatal adverse effects. These cytotoxic effects in the kidney could be due to a membrane or a membrane-protein interaction (Lawrence, 2000).

Although, this study of twenty EOC´s concentrated more on the cytotoxic effects of individual EOC's and some selected combinations, the profiles of many essential oil products

and other individual constituents are as yet not fully understood. As is the maximum dose that can safely be administered (Cragg, 1997; Cassella *et al*., 2002). Infants, children, the elderly, and people with severe illnesses should not use essential oils internally except under the strict supervision of an experienced physician. Healthy adults should use well established products (such as peppermint oil and its constituents) for which safe dosages have been determined (Cassella *et al*., 2002). Topical or inhaled essential oils are considered safer than orally administered oils. However, allergic reactions to inhaled or topical plant fragrances are common. Furthermore, when applied to the skin, some essential oils might also promote hypersensitivity reactions, photosensitization, increase the risk of skin cancer, or be absorbed sufficiently to cause systemic toxic effects (Jirovertz *et al*., 1990; Carson and Riley, 1995; Lis-Balchin *et al*., 1997; WHO, 2003).

In many chemotherapeutic regimens, drugs are used in combination to delay or overcome factors of resistance. If EOC's were to be combined with the standard antimalarial agent, quinine, the combined antimalarial effect would be potentiated ($\sum$FIC = 0.01; Section 3.3.4). However, so would the likelihood of increased adverse effects ($\sum$FIC = 0.001; Table 5.3). But, therapeutically the administration of an EOC, such as *E*- and *Z*-(±)-nerolidol would not compromise patient treatment or recovery.

Thus, it is clear from this study that extensive research still needs to be conducted to determine the toxic profiles of the whole essential oils and the individual EOC's before they are widely administered and combined with other essential oils or "western" drugs. In addition, it is known that the essential oil may exert a severe effect on an individual organism or cell type, but may have no effect on other cell types. Thus, it is essential that the *in vivo* effects be determined where the effects of metabolism and the resultant metabolites may alter the profile observed in this study.

CHAPTER SIX
ANTIMICROBIAL ACTIVITY

6.1 Introduction

The antimicrobial properties of plant essential oils and their constituents from a wide variety of plants have been assessed against a comprehensive range of organisms (Griffin *et al*., 1999; Harris, 2002; Knobloch *et al*., 1989; Lis-Balchin *et al*., 1997; Dormans and Deans, 2000; Kalemba and Kunicka, 2003; Kang *et al*., 1992; Nakatsu *et al*., 2000; Onawumni *et al*., 1984; Ultee *et al*., 2002). It is clear from these studies that these plant volatile oils have potential application in the cosmetic, food and pharmaceutical industries (Halcon, 2002; Ultee *et al*., 2002). Investigations into the antimicrobial activities, mode of action and potential uses of these essential oils have regained momentum (Griffin *et al*., 1999). There appears to be a revival in the use of traditional approaches in the protection of livestock and food from diseases, pests and spoilage (Dormans and Deans, 2000). Essential oils have been used against food spoiling organisms (Ultee *et al*., 2002) and food poisoning organisms (Dormans and Deans, 2000), spoilage by mycotoxigenic filamentous fungi (Knobloch *et al*., 1989) and pathogenic or dimorphic yeasts (Griffin *et al*., 1999).

In addition to these, Kalemba and Kunicka (2003) reported on the usage of tea tree oil in treating mucous membrane infections, including oral bacteria and oropharyngeal candidiasis. Studies have also been conducted in the treatment of *Tinea pedis* and toenail onychomycosis, acne, dandruff and headlice, whereby tea tree oil was found to be as effective or even better than standard treatment and often presented with a lower side effect profile (Halcon, 2002; Lis-Balchin *et al*., 1997). Since 1887, research has shown the effectiveness of volatile oils on the major illnesses afflicting mankind, such as the antibacterial actions of volatile oils on anthrax spores (Knobloch *et al*., 1989; Lis-Balchin *et al*., 1997). Aromatherapists and caregivers also know that the inhalation of essential oils emitted from salve of menthol was one of the best treatments for respiratory infections (Kang *et al*., 1992).

The antibacterial properties of volatile oils were well demonstrated in 1924 on a mixed bacterial culture obtained from the human respiratory tract (Lis-Balchin *et al*., 1997). Later, in 1958 this was confirmed by using the direct contact method to test the antimicrobial activity of these volatile oils against selected bacteria and fungi (Kalemba and Kunicka, 2003;

Lis-Balchin *et al*., 1997). This study showed that 90% or more of the oils were effective in killing *Aspergillus niger* (Kalemba and Kunicka, 2003; Lis-Balchin *et al*., 1997). In 1959, it was again shown that the same volatile oils were effective against *Candida albicans* and *A. niger*, with 82 of the 100 volatile oils inhibiting the growth of *A. niger* (Lis-Balchin *et al*., 1997). In 1960, 133 volatile oils were tested against a number of bacteria including *Escherichia coli, Staphylococcus aureus, Streptococcus faecalis, Salmonella* species and *Mycobacterium avium* (Knobloch *et al*., 1989). The documented data indicated that Gram-positive bacteria where more vulnerable to essential oils than Gram-negative bacteria (Knobloch *et al*., 1989). Inouye *et al*. (2001) reported that essential oils in their gaseous state were effective against the respiratory tract pathogens.

As investigations in this field continued, analysis of the literature revealed that the results were not convincing. It was concluded that the variability in the results was due to the volatility, low water solubility and complexity of the essential oils (Janssen *et al*., 1987). The following main factors which require consideration when testing essential oils are: extraction procedures, dissolution and dispersion of the oils (the purity of the essential oils), the various chemical and biological assay techniques (the growth medium, the microorganisms being tested, the incubation periods) (Lahlou, 2004b; Nakatsu *et al*., 2000). On examination of the literature it is difficult to compare the antimicrobial activity of essential oils (Nakatsu *et al*., 2000). The methods used to assess the biological activities of the essential oils also differed widely, such that it was difficult to reproduce and compare results (Inouye *et al*., 2001). Knobloch *et al*. (1989) highlighted the problems with the solubility of the essential oils and the assay procedures used and that these errors did not lend credibility to the reported results.

6.1.1 Microbial infections

Microorganisms are ubiquitous and can be transmitted from person to person in many ways; direct contact, inhalation of infected air and consumption of contaminated water or food (WHO, 2003). Not all microorganisms cause disease. Bacteria exist on the skin surface or in the bowel without causing ill-effects and their presence is beneficial to the host (Nakatsu *et al*., 2000; WHO, 2003). Infectious diseases occur when the body has little or no natural immunity to the infection in question, or because the number of invading microbes is too great for the immune system to overcome or because the organism is pathogenic (Halcon, 2002; Burt, 2004). Microbial infections span a huge variety of different types of life-threatening diseases, involving bacteria, fungi and viruses. These infections can

effectively be managed, however pathogens constantly adapt and develop resistance to established therapies (Cowan, 1996).

Classic examples of such infectious bacteria include the Gram-positive *S. aureus* which is found mainly on human skin and in skin glands (mucous membrane) and sometimes in the mouth, blood, mammary glands, intestinal, genitourinary and upper respiratory tracts (Cox *et al*, 2001; Jirovertz *et al*., 1990). Pathogenic strains are known to form toxins and their antibacterial resistance has been reported towards β-lactam antibiotics (oxacillin and ampicillin), aminoglycosides (gentamicin), macrolides (erythromycin), quinolones (ciprofloxacin), clindamycin, chloramphenicol, tetracycline and trimethropim, to mention a few (Cox *et al*, 2001; De Smet, 1997).

Another Gram-positive bacterium, *Bacillus cereus* is well known for food spoilage and its pathogenic strains form toxins causing severe illness in the host (Ultee *et al*., 2002). Antibacterial resistance has been reported for β-lactam antibiotics (oxacillin), aminoglycosides (gentamicin), macrolides (erythromycin), clindamycin and chloramphenicol (Gollin, 1999).

Resistance by Gram-negative bacteria, such as *E. coli* have also expanded against various antibacterial drugs such as; β-lactam antibiotics (ampicillin), aminoglycosides, quinolones, chloramphenicol, doxycycline and trimethoprim (Griffin *et al*., 1999; Inouye *et al*., 2001). *Escherichia coli* belongs to the normal intestinal flora of humans, however the enteropathogenic strains are capable of forming toxins that can cause severe diarrhoea (Burt, 2004).

Historically, fungal infections have taken a low priority in the clinical, laboratory and medicinal research settings (De Smet, 1997). However, these infections are now occurring in ever-increasing numbers among neutropenic (low white blood cell count) cancer patients, transplant recipients and patients with AIDS (Kalemba and Kunicka, 2003). These patients are particularly more susceptible to infections, due to the administration of cancer chemotherapeutic or immunosuppressive agents or corticosteroids (Halcon, 2002). Although, many species of yeasts live commensally in and on the human body and lack clinical significance, some of them like *C. albicans* can cause systemic or topical pathological changes in susceptible persons (Tampieri *et al*., 2005).

6.1.2 Microbial resistance

The key message from the market analysis is that over the next two decades resistance will develop to all of the antibacterial and antifungal agents that are currently in use and thus there is a drive to meet this need for new antimicrobial agents (Cragg *et al*., 1997). To be successful in this market, scientists must discover and develop innovative antibacterial and antifungal agents that are effective against resistant strains. Just over a decade ago, it was postulated that bacterial and fungal infections were going to be conditions confined to the medical history textbooks (Cragg *et al*., 1997; De Smet 1997). However, time has proven this not to be the case. Today and for the unforeseeable future, we face the problem of microorganisms evolving and becoming increasingly resistant to the world´s antibacterial and antifungal defences.

Thus, there is an increasing demand to search for novel molecules that can be developed into new antimicrobial drugs to meet this demand. Microbial infections affect everyone and are one of the largest markets for pharmaceutical products (Cragg *et al*., 1997). It has been reported that infectious diseases cause more than 13 million deaths each year, and according to WHO (2003) this is approximately 30% of deaths worldwide. This therapeutic area therefore consists of significant commercial opportunities for companies able to predict future disease patterns and customer needs, and develop innovative anti-infective drug therapies including plant-derived medicines (Cragg *et al*., 1997). Plus, resistance to synthetic drugs leaves a space for the potential of plant-based medicine to be further explored. With this real and growing medical need as the focus in this study, the antimicrobial activity of twenty EOC's from seven different structural chemical groups were investigated. To determine the interaction between some of these EOC's, various combinations were also investigated.

6.2 Methodology

To determine the antimicrobial activity of selected EOC´s, they were initially screened using the disc diffusion assay (Section 6.2.2). Thereafter, their minimum inhibitory concentration (MIC) values were determined using *p*-iodonitrotetrazolium violet (INT) method (Section 6.2.3.4). Various combinations of EOC´s were combined, as well as with standard antimicrobial agents (Section 6.2.3.2).

6.2.1 Preparation of microbial culture

The antimicrobial activity of the 20 EOC´s was determined against two Gram-positive bacteria, *Staphylococcus aureus* ATCC 25923 and *Bacillus cereus* ATCC 11778 and one

Gram-negative, *Eschericia coli* ATCC 11775, as well as the yeast *Candida albicans* ATCC 10231. These reference stock cultures were obtained from the South African Bureau of Standards (SABS), Johannesburg. They were maintained viable in the Pharmaceutical Microbiology Laboratory, University of the Witwatersrand, Johannesburg. For the disc diffusion and MIC assays, the inoculum size of approximately 1 million colony forming units (CFU)/ml, was prepared in Tryptone Soya broth (Oxoid) from the stock microbial culture.

6.2.2 Disc diffusion assay

6.2.2.1 Preparation of individual essential oil constituents

The individual EOC's were prepared by aseptically transferring a 6mm paper disc into a petri dish with a sterile needle and then the disc was saturated with 20µl of the pure (100%) EOC.

6.2.2.2 Preparation of the combinations of two or three essential oil constituents

Based on the values of the zones of inhibition obtained from the individual EOC´s, forty five combinations of the EOC's per each microbial strain mentioned above were performed. When two EOC's or three EOC's were combined, 20µl of two or three EOC´s were combined in a 1:1 or 1:1:1 (v/v) ratio in eppendorfs, respectively. The oils were vigorously mixed before 20µl was applied to the disc paper.

6.2.2.3 Preparation of agar and bioassay agar plates

Agar was prepared by dissolving 30g of Tryptone Soya agar (Oxoid) into 750ml of distilled water and autoclaved at 121ºC for 15min to sterilize the solution. The sterilized agar was poured into bioassay plates in two layers: the first layer served as the base and was half the total volume added to the plate. This was left to solidify before the second layer which was seeded with the test organism was poured on top. Once this had solidified, paper discs impregnated with EOC's (Section 6.2.2.1) were transferred onto each of the four seeded agar plates using a sterile needle. The discs were left to prediffuse for at least 1h at 4ºC before being incubated at 37ºC for 24h (bacteria) and 48h (yeast). Prediffusion is of utmost importance as it enhances diffusion of the EOC into the agar (Janssen *et al*., 1987; Onawumni *et al*., 1984). Neomycin (30µg, Oxoid) and nystatin (100IU) served as positive controls for the bacteria and yeast, respectively. The assay was repeated in duplicate to confirm consistency.

