The Effect of Terpenes on the Life Cycle of the Malaria Parasite

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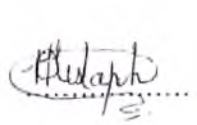
A dissertation submitted to the Faculty of Health Sciences,
University of the Witwatersrand, Johannesburg,
in the fulfilment of the requirements for the degree of
Master of Science in Medicine

Johannesburg, South Africa, 2017

Declaration

I, Obaidiyah Mustapha, 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, South Africa. It has not been submitted before for any degree or examination at this or any other University.



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18 day of June, 2017

Abstract

Malaria is a parasitic infectious disease resulting in high mortality rates especially in sub-Saharan Africa. Vector control and chemoprophylaxis are important aspects in the prevention of malaria. However, due to the emergence of resistance to antimalarial therapies and insecticides as a global issue, new compounds are required to ensure adequate therapy. For centuries, traditional phytomedicines have been used as effective malaria management. African traditional plants are commonly used in South Africa, where the essential oils (EOs) and extracts have been shown to possess promising activity in the control of malaria. As such, the activity of various EOs and essential oil constituents (EOCs) has been investigated on the lifecycle of the parasite.

The *in vitro* parasite lactate dehydrogenase (pLDH) assay determined the antimalarial activity of the EO/EOCs on the asexual stages of the parasite. All five EOs, *Artemisia afra, Lippia javanica, Cymbopogon citratus, Cymbopogon nardus* and *Ocimum basilicum* displayed antimalarial activity, with *C. citratus* (IC_{50} value: 2.00 $x10^{-5}$%) displaying the most activity in comparison to the control, quinine (IC_{50} value 1.71 $x10^{-5}$%; 0.18 µM). Nine of the 22 selected EOCs displayed antimalarial activity with eucalyptol (IC_{50} value: 0.37 µM; 6.19 $x10^{-6}$%) the most active.

The sensitivity of the *Anopheles* vector was assessed by determining the larvicidal activity of the EO/EOCs. Larvicidal activity was displayed by all five EOs and 14 EOCs with LC_{50} values ranging from 0.001 to 0.047%. The EOCs, *cis*-nerolidol and *p*-cymene displayed the most promising larvicidal activity of all tested EO/EOCs with LC_{50} values of 0.001 and 0.004%, respectively. When combined these two EOCs interacted in an additive manner (average ΣFIC: 0.94). It was also determined that the reconstituted crude oils made from the EOCs to replicate the original EO, displayed less larvicidal activities than the original EO. To determine the preliminary toxicological effect of the EO/EOCs, the haemolysis, lipid peroxidation inhibition, tetrazolium and brine shrimp lethality assays were undertaken. Haemolytic activity was not displayed by any of the EO/EOCs, with only *O. basilicum* and eugenol inhibiting lipid peroxidation. Cellular viability was affected by all five EOs (IC_{50} values: 2.4 $x10^{-4}$ - 2.5 $x10^{-1}$%) and fifteen of the selected EOCs (IC_{50} values: 0.2 - 72.4 µM). *Cymbopogon citratus* and *C. nardus* and ten EOCs possessed *Artemia* nauplii lethality activity (LC_{50} values: 6 $x10^{-7}$ - 1.4 $x10^{-2}$%). Varying antimalarial, larvicidal and toxicological properties were observed for the various isomers of nerolidol, geraniol, pinene, linalool, thujone and citronellal. These results showed that the biological activities of the EO/EOCs have the potential to be used as adjuncts in the management of the malaria parasite and vector, as well as the development for novel drugs.

Acknowledgements

Bismillahir Rahmanir Raheem

In the Name of Allah, the Most Gracious, the Most Merciful

I am grateful for the endless bounties, mercy and blessings Allah has bestowed upon me.

I would first like to thank my supervisor Professor Robyn van Zyl, Associate professor of the Pharmacology Division, of the School of Therapeutic Sciences at the University of the Witwatersrand. Her knowledge in the field of malaria parasitology and toxicology has been a great source of expertise to my research. I would like to thank you for your constant support, supervision and patience throughout my project. Your continuous guidance and motivation is highly appreciated.

I would also like to thank my co-supervisor, Professor Maureen Coetzee, director of the Wits Research Institute for Malaria at the University of the Witwatersrand, for your guidance and support. I have been honoured and still in awe to be supervised under a researcher whose accolades in the field of malaria vector control and vector-parasite interactions, has lead to the mosquito subgenus, *Coetzeemyia*, being named after her by the Smithsonian Institution in the USA. I am thankful for your valuable input on this dissertation.