6.2.2.4 Data analysis and interpretation

After the respective incubation periods, the zone of inhibition was measured from the edge of the disc to the edge of the zone in millimetres (mm) using a vernier caliper. From the zones of inhibition (ZoI), the antimicrobial activity of each EOC was determined. The interactions between two EOC's were evaluated and defined as follows: antagonism: $ZoI_{(AB)} < \sum ZoI_{(A+B)}$, synergism: $ZoI_{(AB)} > \sum ZOI_{(A+B)}$ and indifferent includes additive: $ZoI_{(AB)}$ > or < either $ZoI_{(A)}$ or $ZoI_{(B)}$, that is, when the combination of the two EOC's is not clearly defined as one compound may be antagonistic whilst the other synergistic relative to $ZoI_{(AB)}$. For three EOC's the same applies except that there is an extra EOC added, thus the above will be substituted with $\sum ZoI_{(A+B+C)}$, $ZoI_{(ABC)}$ and $ZoI_{(A)}$ or $ZoI_{(B)}$ or $ZoI_{(C)}$. Where $ZoI_{(AB)}$ is the value of the zone of inhibition obtained from the combination of the EOC's, $\sum ZoI_{(A+B)}$ is the sum resulting from adding the individual values of the zones of inhibition and $ZoI_{(A)}$ or $ZoI_{(B)}$ or $ZoI_{(C)}$ refers to the value of the zone of inhibition for each individual EOC.

6.2.3 Minimum inhibitory concentration microplate assay

6.2.3.1 Preparation of individual essential oil constituents

A mass of 128mg of oil was weighed out and dissolved in 1ml of acetone, to obtain a starting concentration of 32mg/ml in the first well, halving the concentration every time the oil was added to the subsequent row (i.e. 100µl of the oil diluted in 100µl of sterile water).

6.2.3.2 Preparation of essential oil constituents or standard antimicrobials for the combination studies

To determine the combined effect of two selected EOC´s, 100µl of one EOC was combined with 100µl of another EOC or the standard antimicrobial. The concentrations used in the combination experiment were based on the MIC values obtained from the individual EOC´s and nine fixed ratios of the two EOC´s were combined as follows: 10:90; 20:80; 30:70; 40:60; 50:50; 60:40; 70:30; 80:20; 90:10 (v/v). The dilutions for each individual EOC were prepared in acetone and diluted two-fold as previously described. To row A, 100µl of the nine combined EOC dilutions were added to the first nine consecutive wells and then serially diluted. Based on the antimicrobial activity of the individual EOC´s, the most active EOC on the respective microorganism was also combined with the standard antimicrobial agent (ciprofloxacin or amphotericin B).

6.2.3.3 Preparation of microtitre plates

Sterile water (100µl) was aseptically added to all wells of a 96 well flat bottom microtitre plate. To this, 100µl of various EOC solutions were added into the wells of row A1 to A10. In contrast, for the combinations, 100µl of each nine EOC dilutions (Section 6.2.3.2) were plated out to the wells of row A1 to A9. Whilst, to the wells of row A10, A11 and A12, 100µl of one EOC, another EOC or standard antimicrobial and microbial culture, respectively, were added. Thereafter, serial dilutions were performed from A to B and B to C until row H, diluting the EOC two folds each time. In row A11, the reference antimicrobial agent (positive control) was added, while water/acetone was added to row A12 and served as the negative control. To all the wells, 100µl of the culture was added and the plates were then incubated for 24h (bacteria) and 48h (yeast) at 37ºC.

6.2.3.4 Determination of minimum inhibitory concentration

The MIC was determined using the INT method according to Eloff (1998). A stock solution of 0.2mg/ml of INT chloride (Sigma-Aldrich) was prepared in sterile water, to make an INT stock solution of 0.04% (= 0.05mM). After the appropriate incubation periods, 40µl INT was added to all inoculated wells. The MIC corresponds to the lowest concentration inhibiting microbial growth seen as a clear well of the microtitre plate.

6.2.3.5 Data analysis and interpretation

6.2.3.5.1 Isobologram construction

To evaluate the interactions between the two EOC's or the EOC and standard antimicrobial drug, the abovementioned MIC assay was modified to accommodate their testing. The drug ratio values (MIC value of combined EOC's/MIC value of individual EOC) were then used to construct an isobologram, from which the nature of the interaction between the two EOC´s could be determined as previously described in Section 3.2.5.1 (Berenbaum, 1989; Meadows *et al.*, 2000). Values on the isobologram lying above the line were interpreted as being antagonistic, below as synergistic and on the line as additive. It must be noted that there may be some points on the isobolograms overlying on each other and sometimes cannot be clearly seen. This can be the case irrespective that nine fixed ratios of the two EOC´s were combined. Each experimental run was done in duplicate to confirm consistency.

6.2.3.5.2 Fractional inhibitory concentration determination

To verify the interaction read from the isobolograms, the sum of fractional inhibitory concentrations (∑FIC or FIC_{AB}) was calculated for each combination according to Equation 6.1.

Equation 6.1:

$$FIC_{AB} = \frac{MIC_A \text{ in combination}}{MIC_A \text{ alone}} + \frac{MIC_B \text{ in combination}}{MIC_B \text{ alone}}$$

Where A and B are the two selected EOC's used in combination.

Values from these ∑FIC or FIC_{AB} calculations were interpreted as follows: the ∑FIC values <0.5 is synergistic, 0.5-1.0 is additive, >1.0 is indifferent, while that >2.0 are antagonistic (Berenbaum, 1989). Combinations were performed in duplicate for both the MIC and disc diffusion assays to ensure consistency between the results.

6.3 Results

6.3.1 Disc diffusion assay

6.3.1.1 The disc diffusion assay: individual essential oil constituents

Table 6.1 summarizes the results of the disc diffusion assay of the 10 individual EOC´s. All the bacterial strains and the yeast demonstrated various degrees of sensitivity to the EOC´s tested. The EOC with the widest spectrum of antimicrobial activity was found to be eugenol and had the highest zones of inhibition in most instances. Its values of the zones of inhibition against *S. aureus, E. coli, B. cereus* and *C. albicans* were 3mm, 3.4mm, 10mm and 11.1mm, respectively.

6.3.1.2 The disc diffusion assay: the combined effects of two essential oil constituents

When the selected EOC´s were combined, the combinations interacted in varying ways (Table 6.2; Tables A.1 and A.2 in Appendix A). The raw data for the disc diffusion assay in Tables A.1 and A.2 are interpreted and summarised in Tables 6.2 and 6.3. In order to highlight some of the interactions between the individual EOC´s that could occur in the whole essential oil mixture, the combined effects of six interactions on various organisms are shown in Figures 6.1 to 6.4. The EOC´s interacted in synergistic, additive, indifferent and antagonistic manners, with (R)-(+)-limonene and *E*- and *Z*-(±)-nerolidol displaying the most synergistic interaction against all test organisms except *B. cereus* (Table 6.2; Figures 6.1, 6.2, 6.3 and 6.4).

Table 6.1: The antimicrobial disc diffusion activity of 10 essential oil constituents (n = 2).

Essential oil constituent	Zones of inhibition (mm)			
	S. aureus	***E. coli***	***B. cereus***	***C. albicans***
Alcohols				
trans-Geraniol	0.9	2.2	12	9.4
(±)-Linalool	0.9	0.9	12	1.6
E- and *Z*-(±)-Nerolidol	0.9	0	0	0
Aldehyde				
(-)-Citronellal	0	0	12	1.5
Esters				
Linalyl acetate	0.9	0	12	0
Geranyl acetate	0.9	0	12	1.2
Ketone				
(-)-Menthone	0	0	12	1.6
Phenol				
Eugenol	3	3.4	10	11.1
Terpene Hydrocarbons				
(+)-α-Pinene	0	0.9	0	2.7
(R)-(+)-Limonene	0	0	12	0
Controls				
Neomycin	15	15	15	-
Nystatin	-	-	-	16

In contrast, (-)-citronellal and eugenol interacted more antagonistically than geranyl acetate with eugenol or *trans*-geraniol against all four test organisms (Table 6.2). As seen in Figure 6.2, the potent antibacterial activity of eugenol against *E. coli* is antagonised when combined with *E*- and *Z*-(±)-nerolidol. In contrast, the potent activity of eugenol against *B. cereus* is potentiated when combined with *E*- and *Z*-(±)-nerolidol (Figure 6.3).

Of the 45 combinations tested against *S. aureus*, 25 were antagonistic and 11 synergistic in nature (Table 6.2). The synergistic interaction between *E*- and *Z*-(±)-nerolidol and (R)-(+)-limonene against *S. aureus* (Figure 6.1), *E. coli* (Figure 6.2) and *C. albicans* (Figure 6.4) was also observed.

Table 6.2: The antimicrobial disc diffusion interactions between two selected EOC's (An: antagonism, I: indifferent and S: synergism, Raw data seen on Table A.1, n = 2).

Essential oil constituent		**Zones of inhibition (mm)**			
		S. aureus	***E. coli***	***B. cereus***	***C. albicans***
(-)-Citronellal	Linalyl acetate	An	I	An	An
	Geranyl acetate	S	I	An	S
	(±)-Linalool	An	I	An	S
	trans-Geraniol	I	An	An	S
	Eugenol	An	An	An	An
	(+)-α-Pinene	I	An	I	An
	E- and *Z*-(±)-Nerolidol	S	I	An	An
	(-)-Menthone	S	I	An	An
	(R)-(+)-Limonene	S	S	An	An
Linalyl acetate	Geranyl acetate	An	I	An	S
	trans-Geraniol	An	An	An	An
	Eugenol	S	An	An	An
	(+)-α-Pinene	An	I	I	An
	E- and *Z*-(±)-Nerolidol	An	I	I	I
	(-)-Menthone	An	I	An	An
	(±)-Linalool	An	An	An	S
	(R)-(+)-Limonene	I	S	An	S
Geranyl acetate	(±)-Linalool	An	I	An	An
	trans-Geraniol	An	An	An	An
	Eugenol	An	An	An	An
	(+)-α-Pinene	An	I	An	An
	E- and *Z*-(±)-Nerolidol	An	I	An	An
	(-)-Menthone	An	I	An	An
	(R)-(+)-Limonene	An	I	An	An
(±)-Linalool	*trans*-Geraniol	S	An	An	An
	Eugenol	S	An	An	An
	(+)-α-Pinene	An	An	I	An
	E- and *Z*-(±)-Nerolidol	An	I	I	An
	(-)-Menthone	An	I	An	An
	(R)-(+)-Limonene	I	S	An	S
trans-Geraniol	Eugenol	An	An	I	An
	(+)-α-Pinene	S	An	I	An
	E- and *Z*-(±)-Nerolidol	An	An	An	An
	(-)-Menthone	An	An	An	An
	(R)-(+)-Limonene	S	An	An	An
Eugenol	(+)-α-Pinene	An	An	S	An
	E- and *Z*-(±)-Nerolidol	An	An	S	An
	(-)-Menthone	An	An	An	An
	(R)-(+)-Limonene	S	An	An	An
(+)-α-Pinene	*E*- and *Z*-(±)-Nerolidol	I	I	S	An
	(-)-Menthone	I	An	An	An
	(R)-(+)-Limonene	I	S	An	S
E- and *Z*-(±)-Nerolidol	(-)-Menthone	I	I	An	An
	(R)-(+)-Limonene	S	S	I	S
(-)-Menthone	(R)-(+)-Limonene	I	S	An	An

In addition, the combinations of EOC's against *E. coli* showed 21 antagonistic interactions of the 45 tested combinations (Table 6.2). While synergism was only observed six times when (R)-(+)-limonene interacted with one of the following EOC's: *E*- and *Z*-(±)-nerolidol, (+)-α-pinene (Figure 6.2), (-)-citronellal, (-)-menthone, (±)-linalool and linalyl acetate (Table 6.2).

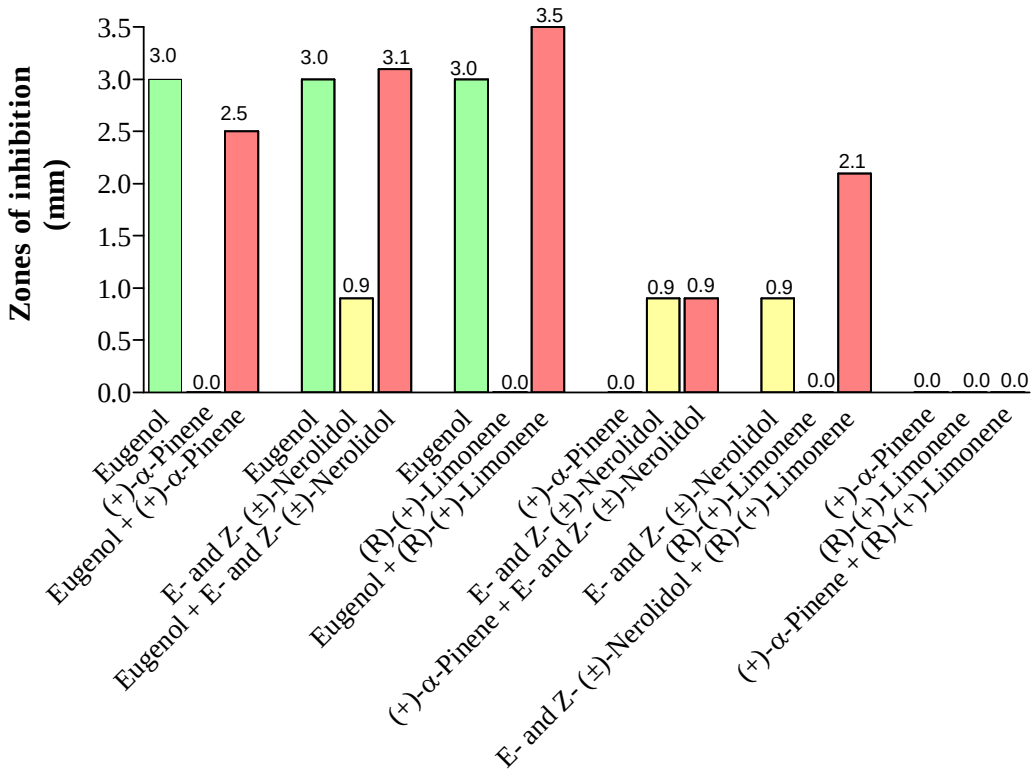


Figure 6.1: The antimicrobial disc diffusion activity of six selected combinations of essential oil constituents against *Staphylococcus aureus*.

The combination of (+)-α-pinene and *E*- and *Z*-(±)-nerolidol was extremely synergistic against *B. cereus* (Figure 6.3), while individually both EOC's did not show any antibacterial activity (Table 6.1). Only three synergistic interactions were observed of the 45 combinations, with eugenol interacting favourably with (+)-α-pinene or *E*- and *Z*-(±)-nerolidol (Figure 6.3). Thirty-four antagonistic interactions were recorded with the more antagonistic interaction observed between (+)-α-pinene and (R)-(+)-limonene (Figure 6.3), (-)-menthone along with either (R)-(+)-limonene, geranyl acetate and linalyl acetate; as well as between (±)-linalool and *trans*-geraniol (Table 6.2).

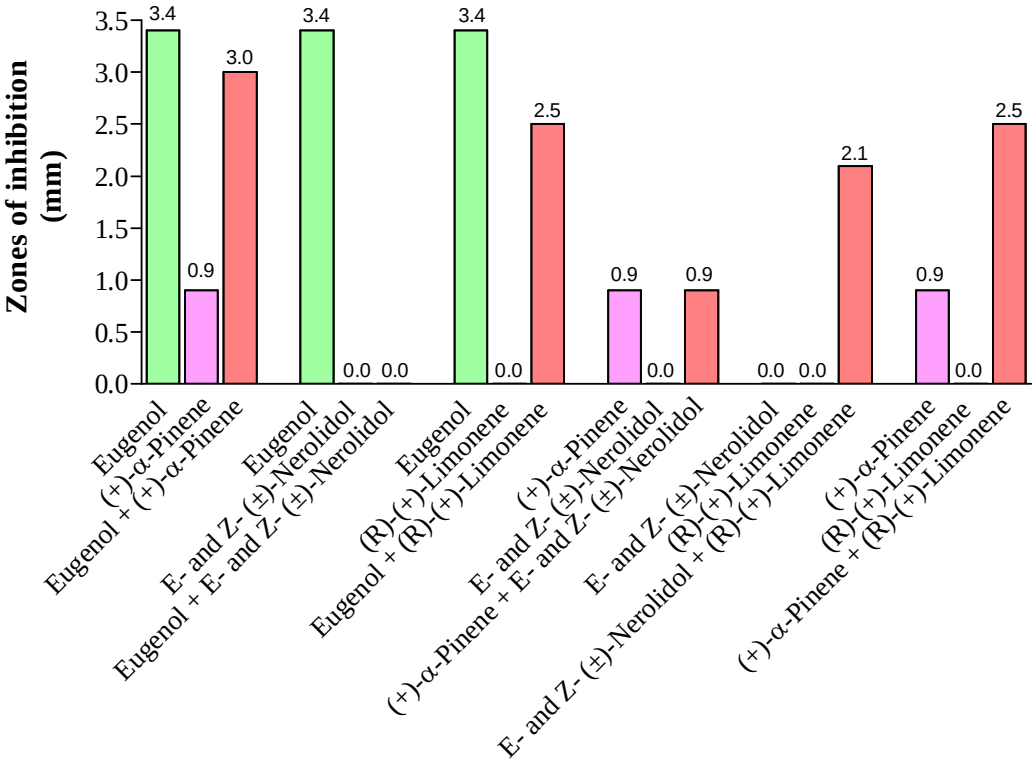


Figure 6.2: The antimicrobial disc diffusion activity of six selected combinations of essential oil constituents against *Eschericia coli*.

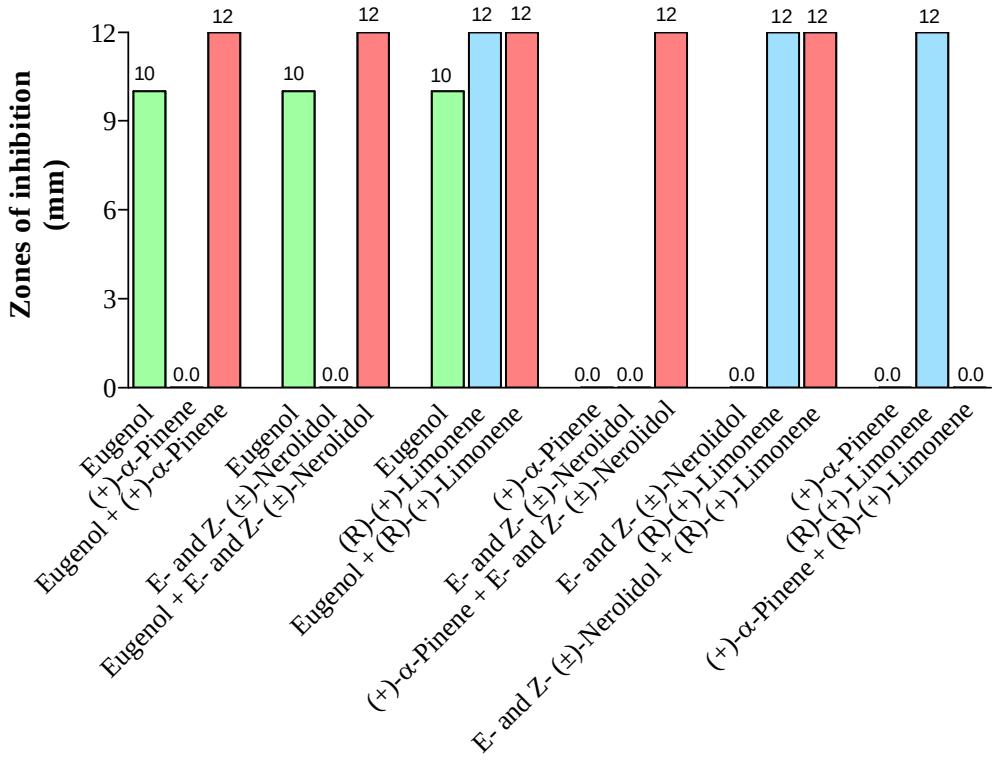


Figure 6.3: The antimicrobial disc diffusion activity of six selected combinations of essential oil constituents against *Bacillus cereus*.

Furthermore, the combinations of EOC's against *C. albicans* were dominated by mostly antagonistic interactions with 35 of 45 combinations being antagonistic. While nine synergistic interactions were observed when (R)-(+)-limonene interacted with either *E*- and *Z*-(±)-nerolidol, (+)-α-pinene, (±)-linalool or linalyl acetate) (Table 6.2 and Figure 6.4). Moreover, when geranyl acetate interacted synergistically with either (-)-citronellal or *trans*-geraniol or linalyl acetate or (±)-linalool and linalyl acetate with the latter EOC against *C. albicans* (Table 6.2).

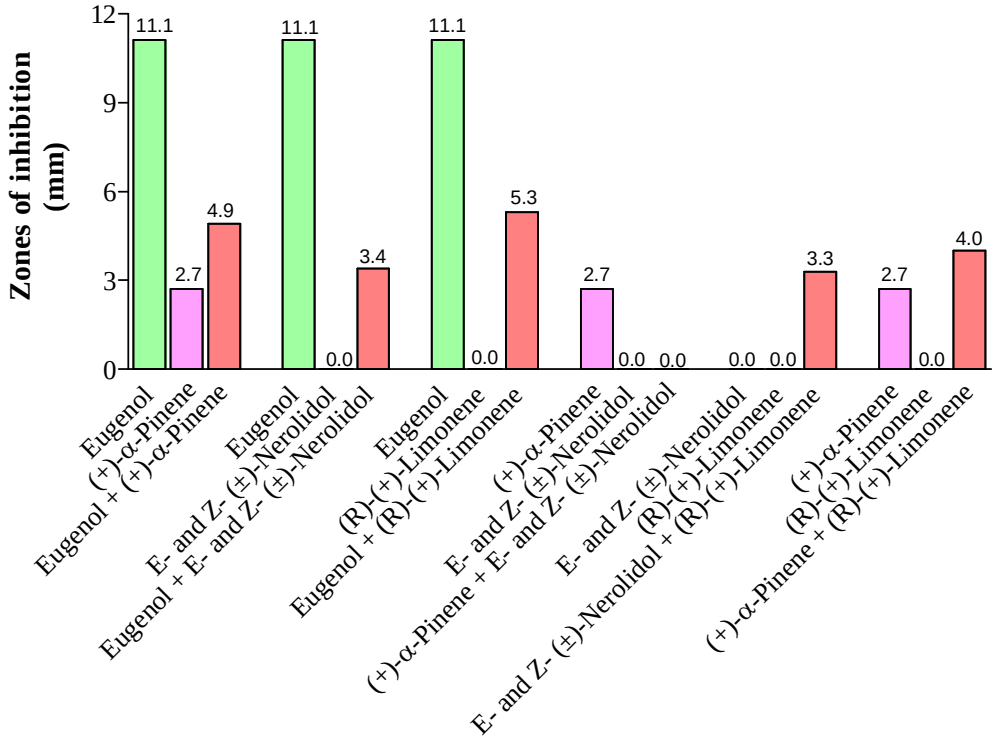


Figure 6.4: The antimicrobial disc diffusion activity of six selected combinations of essential oil constituents against *Candida albicans.*

6.3.1.3 The disc diffusion assay: the combined effects of three essential oil constituents

Due to the complexity of the essential oils, three EOC's were combined and tested against the four test microorganisms (Table 6.3; Table A.2 in Appendix A; Figure 6.5). The antagonistic interactions among the following EOC's were observed against all test organisms: (-)-citronellal, *trans*-geraniol and geranyl acetate; linalyl acetate, (±)-linalool and (R)-(+)-limonene; (R)-(+)-limonene, *trans*-geraniol and (-)-menthone; eugenol, *E*- and *Z*-(±)-nerolidol and (±)-linalool. The only exception were indifferent interactions among linalyl acetate, (±)-linalool and (R)-(+)-limonene against *E. coli* and (R)-(+)-limonene, *trans*-geraniol and (-)-menthone against *S. aureus*.

Table 6.3: The antimicrobial disc diffusion interactions among three selected essential oil constituents (Raw data seen on Table A.2 in Appendix A, n = 2).

Essential oil constituents	Zones of inhibition (mm)			
	S. aureus	***E. coli***	***B. cereus***	***C. albicans***
(-)-Citronellal + *trans*-Geraniol + Geranyl acetate	**An**	**An**	**An**	**An**
Linalyl acetate + (±)-Linalool + (R)-(+)-Limonene	**An**	**I**	**An**	**An**
(R)-(+)-Limonene + *trans*-Geraniol + (-)-Menthone	**I**	**An**	**An**	**An**
Eugenol + *E*- and *Z*-(±)-Nerolidol + (±)-Linalool	**An**	**An**	**An**	**An**

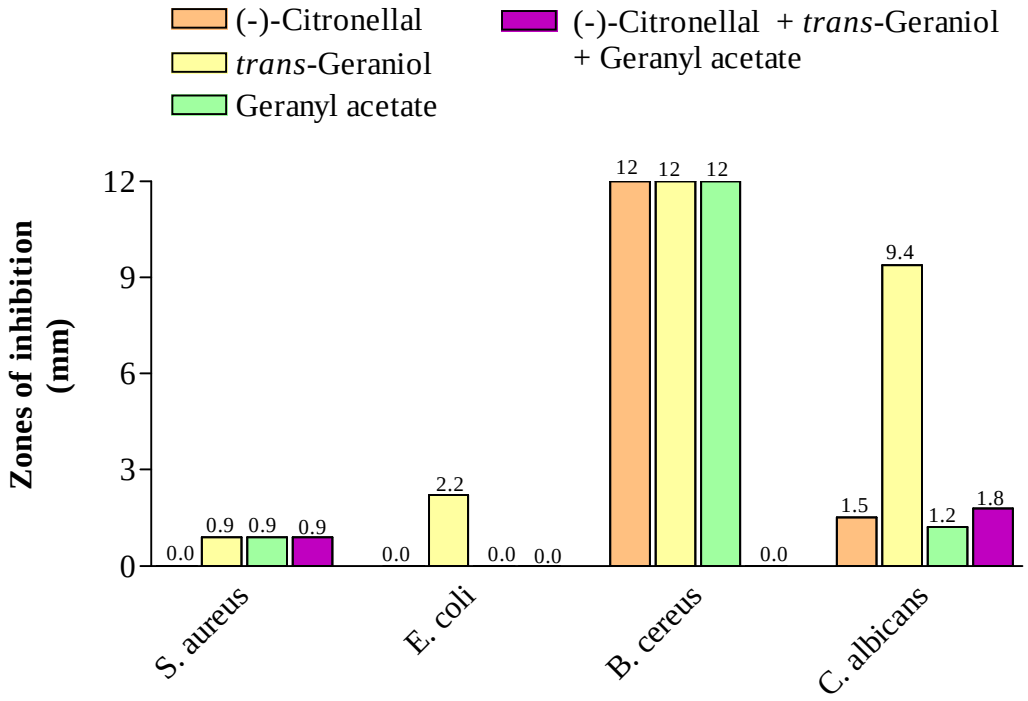


Figure 6.5: The antimicrobial disc diffusion activity of four selected combinations of essential oil constituents against four test microorganisms.

6.3.2. The minimum inhibitory concentration micropate assay

6.3.2.1 The minimum inhibitory concentration assay: individual essential oil constituents

The *in vitro* antimicrobial activity of the 20 EOC´s are shown in Table 6.4 along with the antibacterial and antifungal controls. The EOC´s inhibited the growth of Gram-positive and Gram-negative bacteria, with MIC values ranging from 12.2 to >238.4mM.

Table 6.4: *In vitro* antimicrobial minimum inhibitory concentration activity of 20 individual essential oil constituents and the standard antimicrobial agents (n = 2).