My appreciation towards my parents, Abba and Ammi (Mohammed Khalil and Zaiboon Mustapha) is beyond recognition. I am immensely thankful for your constant love, motivation and understanding. This journey would have not been possible without your exceptional support and encouragement. "A father is neither an anchor to hold us back, nor a sail to take us there, but a guiding light whose love shows us the way". With this said, I would like to thank you, Abba, for all the love, the amazing opportunities you have given me and for being a pillar of strength. Thank you for always believing in me and constantly motivating me. To Ammi; certainly, I am a strong woman because a strong woman has raised me. I shall always cherish your love and support. Thank you for always having faith in me. I have truly been blessed with your kindness and pray that you both are continued to be blessed with happiness, contentment and all life's beauties.

A special thanks to my family; Mohammed, Qudsiyyah, Raeesah, Jameel Ahmed and Muhammad Tahmeed. Thank you for all your love, patience and support. Thank you for adding the extra sun-shine and joy to my life.

Thank you to my fiancé, Muhammad. Thank you for being there for me. Your patience, love, understanding and encouragement are highly appreciated. Thank you for making my life so special.

I would like to thank my new family, Mahomed Iqbal and Nashreen Hassim, Mohamed Shahabuddin and Tasleem for your warmth, love and support.

To my Mustapha and Khan family; I am blessed to have all of you. Thank you.

I would also like to thank Fatima Kathrada. Thank you for always going the extra mile, for your valuable friendship and support. I would also like to thank Natasha Jansen van Vuuren and Chieng-Teng Chen for your continuous advice, knowledge and help throughout my dissertation. I appreciate you all.

Thank you to everyone else in the Department of Pharmacy and Pharmacology.

Thank you to Shüné Oliver from the National Institute for Communicable Diseases. Your assistance, vast skills and knowledge in the vector field has been tremendously helpful.

I would like to thank those involved in the funding of this project, including the National Research Foundation and the Faculty Research Committee.

Last but not least, I'd like to thank all my friends for your utmost support and care.

"Acquire knowledge: it enables its possessor to distinguish right from the wrong, it lights the way to heaven; it is our friend in the desert, our society in solitude, our companion when friendless - it guides us to happiness; it sustains us in misery; it is an ornament among friends and an armor against enemies."

"Seek knowledge from the cradle to the grave."

-Prophet Muhammad S.A.W.

List of Congresses Attended

O. Mustapha, R.L. van Zyl, M. Coetzee and S. Oliver. THE EFFECT OF *OCIMUM BASILICUM* AND ITS CONSTITUENT TERPENES ON THE LIFE CYCLE OF MALARIA. South African Society of Basic and Clinical Pharmacology (SASBCP) and Toxicology SA (ToxSA) Congress 2015. University of the Witwatersrand, Johannesburg, South Africa. 31st August – 2nd September 2015. (Poster presentation) (*Southern African Journal of Infectious Diseases*, 2015, 30 (Supp1):S34).

O. Mustapha, RL van Zyl, M Coetzee, S Oliver. THE EFFECT OF *OCIMUM BASILICUM* AND ITS CONSTITUENT TERPENES ON THE LIFE CYCLE OF MALARIA. WITS School of Therapeutic Sciences Research Day, Parktown, South Africa, 8 September 2015. (Poster presentation).

O. Mustapha, R.L. van Zyl, M. Coetzee, S. Oliver. THE ISOMERIC EFFECTS OF ESSENTIAL OIL CONSTITUENTS ON *ANOPHELES ARABIENSIS* LARVAE AND *ARTEMIA FRANCISCANA* NAUPLII. All Africa Congress On Pharmacology And Pharmacy. Misty Hills Hotel and Conference Centre, Muldersdrift, Gauteng, South Africa. 5-8 October 2016. (Poster presentation).

O. Mustapha, R.L. Van Zyl, M. Coetzee, S. Oliver. THE ISOMERIC EFFECT OF ESSENTIAL OIL CONSTITUENTS ON *ANOPHELES ARABIENSIS* LARVAE AND *ARTEMIA FRANCISCANA* NAUPLII. 2nd South African Malaria Research Conference. University of Pretoria, Pretoria, South Africa. 31st July – 2 August 2016. (Poster presentation).