Essential oil constituent	Antimicrobial activity (mM)			
	S. aureus	***E. coli***	***B. cereus***	***C. albicans***
Alcohols				
(-)-α-Bisabolol	143.9±0.0	143.9±0.0	143.9±0.0	36.0±0.0
trans-Geraniol	38.9±18.2	25.9±0.0	51.9±0.0	19.5±0.0
(±)-Linalool	77.8±37.0	51.9±29.8	103.7±29.8	38.9±0.0
E-and *Z*-(±)-Nerolidol	143.9±0.0	143.9±24.7	143.9±24.7	143.9±0.0
Aldehyde				
(-)-Citronellal	129.7±55.1	207.5±45.4	207.5±64.8	77.8±0.0
Esters				
Geranyl acetate	163.0±35.7	163.0±29.0	163.0±51.0	163.0±0.0
Linalyl acetate	163.0±0.0	163.0±0.0	163.0±51.0	163.0±0.0
Ketones				
(*cis*+*trans*)-(±)-α+β Thujone	210.1±0.0	210.2±0.0	210.2±0.0	157.7±0.0
(+)-Carvone	53.3±0.0	106.5±0.0	106.5±0.0	106.51±0.0
(-)-Fenchone	105.1±0.0	210.2±0.0	210.2±0.0	210.2±0.0
(-)-Menthone	207.5±59.6	207.5±0.0	207.5±0.0	6.48±0.0
(-)-Pulegone	157.7±36.1	157.7±36.1	105.1±0.0	26.3±0.0
Phenols				
Carvacrol	13.3±0.0	20.0±9.3	13.3±0.0	1.66±0.0
Eugenol	194.9±6.7	12.2±0.0	12.2±0.0	79.2±0.0
Terpene Hydrocarbons				
(+)-α-Pinene	234.9±0.0	234.9±0.0	234.9±0.0	88.1±0.0
(+)-β-Pinene	22.0±1.5	117.4±0.0	117.4±0.0	7.3±0.0
γ-Terpinene	234.9±0.0	234.9±0.0	234.9±0.0	44.0±0.0
(R)-(+)-Limonene	176.2±40.4	176.2±40.4	234.9±0.0	73.4±0.0
p-Cymene	238.4±0.0	238.4±0.0	238.4±0.0	238.4±0.0
Oxide				
1,8-Cineole	207.5±0.0	207.5±0.0	207.5±0.0	207.5±0.0
Controls				
Ciprofloxacin	0.91±0.0	1.8±0.0	0.3±0.0	-
Amphotericin B	-	-	-	0.7±0.0

The MIC values for *C. albicans* ranged from <1.66 to >238.4mM. Carvacrol displayed the broadest spectrum of activity, while *p*-cymene possessed the weakest antimicrobial activity against Gram-negative and Gram-positive bacteria and *C. albicans*. In addition, to carvacrol, (-)-menthone and (+)-β-pinene were very active against both *E. coli* and *B. cereus* (Table 6.4). Of note, (+)-β-pinene was approximately 10x more active than (+)-α-pinene against *S. aureus* and *C. albicans* (Table 6.4).

6.3.2.2 The minimum inhibitory concentration assay: combined effects of two selected essential oil constituents

After investigating the antimicrobial activity of individual EOC´s, twelve various combinations were duplicated (Table 6.5; Figures 6.6 to 6.8 and Figures B.1 to B.6 in Appendix B). The interactions are depicted as isobolograms: two of the most active EOC´s against *C. albicans*, namely (+)-β-pinene and (-)-menthone (Figure 6.6) greatly antagonised each other´s antifungal activity to a much greater degree than when (+)-β-pinene was combined with 1,8-cineole (Table 6.5 and Figure B.2 in Appendix B).

The synergistic interaction between *E*- and *Z*-(±)-nerolidol and γ-terpinene against *B. cereus* (Figure 6.7) was observed. Contrasting interactions were observed against *E. coli*, with an indifferent interaction between *E*- and *Z*-(±)-nerolidol and eugenol; compared to the additive interaction between *E*- and *Z*-(±)-nerolidol and geranyl acetate (Figure 6.8).

6.3.2.3 The minimum inhibitory concentration assay: the combined effect of selected essential oil constituents and standard antimicrobial agents

The synergistic interactions observed when an EOC is combined with a standard antibacterial or antifungal agent are indicated in Table 6.6; Figures 6.9, 6.10 and Figure B.6 in Appendix B.

Table 6.5: The combined antimicrobial minimum inhibitory concentration activity of selected essential oil constituents (n = 2).

Micro-organism	EOC_1	EOC_2	∑ FIC value	Interaction	Figure / Appendix
S. aureus	Carvacrol	(+)-β-Pinene	0.33	Synergistic	B.1
	γ-Terpinene	(+)-β-Pinene	0.34	Synergistic	B.4
	γ –Terpinene	Geranyl acetate	0.31	Synergistic	B.4
B. cereus	Eugenol	*trans*-Geraniol	0.32	Synergistic	B.3
	trans-Geraniol	*E*- and *Z*-(±)-Nerolidol	0.36	Synergistic	B.5
	E- and *Z*-(±)-Nerolidol	γ –Terpinene	0.36	Synergistic	6.7
E. coli	Eugenol	*trans*-Geraniol	0.60	Additive	B.3
	E- and *Z*-(±)-Nerolidol	Geranyl acetate	1.04	Additive	6.8
	E- and *Z*-(±)-Nerolidol	Eugenol	1.44	Indifferent	6.8
C. albicans	1,8-cineole	Geranyl acetate	0.90	Additive	B.2
	(+)-β-Pinene	(-)-Menthone	9.80	Antagonistic	6.6
	1,8-cineole	(+)-β-Pinene	0.35	Synergistic	B.2

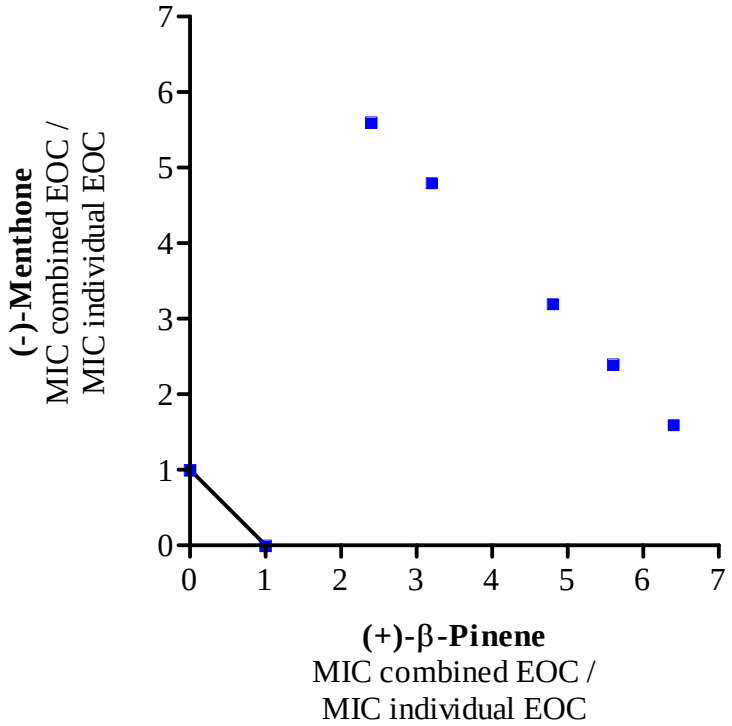


Figure 6.6: An antagonistic interaction between (+)-β-pinene and (-)-menthone against *C. albicans*.

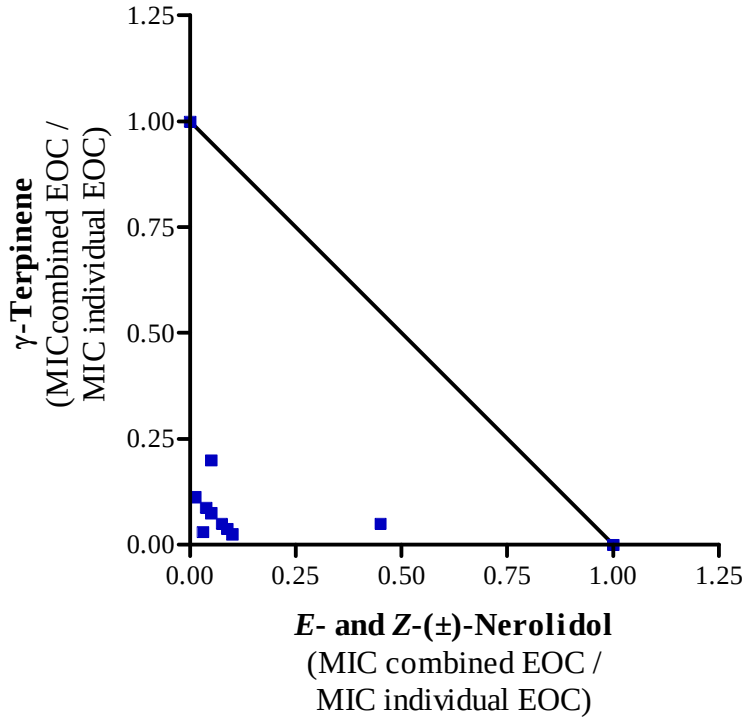


Figure 6.7: A synergistic interaction between *E*- and *Z*-(±)-nerolidol and γ-terpinene against *B. cereus*.

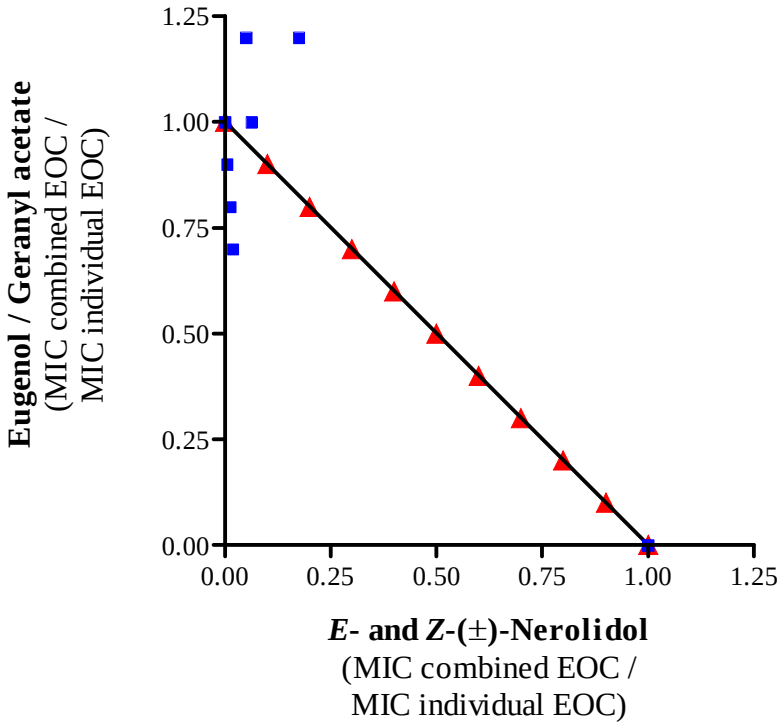


Figure 6.8: Contrasting interactions against *E. coli*, with an indifferent interaction between *E*- and *Z*-(±)-nerolidol and eugenol (■); compared to the additive interaction between *E*- and *Z*-(±)-nerolidol and geranyl acetate (▲).

Table 6.6: The antimicrobial minimum inhibitory concentration activity of an essential oil constituent combined with a standard antimicrobial agent (n = 2).

Microorganism	Standard antimicrobial agent	EOC	∑FIC Value	Interaction	Figure / Appendix
S. aureus	Ciprofloxacin	Eugenol	0.35	Synergistic	6.9
B. cereus	Ciprofloxacin	Carvacrol	0.22	Synergistic	6.9
E. coli	Ciprofloxacin	Eugenol	0.23	Synergistic	B.6
C. albicans	Amphotericin B	Carvacrol	0.41	Synergistic	6.10

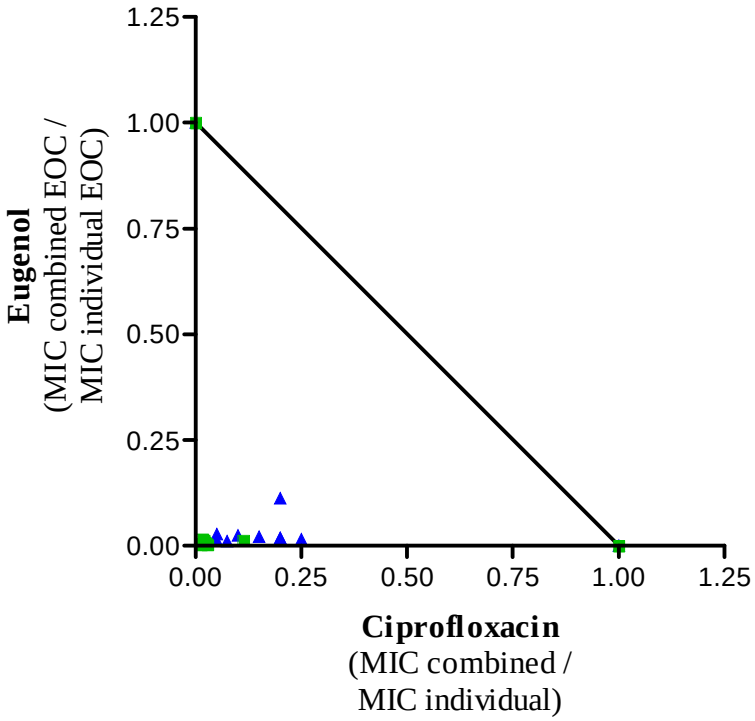


Figure 6.9: A synergistic interaction between ciprofloxacin and eugenol against *S. aureus* (■) and *E. coli* (▲).

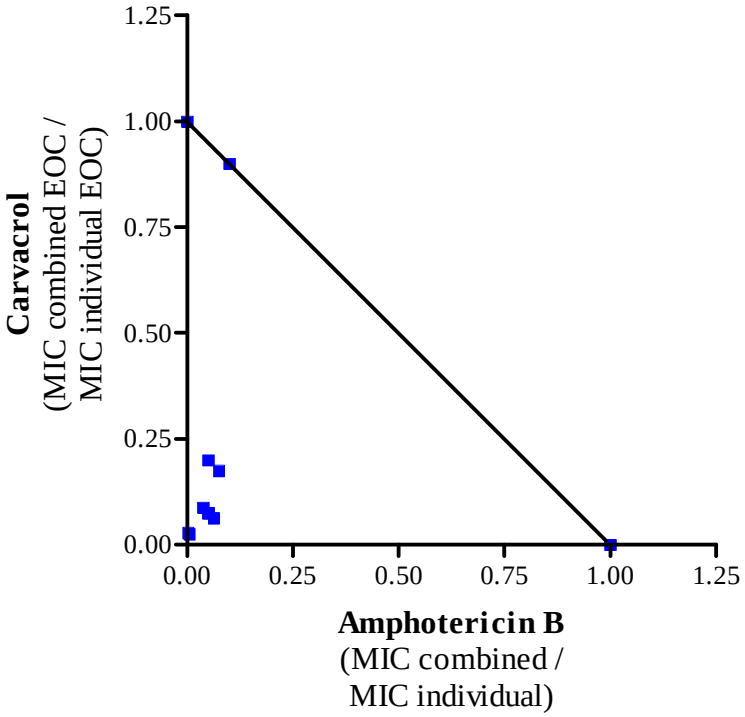


Figure 6.10: A synergistic interaction between amphotericin B and carvacrol against *C. albicans.*

6.4 Discussion

6.4.1 The disc diffusion assay: the antimicrobial activity of 10 individual essential oil constituents

These results (Table 6.1) are not unexpected as the phenol eugenol, has previously been reported to be highly active against various test organisms (Dormans and Deans, 2000, Kalemba and Kunicka, 2003). Members of this class are known to be either bacteriostatic or bactericidal, depending upon the concentration used (Dormans and Deans, 2000). Dormans and Deans (2000), Knobloch *et al*. (1989) and Ultee *et al*. (2002) have shown that the hydroxyl group in the phenols (Figure 2.1, m and n) influence their activity against Gram-positive, Gram-negative bacteria and yeast. Thus, the highest activity of phenols is explained by the acidic nature of the hydroxyl group, forming a hydrogen bond with an enzyme active centre of the microorganism (Kalemba and Kunicka, 2003). These latter reports are in agreement with the results of the present study (Table 6.1). Eugenol (11.1mm) and the alcohol *trans*-geraniol (9.4mm) displayed good antimicrobial activity against the yeast *C. albicans*. Kang *et al*. (1992) however, reported that these EOC´s showed little or no activity against another strain of *Candida*.

The ketone, (-)-menthone has demonstrated that the presence of an oxygen function in its framework (Figure 2.1k) increases its antimicrobial activity (Knobloch *et al*., 1989). This study revealed that (-)-menthone (1.6mm) has the modest antimicrobial activity against *C. albicans*, whilst *B. cereus* is the most significantly affected (Table 6.1). However, the results in the disc diffusion assay of the study by Dormans and Deans (2000) revealed that among the test organisms used, *S. aureus* was significantly affected, which is not the case in the present study. Notably, it is not clear in the study by Dormans and Deans (2000) which strain they used.

The terpene hydrocarbon, (R)-(+)-limonene demonstrated little or no activity against all the test micoorganisms, when using the disc diffusion assay (Table 6.1). However, when the MIC assay was used it showed some antimicrobial activity, suggesting that the method employed in the study can and does influence the results. Individually, the EOC´s demonstrate antimicrobial activity, although their combined properties are poorly studied and understood.

6.4.2 The disc diffusion assay: the combined effects of two essential oil constituents

The interactions between the combined effects of two EOC´s were diverse and can be categorised as discussed below (Figure 6.2 to 6.4; Table 6.2 and Table A.1 in Appendix A). Antagonism was observed between the following EOC´s against all four test organisms: (-)-citronellal and eugenol; (-)-menthone and eugenol; *trans*-geraniol and linalyl acetate; *trans*-geraniol and *E*- and *Z*-(±)-nerolidol; *trans*-geraniol and (-)-menthone; *trans*-geraniol and geranyl acetate. Notably, individually *trans*-geraniol was the second most active, while eugenol was the most active against all test organisms, except *B. cereus*; where eugenol was 83% less active compared with *trans*-geraniol and other EOC´s, except (+)-α-pinene and *E*- and *Z*-(±)-nerolidol (Table 6.1).

Upon selecting the most active EOC´s when used individually namely, *trans*-geraniol and eugenol, one would have expected a synergistic interaction. However, their observed interaction was antagonistic against all four test organisms, irrespective of their potent individual antimicrobial activity (Table 6.2 and Table A.1 in Appendix A). It was also clearly observed that against two Gram-positive bacteria, *S. aureus* and *B. cereus* the obtained $ZoI_{(AB)}$ values (i.e. from the results) of the combined effect of these two EOC´s were 82.5% and 54.5% less than the expected $\sum ZoI_{(A+B)}$ values (i.e. the added sum of the values of their zones of inhibition), respectively. In addition, against the Gram-negative *E. coli* bacteria and *C.*

albicans yeast, the obtained $ZoI_{(AB)}$ values were 46.4% and 22.4% less than the expected $\sum ZoI_{(A+B)}$ values, respectively (Table 6.1 and 6.2).

It has been mentioned (Section 6.4.1) that apart from the influence of the hydroxyl group in the structure of eugenol, the high activity of the phenolic components may be further explained in terms of the alkyl substitution into the phenol nucleus (Figure 2.1, m; for example the presence of a methyl group), which is known to enhance the antimicrobial activity of phenols (Dormans and Deans, 2000). Futhermore, the distribution of alkylation has been proposed to alter the distribution ratio between the aqueous and the nonaqueous phases (including bacterial phases) by reducing surface tension or altering the species selectivity (Dormans and Deans, 2000). While alcohols including *trans*-geraniol are known to possess bactericidal rather than bacteriostatic activity against vegetative cells (Griffin *et al*., 1999), they act either as protein denaturating agents, solvents or dehydrating agents (Kalemba and Kunicka, 2003). It is possible that these two EOC´s (*trans*-geraniol and eugenol) by attacking the same target sites on the microbial cell membrane, decreased each other´s effect in an antagonistic manner (Table 6.2 and Table A.1 in Appendix A).

While antagonism was observed for most combinations, some combinations such as linalyl acetate and eugenol; *trans*-geraniol and (±)-linalool; (±)-linalool and eugenol; eugenol and (R)-(+)-limonene, showed synergism against *S. aureus* and antagonism against *E. coli, B. cereus* and *C. albicans* (Table 6.2). The advantageous synergistic effects of the EOC´s have been observed before (Dormans and Deans, 2000; Griffin *et al*., 1999). However, if eugenol and (R)-(+)-limonene were to be chosen as the most and least active EOC´s, respectively, irrespective that (R)-(+)-limonene had potent activity against *B. cereus*, they interacted antagonistically against all test organisms except *S. aureus*. When (R)-(+)-limonene was combined with (±)-linalool or linalyl acetate (an ester enantiomer of (±)-linalool), synergism against *E. coli* and *C. albicans*; indifference against *S. aureus* and antagonism against *B. cereus* were seen. The combination of (±)-linalool and linalyl acetate interacted synergistically against *C. albicans* and antagonistically against *S. aureus*, *E. coli* and *B. cereus*. The interaction of these two enantiomers appeared to be more active against the yeast than against Gram-positive and Gram-negative bacteria. It is quite interesting though, as it has been reported before that Gram-positive bacteria are more susceptible to EOC's than Gram-negative bacteria (Burt, 2004), as both bacteria appeared to be less susceptible in this instance. Linalyl acetate with *E*- and *Z*-(±)-nerolidol showed an indifferent interaction against *E. coli*

and *C. albicans*; and an antagonistic interaction against *S. aureus* (Table 6.2 and Table A.1 in Appendix A). These interactions between an ester (linalyl acetate) and the alcohols ((±)-linalool or *E*- and *Z*-(±)-nerolidol), further show that when these two chemical groups are combined they have no potent inhibitory effect on Gram-positive bacteria.

When the two alcohols, namely (±)-linalool and *E*- and *Z*-(±)-nerolidol were combined antagonism was observed against *S. aureus* and *C. albicans*; and an indifferent manner against *E. coli* and *B. cereus* (Table 6.2 and Table A.1 in Appendix A). In contrast, when another set of alcohols, namely *E*- and *Z*-(±)-nerolidol and *trans*-geraniol were combined, they interacted in an antagonistic manner against all four test organisms. Here the interactions observed cannot be associated with the Gram-reaction of the microorganism, that is, whether the combination affected Gram-positive bacteria more than Gram-negative bacteria or vice versa. In contrast, when two esters (linalyl acetate and geranyl acetate) were combined, antagonism, indifference and synergism were observed against *S. aureus* and *B. cereus*; *E. coli* and *C. albicans*, respectively (Table 6.2). In this scenario, the reported observation (Burt, 2004), that Gram-positive bacteria are more susceptible than Gram-negative bacteria has been verified.

The combination between *E*- and *Z*-(±)-nerolidol with (+)-α-pinene resulted in synergism against *B. cereus*, indifference against *S. aureus* or *E. coli* and antagonism against *C. albicans*. When (+)-α-pinene and R-(+)-limonene were combined, synergism against *E. coli* (Figure 6.2) and *C. albicans*; antagonism against *B. cereus* (Figure 6.3) and indifference against and *S. aureus*. The indifferent interaction between (+)-α-pinene and (R)-(+)-limonene against *S. aureus* (Figure 6.1 and Table 6.2) may be due to the fact that these two terpene hydrocarbons appear to act at the same target site where they both access and accumulate in the microbial membrane and exhibit their antimicrobial activity, but failed to increase each other´s effect when combined. Both molecules have carbon to carbon double bonds, where this feature of conjugation has been described as having an increase electronegativity arrangement which increases the antibacterial activity (Dormans and Deans, 2000). Electronegative compounds may interfere in biological processes involving electron transfer and react with vital nitrogen components, e.g. proteins and nucleic acids and therefore inhibit microbial growth (Dormans and Deans, 2000; Griffin *et al.*, 1999; Kang *et al.*, 1992). Moreover, the alkylation of (R)-(+)-limonene [1-methyl-4-(1-methylethenyl)-cyclohexene] (Table 2.2 and Figure 2.1, r), could influence its antibacterial activity, as seen against *E. coli*

(Kang *et al*., 1992) especially when combined with (+)-α-pinene (Table 6.2 and Table A.1 in Appendix A).

The combinations of the EOC´s in varying ratios produced synergistic, indifferent or antagonistic interactions (Figures 6.1 to 6.4 and Table 6.2). However, the mechanism of action of these combinations is not yet fully understood, and warrants further detailed investigation.

6.4.3 The disc diffusion assay: the combined effects of three essential oil constituents

Essential oil constituents in triple combinations (Figure 6.5; Table 6.3 and Table A.2 in Appendix A) failed to show potent antimicrobial activity. Antagonistic interactions were observed when (-)-citronellal, *trans*-geraniol and geranyl acetate; linalyl acetate, (±)-linalool and (R)-(+)-limonene; (R)-(+)-limonene, *trans*-geraniol and (-)-menthone; eugenol, *E*- and *Z*-(±)-nerolidol and (±)-linalool were combined against all four test organisms. It is unknown why such an observation occurred. It is proposed that there is an optimal ratio at which the EOC´s interact such that there is no interference with their uptake and incorporation into the bacteria or fungi. In these triple combination studies, the three EOC´s were combined in 1:1:1 ratio, however, it should be noted that the ratio at which an optimal effect is achieved can be quite variable and specific against a particular microorganism. However, indifferent interactions were noted for linalyl acetate, (±)-linalool and (R)-(+)-limonene against *E. coli* and (R)-(+)-limonene, *trans*-geraniol and (-)-menthone against *S. aureus*. In contrast, the yeast *C. albicans* was relatively susceptible to all of the combinations tested (Table 6.3 and Table A.1 in Appendix A), where the interaction with the cell wall structure and cellular target(s) could have influenced this observation.

6.4.4 Minimum inhibitory concentration assay: the antimicrobial activity of the individual essential oil constituents

Gram-negative bacteria are generally less susceptible to the action of essential oils due to the presence of the outer membrane surrounding the cell wall, which restricts the diffusion of lipophilic compounds through the lipopolysaccharide covering (Burt, 2004). While this was the general trend for the tested constituents, eugenol, carvacrol, *trans*-geraniol and (±)-linalool showed promising activity against *E. coli* and Gram-positive bacteria (Table 6.4).

Carvacrol is a major constituent in the essential oils of thyme and oregano (Burt, 2004; Vardar-Ünlü *et al*., 2003) and displayed the broadest spectrum of activity against Gram-

negative and Gram-positive bacteria and *C. albicans*. It has been reported that the activity of carvacrol against the food-borne pathogen, *B. cereus* is ascribed to the interaction of the phenolic hydroxyl group with the bacterial cell membrane (Ultee *et al*., 2002). Where it aligns between the fatty acid chains in the phospholipid bilayer increasing membrane permeability and impairing the pH gradient that is required for essential processes in the cell, ultimately resulting in cell death (Ultee *et al*., 2002). In contrast, *p*-cymene which is the biological precursor of carvacrol possessed weak antimicrobial activity which is possibly linked to the absence of the phenolic hydroxyl group (Figure 2.1, m and r) (Burt, 2004; Ultee *et al*., 2002; Vardar-Ünlü *et al*., 2003). Interestingly, carvacrol (Figure 2.1, m) was only a tenth more active than eugenol (Figure 2.1, n) against *B. cereus*. The activity of both these two phenols compared favourably against this microorganism and may be attributed to the presence of the hydroxyl groups and their lipophilic property (Knobloch *et al*., 1989; Ultee *et al*., 2002). In addition, the high activity of phenolic components may also be explained in terms of the alkyl substitution into the phenol nucleus, which is known to enhance the antimicrobial activity of phenols (Dormans and Deans, 2000). Along with other studies, these results support the fact that alone *p*-cymene is ineffective; whilst carvacrol or eugenol could be applied therapeutically as an antibacterial or antifungal agent (Griffin *et al*., 1999; Ultee *et al*., 2002).

The non-phenolic alcohols such as *trans*-geraniol (Figure 2.1, b) and (±)-linalool (Figure 2.1, c) were less active than the above phenolic alcohols. Griffin *et al*. (1999) showed that in alcohols the presence of a functional group rather than its location along the carbon chain, is more crucial for the antimicrobial activity against *E. coli*, *S. aureus* and *C. albicans*. In the present study, among the four alcohols tested in Table 6.4, *trans*-geraniol was the most active against all four test microbes. While, (±)-linalool was half as active as *trans*-geraniol against all these microbes. The antimicrobial activity of the acyclic monoterpenoid alcohol *trans*-geraniol, found mainly in the oils of citrus fruits (Choi *et al*., 2000), concurs with that reported for *S. aureus* and various fungi including *C. albicans* (Tampeiri *et al*., 2005) and *Aspergillus* (Shin and Kang, 2003). Similarly, the activity of (±)-linalool, the major constituent in the oils of *Perilla frutescens*, *Lavandula angustifolia* and *Melaleuca alternifolia* correlates with that reported for *S. aureus* and *E. coli* (Carson and Riley, 1995; Kang *et al*., 1992; Knobloch *et al*., 1989; Nakatsu *et al*., 2000). The oil of *P. frutescens* (Lamiaceae) forms part of the traditional Chinese herbal medicine prescribed for colds, coughs and for promoting digestion (Kang *et al*., 1992). However, this study does not confirm the activity of γ-terpinene against *E. coli* and *S. aureus* (Kang *et al*., 1992).

Carvacrol was the most active against *C. albicans* with (-)-menthone, (+)-β-pinene, *trans*-geraniol, (-)-pulegone, (-)-α-bisabolol, (±)-linalool and γ-terpinene also displaying favourable activity, as previously reported (Constantin *et al*., 2001; Tampeiri *et al*., 2005). Interestingly, of the two structural isomers, (+)-β-pinene (Figure 2.1, o) was approximately two to twelve times more active than (+)-α-pinene (Figure 2.1, p) against both Gram-positive and Gram-negative bacteria as well as *C. albicans* (Table 6.4). There is corroborative evidence that beta-isomers are relatively more microbiologically active than alpha-isomers (Carson and Riley, 1995). These two isomers predominate in the oils of the leaves and flowers of *Hedychium gardnerianum* and show antimicrobial activity against *S. aureus* and *Pseudomonas aeruginosa* (Medeiros *et al*., 2003). Terpinene, found in tea tree (*M. alternifolia*) and citrus fruit oils is active against *C. albicans*, but the results of this MIC assay unlike those of disc diffusion assay, do not confirm this antifungal activity of eugenol (Griffin *et al*., 1999; Tampeiri *et al*., 2005). Linalool has been shown to suppress spore formation, while linalyl acetate inhibited spore germination and fungal growth (Kang *et al*., 1992).

The aldehyde, (-)-citronellal, most ketones including (±)-α+β-thujone, the hydrocarbons, *p*-cymene and 1,8-cineole (Table 6.4) all showed minimal antimicrobial activity, although citronellal has been reported to be active against *A. niger* (Shin and Kang, 2003). The relative inactivity of (-)-citonellal, (±)-α+β-thujone, *p*-cymene and 1,8-cineole has been associated with their low water solubility and hydrogen bonding capacity, thus limiting their entry into the Gram-negative organisms that possess inefficient hydrophobic pathways in the outer membrane (Griffin *et al*., 1999). 1,8-Cineole (eucalyptol), a major constituent (60%) in the oil of *Osmitopsis asteriscoides* (Asteraceae) has been shown to be active against *C. albicans* (Viljoen *et al*., 2003), *E. coli* and *S. aureus* (Nakatsu *et al*., 2000). A possible reason for this disparity is that Tween™ was used as an oil-in-water emulsifier that could facilitate the uptake of EOC´s which was not the case in this study.

Geranyl acetate (3,7-dimethyl-2,6-octadiene-1-ol acetate) a colourless liquid prepared by acetylation of geraniol (Nakatsu *et al*., 2000) was five fold less active than *trans*-geraniol. The addition of an acetate moiety to the molecule appeared to decrease the antibacterial activity. This effect is mirrored with linalyl acetate and (±)-linalool (Table 6.4).

These antimicrobial results are not unexpected as it has been reported that terpene aldehydes, terpene hydrocarbons, terpene ketones and terpene esters are generally less active in

comparison with the terpene alcohols (Burt; 2004; Griffin *et al*., 1999; Knobloch *et a*l., 1989). In contrast to the former compounds the terpene alcohols, *trans*-geraniol and (±)-linalool also possessed a broad spectrum of activity against both Gram-positive, Gram-negative bacteria and *Candida*.

6.4.5 Minimum inhibitory concentration assay: the antimicrobial activity of the two combined essential oil constituents

This study investigated the antimicrobial activity of EOC′s in combinations against three bacteria and a yeast (Table 6.5). The isobolograms of the combinations of the selected EOC´s for each microorganism are given in Figure 6.6 to 6.8. The following isobolograms show antagonism (Figure 6.6), synergism (Figure 6.7), indifference and additivity (Figure 6.8).

Based on previous studies (Griffin *et al*, 1999; Kalemba and Kunicka, 2003) the combined antimicrobial action of essential oils and their constituents are prposed to take place through two stages. Firstly, the transfer of the terpenoids into a bioavailable form, followed by penetration into the lipid bilayer of the cytoplasmic membrane in sufficient amounts to instigate membrane disruption (Griffin, *et al*, 1999). For Gram-negative bacteria, entry of the terpenoids into the lipid bilayer depends on the protective cellular structures, molecular size and hydrogen bonding parameters of these organisms (Kalemba and Kunicka, 2003).

In contrast, for other organisms like fungi and Gram-positive bacteria antimicrobial potency depends on log k_{ow} (the solubility factor). Thus, terpenoids require properties that will allow them to enter and accumulate in bioavailable forms to cause membrane disruption (Griffin *et al*., 1999). Thus, terpene alcohols (*trans*-geraniol and *E*- and *Z*-(±)-nerolidol), ketones ((-)-methone) and oxides (1,8-cineole) possess the essential balance of properties to exhibit promising antimicrobial activity.

For example, when (+)-β-pinene was combined with 1,8-cineole against *C. albicans* (Figure B.2 in Appendix B and Table 6.5) and γ–terpinene with *E*- and *Z*-(±)-nerolidol against *B. cereus* (Figure 6.7 and Table 6.5), synergistic interactions were observed. Likewise, when geranyl acetate was combined with 1,8-cineole against *C. albicans* (Figure B.2 in Appendix B and Table 6.5) an additive interaction was observed. While an indifferent interaction between *E*- and *Z*-(±)-nerolidol and eugenol was observed against *E. coli* (Figure 6.8 and Table 6.5).

However, the combination of geranyl acetate with *E*- and *Z*-(±)-nerolidol against *E. coli* (Figure 6.8) showed an additive interaction. It is possible that these two EOC´s might have had simultaneous inhibitory effect at the same microbial cell targets or acting on different targets in the microbial cell membrane (Halcon, 2002).

The other mechanism by which terpenoids destroy the microorganism is by accumulating in the microbial cytoplasmic membrane, resulting in the inhibition of cell growth (Kalemba and Kunicka, 2003). This is attributed to an increased bilayer disorder and direct bilayer damage, as well as K^+ leakage as shown with *E. coli* (Griffin *et al*., 1999). Once the osmotic balance of the microbial cell due to loss of small ions is disturbed, the microbial cell energy stores become depleted. Thus, the microbial structural integrity becomes compromised resulting in a detrimental effect on the cytoplasm and nuclear material, eventually leading to cell death or inhibition of cell growth (Halcon, 2002).

This study showed that if at any given dose the EOC is unable to reach the targeted site in sufficient amounts, it cannot exert a desired effect of microbial cell death. When combining two EOC´s targeting the same site or different sites, the chance of killing a microbe is increased. This is due to the synergistic or additive interaction that occurs between them (Figure 6.7 and Table 6.5). It is important to note however, that the activity of two active EOC´s can be compromised when they are combined. For example the combination of a ketone, (-)-menthone and a monterpene, (+)-β-pinene showed antagonism against *S. aureus* (Table 6.4 and Figure 6.5). (+)-β-Pinene like other terpene hydrocarbons has been reported to disrupt microbial membranes (Griffin *et al*., 1999; Knobloch *et al*., 1989). However, together with (-)-menthone they are precluded from accessing the membrane in abundance to exhibit antimicrobial activity, hence failure to eradicate the microbial cell.

6.4.6 Minimum inhibitory concentration assay: the antimicrobial activity of essential oil constituents with the standard antimicrobial agents

Potential interactions of EOC´s with antibiotics is another area where essential oils hold a great promise. In an age of increasing microbial resistance to drugs, there is a trend to look for ways to minimise the development of resistance to existing drugs instead of seeking new and stronger antibiotics. The benefit is that due to the chemical complexity of the combination, the chance of developing resistance is significantly reduced. Harris (2002) reported that the combination of chloramphenicol and essential oils from Korean aromatic plants, were effective in a synergistic manner against *S. aureus*. The present study shows that the

combination of eugenol with ciprofloxacin is also effective against *S. aureus* and *E. coli* in a synergistic way (Table 6.6 and Figure 6.9). Similarly, when another phenol namely, carvacrol was combined with ciprofloxacin and amphotericin B against *B. cereus* (Figure B.6 in Appendix A and Table 6.6) and *C. albicans* (Figure 6.10 and Table 6.6), respectively, synergistic interactions were observed.

6.4.7 The correlation between the disc diffusion and minimum inhibitory concentration assay

There was no correlation found between the results of the disc diffusion and MIC assays. The more contrasting observation was when for example, *trans*-geraniol was combined with eugenol or *E*- and *Z*-(±)-nerolidol against *B. cereus*, indifference and antagonism were observed, respectively, in the disc diffusion assay (Table 6.2); whilst in the MIC assay (Table 6.5) synergistic interactions were observed. Generally these differences in the results could be accounted for by the different methodological conditions used. Where the main contributing factors have proposed to be: assay technique, the growth medium, the microorganism and the essential oil (Nakatsu *et al.*, 2000). However, under the experimental conditions and environment of this study, the former three factors can be eliminated as the growth media was the same in both assays, as was the source or batch for the microorganism and essential oil. Lahlou (2004b) reported that laboratory conditions and the techniques used to evaluate the pharmacological activities of essential oils should be standardized. The lipophilicity and volatility of the EOC´s are a major concern when evaluating these compounds, but these too can influence the observed results. Solvents such as Tween™ can be used to solubilise and facilitate incorporation of the EOC. However, upon incubation at 37°C a certain percentage of the EOC is inevitably going to escape into the atmosphere and may contribute to any observed discrepancies.

In conclusion, the antimicrobial results of this study show that EOC´s in combination have the ability to potentiate each others mechanisms of action, and can be an advantage when treating microorganisms resistant to standard antimicrobial agents (Table 6.5). However, the dosage and route of administration of the EOC together with a standard drug still require further research to support their credibility as a plausible therapeutic combination to eradicate resistant infectious microorganisms.

Essential oil constituents individually and in combinations clearly demonstrate promising antimicrobial activity, but efforts to find a correlation between their structure, potency and the

mechanism by which they affect the microbial cell, has yet not been brought to a satisfactory conclusion (van Zyl *et al*., 2006). In the antimicrobial action of EOC´s, the lipophilic character of their hydrocarbon skeleton and the hydrophilic character of their functional groups are of main importance (Kang *et al*., 1992). The potency rank of the antimicrobial activity of the essential oil components is as follows: phenols > aldehydes > ketones > alcohols > esters > hydrocarbons (Kalemba and Kunicka, 2003). However, the general trend observed in the seven chemical group in this study is as follows phenols > alcohols > ketones > aldehydes > esters > hydrocarbons > oxides. However, the antimicrobial activity of essential oils is not only dependent on the EOC´s present, but on their percentage composition, as well as the degree of resistance of the microbial strain (Burt, 2004; Kalemba and Kunicka, 2003).

CHAPTER SEVEN
CONCLUSIONS

- Essential oil constituents individually and in combination showed promising biological activities including antimalarial, anti-oxidant, anticholinesterase and antimicrobial activity. However, the *in vitro* data suggests that some oil constituents may be toxic and thus may be dangerous to human health.
- Individually, linalyl acetate, (-)-pulegone, (+)-α-pinene and *E*- and *Z*-(±)-nerolidol were the most active EOC's with potent antiplasmodial effects, while *p*-cymene was the least active EOC. Of the two structural isomers, (+)-α-pinene had superior antimalarial activity compared to (+)-β-pinene. Some terpenes including *E*- and *Z*-(±)-nerolidol, (R)-(+)-limonene and (±)-linalool have chemical structures similar to some intermediates of the isoprenoid pathway such as farnesyl pyrophophate and geranylgeranyl pyrophosphate. The difference in activity between *E*- and *Z*-(±)-nerolidol and (R)-(+)-limonene is probably due to the difference in their chemical structures, where the hydroxyl group and linear structure of *E*- and *Z*-(±)-nerolidol allows better interaction with the target enzyme compared to the six-member ring of (R)-(+)-limonene. Even though (±)-linalool is similar in structure to *E*- and *Z*-(±)-nerolidol and contains a hydroxyl group, the shorter chain length greatly reduces the antimalarial activity of (±)-linalool. In combination, *p*-cymene (the least active) and *E*- and *Z*-(±)-nerolidol (the most active) displayed significant synergistic interaction. The antimalarial activity of this combination was higher when compared to the interaction between *E*- and *Z*-(±)-nerolidol and the standard antimalarial, quinine.
- This study revealed that eugenol (a phenol), possessed comparable anti-oxidant activity to vitamin C, while another phenol, carvacrol, lacked anti-oxidant activity. Both carvacrol and eugenol have hydroxyl groups. However, the potent anti-oxidant activity of eugenol is attributed to the alkyl substitution (the presence of a methyl group) in the phenol nucleus.
- The degree of saturation and the position of the C=C double bond, affected the inhibitory activity of acetylcholinesterase. The more saturated the EOC the better the strength or potency as an anticholinesterase. For example, the activity of eugenol, 1,8-cineole, (±)-α+β-thujone, *trans*-geraniol, (+)-α-pinene and geranyl acetate were comparable to galanthamine. EOC's like (R)-(+)-limonene, carvacrol, linalyl acetate

and *E*- and *Z*-(±)-nerolidol showed equipotent activity, irrespective of the chemical class each belongs to. The alcohol, *trans*-geraniol showed moderate anticholinesterase activity.

- From the results obtained in this study an inference can be drawn that some EOC's are not ideal candidates to form an integral part of the combination therapy, due to their increased toxicity profiles. For example, *E*- and *Z*-(±)-nerolidol was the most active antimalarial and also the most toxic. In combination *E*- and *Z*-(±)-nerolidol with (-)-pulegone or quinine had high toxicity profiles.
- The observed results indicated that for the two structural isomers, (+)-β-pinene was approximately two to twelve times more active than (+)-α-pinene against both Gram-positive and Gram-negative bacteria as well as *C. albicans*. Overall, carvacrol displayed the broadest spectrum of activity against Gram-negative and Gram-positive bacteria and *C. albicans.* Among the phenolic alcohols tested, *trans*-geraniol was the most active against all four test micro-organisms. While (±)-linalool was half as active as *trans*-geraniol against all the tested microorganisms. The reduced antimicrobial activity of (±)-linalool may be attributed to its shorter chain when compared to *trans*-geraniol. When using disc diffusion assay, in combination, *trans*-geraniol and eugenol displayed an antagonistic interaction against all test organisms except *B. cereus*, where an indifferent interaction was observed. While when using the MIC assay a synergistic interaction was observed against *B. cereus*. Alcohols like *trans*-geraniol possess bactericidal activity, while alkylation enhances the antimicrobial activity of phenols. However, the interaction between these compounds suggests that they may be competing for the same target site. In triple combinations, EOC's failed to show antimicrobial activity against all test microorganisms. When the most active EOC, carvacrol was combined with ciprofloxacin against *B. cereus* and amphotericin B against *C. albicans*, synergistic interactions were observed.
- Most EOC's had favourable antimalarial (e.g. *E*- and *Z*-(±)-nerolidol), antimicrobial (e.g. carvacrol) anti-oxidant (e.g. eugenol) and anticholinesterase activities (e.g. eugenol and 1,8-cineole). Furthermore, in combinations some EOC's potentiate each others' mechanisms of action which may contribute to the management and treatment of microorganisms resistant to standard drugs. However, their toxicity profiles reveal that they cannot be applied safely, unless they are thoroughly scientifically investigated.

CHAPTER EIGHT
RECOMMENDATIONS

- There are several 100's of essential oil constituents known today. However, this study acutely focussed on only 20 EOC's, such that the balance still needs to be assayed in similar studies.
- Furthermore, regarding their antimalarial activity only two combinations were performed between two EOC's and one EOC with the standard antimalarial. It is here were three or more combinations are required to mimic the activities of the crude essential oils. Moreover, extra combinations between EOC's and other different standard antimalarial agents (chloroquine, artemisinin, halofantrine, sulfadoxine, pyremethamine and mefloquine) warrant further investigation.
- To mimic the anti-oxidant activities of the crude essential oils, various combinations of EOC's are also required.
- Moreover, a qualitative method should be used to determine the anticholinesterase activity of the individual EOC's.
- To determine a more complete toxicity profile of the EOC's, other cell lines need to be considered (e.g. liver and lung cells).
- The promising antimicrobial results of these EOC's alone, in double combinations with themselves or standard antimicrobials and triple combinations suggest that further combination variations with other standard antimicrobials demand further investigation. More importantly, the spectrum of microorganisms needs to be expanded to further determine the bacteriostatic, bactericidal and antifungal effects of these EOC's.
- It is clear from this project that the test methods have the potential to influence the results and it is a prerequisite to use standardized, established and convenient test method if excellent reproducible results are to be obtained. In addition, other factors like the mechanism of actions of essential oil constituents are very important and still require thorough investigation. To safely use essential oils alone or in combinations, further research on their safety profiles is required. Thus, *in vivo* studies to address issues like safe routes of administration are important.

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APPENDICES

APPENDIX A: The combined antimicrobial disc diffusion activity of selected essential oil constituents.

Table A.1: The interaction between two selected essential oil constituents as determined by the disc diffusion assay (Raw data of Table 6.2, n = 2).

Essential oil constituent	Zones of inhibition (mm)			
	S. aureus	***E. coli***	***B. cereus***	***C. albicans***
(-)-Citronellal	0	0	12	1.5
Linalyl acetate	0.9	0	12	0
(-)-Citronellal + Linalyl acetate	0	0	12	0.9
(-)-Citronellal	0	0	12	1.5
Geranyl acetate	0.9	0	12	1.2
(-)-Citronellal + Geranyl acetate	1	0	12	2.9
(-)-Citronellal	0.9	0	12	1.5
(±)-Linalool	0.9	0.9	12	1.6
(-)-Citronellal + (±)-Linalool	0	0.9	12	3.2
(-)-Citronellal	0	0	12	1.5
trans-Geraniol	0.9	2.2	12	9.4
(-)-Citronellal + *trans*-Geraniol	0.9	0.9	12	12
(-)-Citronellal	0	0	12	1.5
Eugenol	3	3.4	10	11.1
(-)-Citronellal + Eugenol	1.5	2.1	8.1	3.6
(-)-Citronellal	0	0	12	1.5
(+)-α-Pinene	0	0.9	0	2.7
(-)-Citronellal + (+)-α-Pinene	0	0	0	2.2
(-)-Citronellal	0	0	12	1.5
E- and *Z*-(±)-Nerolidol	0.9	0	0	0
(-)-Citronellal + *E*- and *Z*-(±)-Nerolidol	1.2	0	8.4	0.9
(-)-Citronellal	0	0	12	1.5
(-)-Menthone	0	0	12	1.6
(-)-Citronellal + (-)-Menthone	0.9	0	4.5	1.5
(-)-Citronellal	0	0	12	1.5
(R)-(+)-Limonene	0	0	12	0
(-)-Citronellal + (R)-(+)-Limonene	0.9	0.9	12	3.2
Linalyl acetate	0.9	0	12	0
Geranyl acetate	0.9	0	12	1.2
Linalyl acetate + Geranyl acetate	0	0	12	1.5
Linalyl acetate	0.9	0	12	0
(±)-Linalool	0.9	0.9	12	1.6
Linalyl acetate + (±)-Linalool	0	0	12	1.9
Linalyl acetate	0.9	0	12	0
trans-Geraniol	0.9	2.2	12	9.4
Linalyl acetate + *trans*-Geraniol	0.9	0	12	2.2
Linalyl acetate	0.9	0	12	0
Eugenol	3	3.4	10	11.1
Linalyl acetate + Eugenol	4.3	3.3	5.7	4.1

Table A.1 (continued): The interaction between two selected essential oil constituents as determined by the disc diffusion assay (Raw data of Table 6.2).

Essential oil constituent	Zones of inhibition (mm)			
	S. aureus	***E. coli***	***B. cereus***	***C. albicans***
Linalyl acetate	0.9	0	12	0
(+)-α-Pinene	0	0.9	0	2.7
Linalyl acetate + (+)-α-Pinene	0	0.9	0	2.1
Linalyl acetate	0.9	0	12	0
E- and *Z*-(±)-Nerolidol	0.9	0	0	0
Linalyl acetate + *E*- and *Z*-(±)-Nerolidol	0.9	0	0	0
Linalyl acetate	0.9	0	12	0
(-)-Menthone	0	0	12	1.6
Linalyl acetate + (-)-Menthone	0	0	0	0.9
Linalyl acetate	0.9	0	12	0
(R)-(+)-Limonene	0	0	12	0
Linalyl acetate + (R)-(+)-Limonene	0.9	2.5	6.6	3.9
(±)-Linalool	0.9	0.9	12	1.6
Geranyl acetate	0.9	0	12	1.2
Geranyl acetate + (±)-Linalool	0	0.9	12	2.5
Geranyl acetate	0.9	0	12	1.2
trans-Geraniol	0.9	2.2	12	9.4
Geranyl acetate + *trans*-Geraniol	0.9	0	12	3.1
Geranyl acetate	0.9	0	12	1.2
Eugenol	3	3.4	10	11.1
Geranyl acetate + Eugenol	1.5	2.6	12	3.4
Geranyl acetate	0.9	0	12	1.2
(+)-α-Pinene	0	0.9	0	2.7
Geranyl acetate + (+)-α-Pinene	0	0.9	6.5	1.6
Geranyl acetate	0.9	0	12	1.2
E- and *Z*-(±)-Nerolidol	0.9	0	0	0
Geranyl acetate + *E*- and *Z*-(±)-Nerolidol	0.9	0	4.6	0
Geranyl acetate	0.9	0	12	1.2
(-)-Menthone	0	0	12	1.6
Geranyl acetate + (-)-Menthone	0	0	0	1.9
Geranyl acetate	0.9	0	12	1.2
(R)-(+)-Limonene	0	0	12	0
Geranyl acetate + (R)-(+)-Limonene	0	0	0	0.9
(±)-Linalool	0.9	0.9	12	1.6
trans-Geraniol	0.9	2.2	12	9.4
(±)-Linalool + *trans*-Geraniol	2.5	0.9	0	4.7
(±)-Linalool	0.9	0.9	12	1.6
Eugenol	3	3.4	10	11.1
(±)-Linalool + Eugenol	4.7	2.8	12	6.9
(±)-Linalool	0.9	0.9	12	1.6
(±)-α-Pinene	0	0.9	0	2.7
(±)-Linalool + (+)-α-Pinene	0	0.9	12	2.1

Table A.1 (continued): The interaction between two selected essential oil constituents as determined by the disc diffusion assay (Raw data of Table 6.2).

Essential oil constituent	Zones of inhibition (mm)			
	S. aureus	***E. coli***	***B. cereus***	***C. albicans***
(±)-Linalool	0.9	0.9	12	1.6
E- and *Z*-(±)-Nerolidol	0.9	0	0	0
(±)-Linalool + *E*- and *Z*-(±)-Nerolidol	0.9	0.9	12	0.9
(±)-Linalool	0.9	0.9	12	1.6
(-)-Menthone	0	0	12	1.6
(±)-Linalool + (-)-Menthone	0	0.9	12	0
(±)-Linalool	0.9	0.9	12	1.6
(R)-(+)-Limonene	0	0	12	0
(±)-Linalool + (R)-(+)-Limonene	0.9	4.1	12	4.5
trans-Geraniol	0.9	2.2	12	9.4
Eugenol	3	3.4	10	11.1
***trans*-geraniol + Eugenol**	3.2	2.6	12	4.6
trans-Geraniol	0.9	2.2	12	9.4
(+)-α-Pinene	0	0.9	0	2.7
***trans*-Geraniol + (+)-α-Pinene**	2	0.9	12	3.4
trans-Geraniol	0.9	2.2	12	9.4
E- and *Z*-(±)-Nerolidol	0.9	0	0	0
***trans*-Geraniol + *E*- and *Z*-(±)-Nerolidol**	0.9	0.9	0	2.6
trans-Geraniol	0.9	2.2	12	9.4
(-)-Menthone	0	0	12	1.6
***trans*-Geraniol + (-)-Menthone**	0	0	6.6	4.6
trans-Geraniol	0.9	2.2	12	9.4
(R)-(+)-Limonene	0	0	12	0
***trans*-Geraniol + (R)-(+)-Limonene**	2.4	0.9	12	3.1
Eugenol	3	3.4	10	11.1
(+)-α-Pinene	0	0.9	0	2.7
Eugenol + (+)-α-Pinene	2.5	3	12	4.9
Eugenol	3	3.4	10	11.1
E- and *Z*-(±)-Nerolidol	0.9	0	0	0
Eugenol + *E*- and *Z*-(±)-Nerolidol	3.1	0	12	3.4
Eugenol	3	3.4	10	11.1
(-)-Menthone	0	0	12	1.6
Eugenol + (-)-Menthone	2.3	0	12	4.1
Eugenol	3	3.4	10	11.1
(R)-(+)-Limonene	0	0	12	0
Eugenol + (R)-(+)-Limonene	3.5	2.5	12	5.3
(+)-α-Pinene	0	0.9	0	2.7
E- and *Z*-(±)-Nerolidol	0.9	0	0	0
(+)-α-Pinene + *E*- and *Z*- (±)-Nerolidol	0.9	0.9	12	0

Table A.1 (continued): The interaction between two selected essential oil constituents as determined by the disc diffusion assay (Raw data of Table 6.2).

Essential oil constituent	Zones of inhibition (mm)			
	S. aureus	***E. coli***	***B. cereus***	***C. albicans***
(+)-α-Pinene	0	0.9	0	2.7
(-)-Menthone	0	0	12	1.6
(+)-α-Pinene + (-)-Menthone	0	0	0	0
(+)-α-Pinene	0	0.9	0	2.7
(R)-(+)-Limonene	0	0	12	0
(+)-α-Pinene + (R)-(+)-Limonene	0	2.5	0	4
E- and *Z*-(±)-Nerolidol	0.9	0	0	0
(-)-Menthone	0	0	12	1.6
***E*- and *Z*-(±)-Nerolidol + (-)-Menthone**	0.9	0	0	0
E- and *Z*-(±)-Nerolidol	0.9	0	0	0
(R)-(+)-Limonene	0	0	12	0
***E*- and *Z*-(±)-Nerolidol + (R)-(+)-Limonene**	2.1	2.1	12	3.3
(-)-Menthone	0	0	12	1.6
(R)-(+)-Limonene	0	0	12	0
(-)-Menthone + (R)-(+)-Limonene	0	0.9	0	0.9
		Controls		
Neomycin	15	15	15	-
Nystatin	-	-	-	16

Table A.2: The interaction among three selected essential oil constituents as determined by the disc diffusion assay (Raw data of Table 6.3, n = 2).

Essential oil constituent	Zones of inhibition (mm)			
	S. aureus	***E. coli***	***B. cereus***	***C. albicans***
(-)-Citronellal	0	0	12	1.5
trans-Geraniol	0.9	2.2	12	9.4
Geranyl acetate	0.9	0	12	1.2
(-)-Citronellal + *trans*-Geraniol + Geranyl acetate	0.9	0	0	1.8
Linalyl acetate	0.9	0	12	0
(±)-Linalool	0.9	0.9	12	1.6
(R)-(+)-Limonene	0	0	12	0
Linalyl acetate + (±)-Linalool + (R)-(+)-Limonene	0	0.9	0	0.9
(-)-Menthone	0	0	12	1.6
trans-Geraniol	0.9	2.2	12	9.4
(-)-Menthone	0	0	12	1.6
(R)-(+)-Limonene + *trans*-Geraniol + (-)-Menthone	0	0	0	2.9
Eugenol	3	3.4	10	11.1
E- and *Z*-(±)-Nerolidol	0.9	0	0	0
(±)-Linalool	0.9	0.9	12	1.6
Eugenol + *E*- and *Z*-(±)-Nerolidol + (±)-Linalool	2.4	1.8	3.3	3.4
		Controls		
Neomycin	15	15	15	-
Nystatin	-	-	-	16

APPENDIX B: The combined antimicrobial minimum inhibitory concentration activity of selected essential oil constituents and standard antimicrobials.

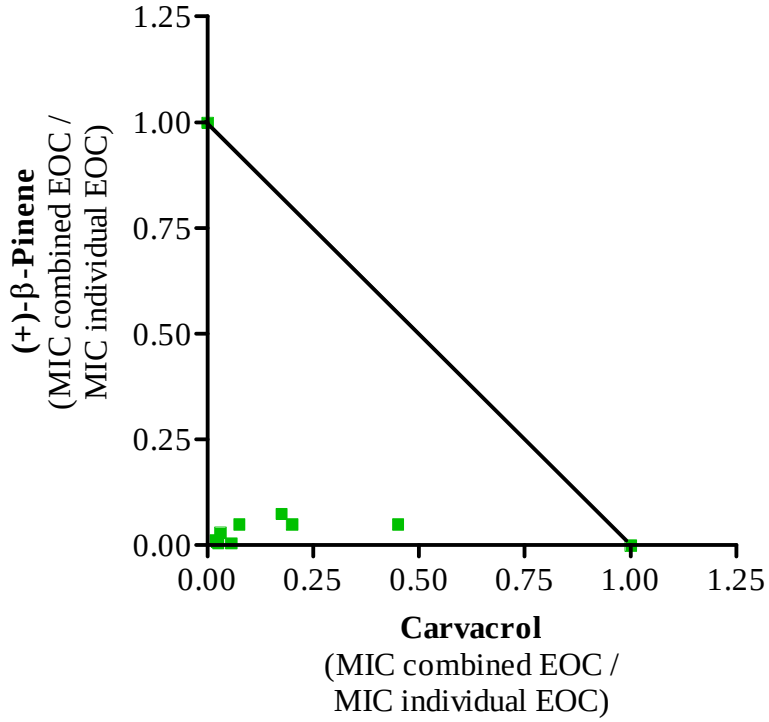


Figure B.1: A synergistic interaction between carvacrol (+)-β-pinene against *S. aureus*.

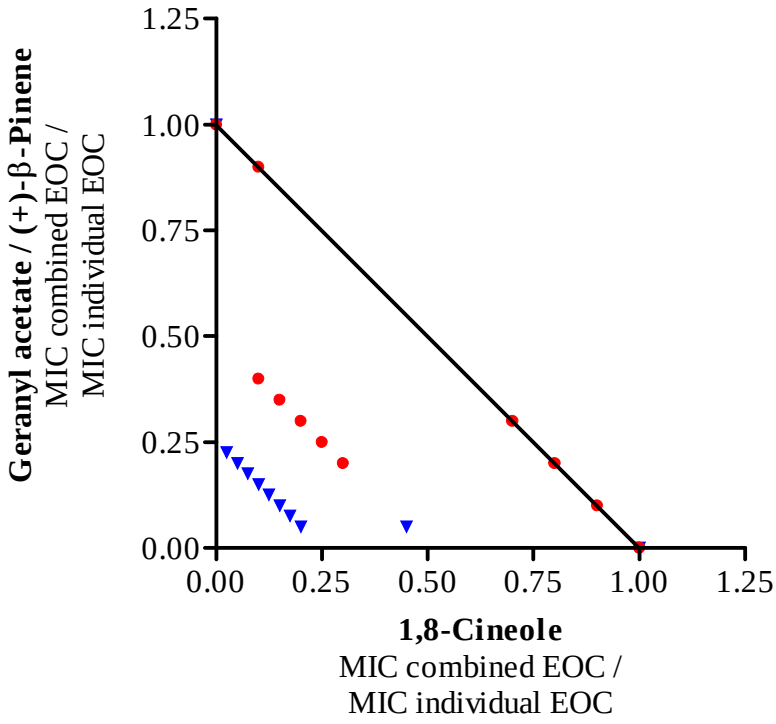


Figure B.2: An additive interaction was observed between 1,8-cineole and geranyl acetate (●) compared to a synergistic interaction between 1,8-cineole and (+)-β-pinene (▼) against *C. albicans*.

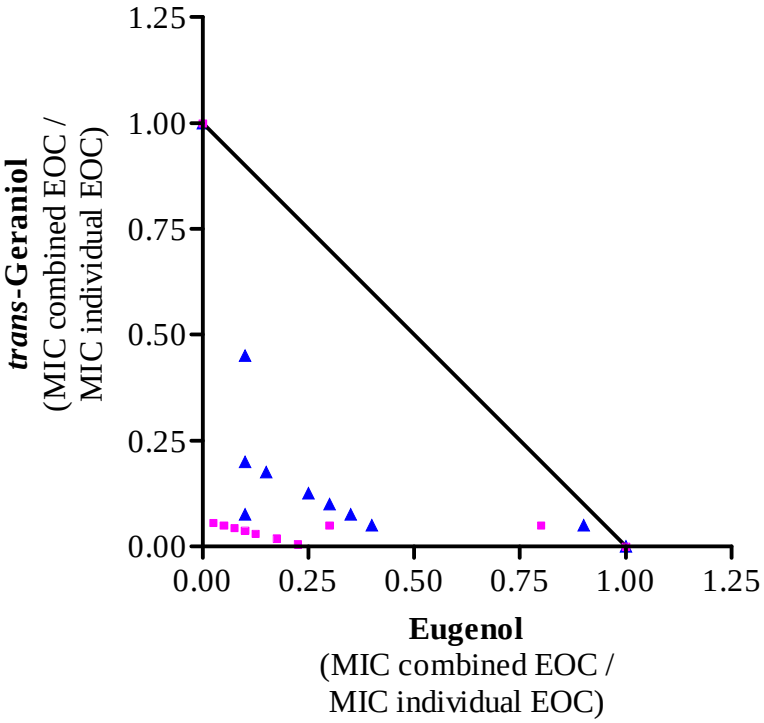


Figure B.3: The synergistic and additive interactions were observed between eugenol and *trans*-geraniol against *B. cereus* (■) and *E. coli* (▲), respectively.

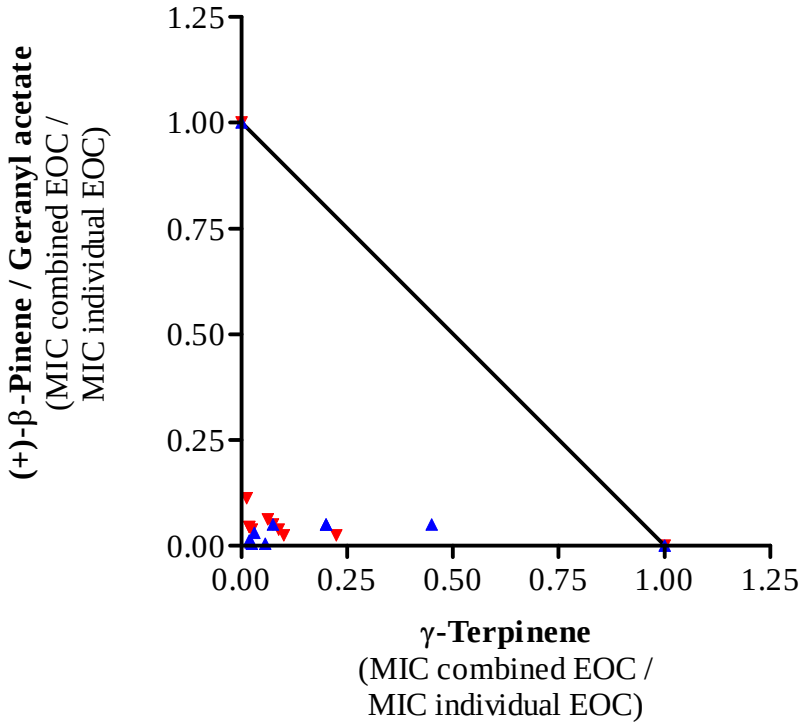


Figure B.4: γ-Terpinene interacted in a synergistic manner with both (+)-β-pinene (▲) and geranyl acetate (▼) against *S. aureus*.

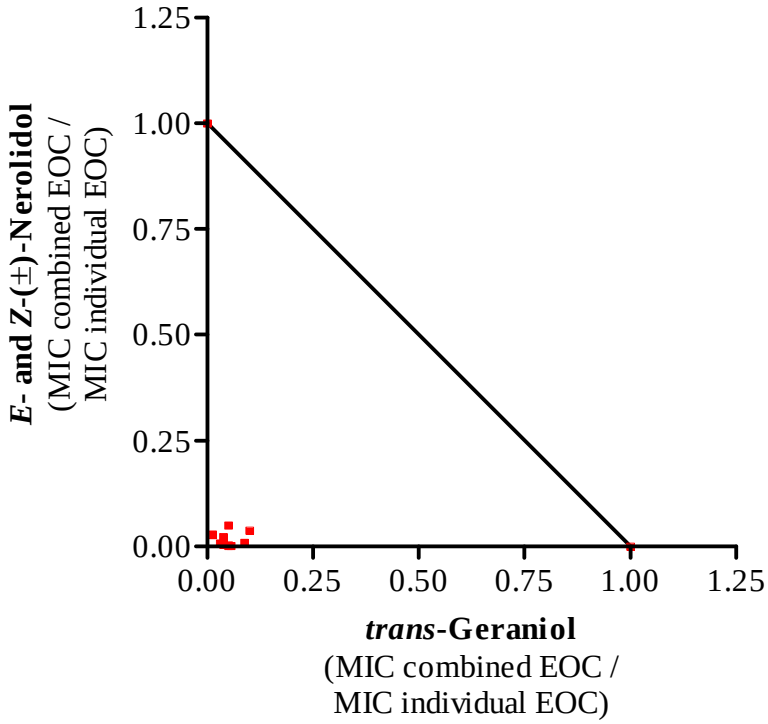


Figure B.5: A synergistic interaction between *trans*-geraniol and *E*- and *Z*-(±)-nerolidol against *B. cereus*.

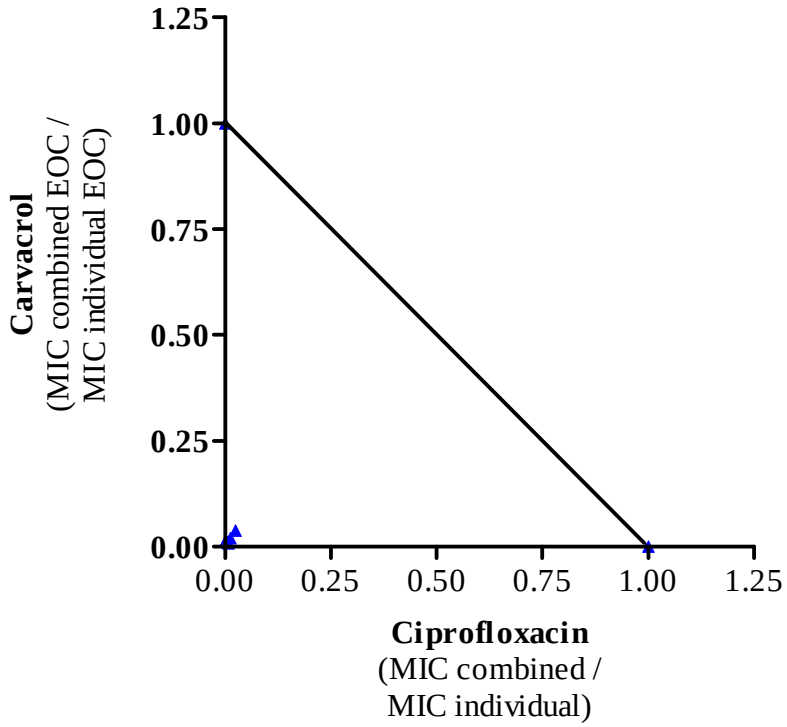


Figure B.6: A synergistic interaction observed between ciprofloxacin and carvacrol against *B. cereus*.