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LIST OF ABBREVIATIONS

Abs	Absorbance
ACD-B	Acid citrate dextrose solution B
ACT	Artemisinin-based combination therapy
APAD	3-Acetylpyridine-adenine dinucleotide
APADH	3-Acetylpyridine-adenine dinucleotide (reduced form)
ATP	Adenosine triphosphate
BSLA	Brine shrimp lethality assay
CDC	Centers for Disease Control and Prevention
CHO	Chinese Hamster Ovary
cm	Centimetre
°C	Degree celcius
DCM	Dichloromethane
DDT	Dichlorodiphenyltrichloroethane
DMAPP	Dimethylallyl pyrophosphate
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DOXP	1-Deoxy-D-xylulose-5-phosphate
e.g.	Example
EO	Essential oil
EOC	Essential oil constituent
EPA	Environmental Protection Agency
FBS	Foetal bovine serum
ΣFIC	Sum of the fractional inhibitory concentration
g	Gravitational constant/ G-force
g	Gram
GC-MS	Gas chromatography mass spectroscopy
GCxGC-TOFMS	Two dimensional gas chromatography time-of-flight mass spectrometry
g/dL	Gram per decilitre
G6PD	Glucose-6-phosphatase dehydrogenase
HeLa	Human cervical cancer cell
HEK-293	Human kidney epithelial cell
HEp-2	Human laryngeal epithelial carcinoma cell
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid
Hep-G2	Human hepatocellular epithelial carcinoma cell
IC_{50}	Concentration required to inhibit 50% of parasite/cell growth

IPP	Isopentenyl pyrophosphate
IRS	Indoor residual spraying
ITN	Insecticide-treated mosquito net
KGB	*Kanyemba Gambia* Species B
L	Litre
LC_{50}	Lethal concentration required to kill 50% of the population
MEP	2-C-methyl-D-erythritol-4-phosphate
m	Metre
M	Molar
mL	Millilitre
mM	Millimolar
µl	Microlitre
µM	Micromolar
mg	Milligram
MMF	Monomolecular films
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
nAchR	Nicotinic acetylcholine receptor
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NBT	Nitroblue tetrazolium
n.d	Not determined
NHLS	National Health Laboratory Service
nm	Nanometre
#	Number
PBS	Phosphate buffered saline
PES	Phenazine ethosulfate
pLDH	Parasite lactate dehydrogenase
RBC	Red blood cell
RDT	Rapid diagnostic test
rpm	Revolutions per minute
RPMI-1640	Roswell Park Memorial Institute medium formula number 1640
SANBI	South African National Biodiversity Institute
s.d.	Standard deviation
SI	Safety index
UV-VIS	Ultraviolet-visible

%	Percentage
% v/v	Percentage volume/volume
% w/v	Percentage weight/volume
Versene- EDTA	Verseneethylenediaminetetraacetic acid
WHO	World Health Organization

CHAPTER ONE – INTRODUCTION

1 Malaria

Malaria is a life-threatening disease caused by the protozoan *Plasmodium* parasite in tropical and subtropical areas. According to the latest World Malaria Report, there have been an estimated 212 million reported cases of malaria in 2015 and 429,000 deaths. Reports show a 41% decline in case incidence rates from the year 2000 to 2015 and a 62% decline in number of deaths occurring globally. Sub-Saharan Africa accounts for the majority of mortality due to malaria. Children under the age of 5 years are most susceptible to malaria, however as a result of the decline in mortality rates during 2000 and 2015, malaria is no longer the leading cause of death in sub-Saharan Africa in that age group (WHO, 2016). Malaria is the infection of red blood cells (RBCs) by the *Plasmodium* parasite, which is transmitted through the bites of infected *Anopheles* female mosquitoes. There are five species of *Plasmodium* namely; *P. falciparum, P. vivax, P. malariae, P. ovale* and *P. knowlesi* (Trampuz, 2003). *Plasmodium falciparum* and *P. vivax* malaria are the largest threat to public health. *Plasmodium vivax* malaria accounts for the majority of all malaria cases outside Africa, whereas *P. falciparum* is most prevalent in Africa (WHO, 2016). Inoculation of infected blood, the use of contaminated needles and the transmission from an infected mother to foetus during pregnancy are less common modes of malaria transmission (Nchinda, 1998).

Malaria has an incubation period of 7 days and presents as an acute febrile illness. The possibility of *P. falciparum* malaria infection is considered any time between 7 days to 3 months after the first possible exposure. Initial symptoms may be mild and not easily recognised as malaria. Severe cases due to *P. falciparum* present with clinical features including fever, headache, muscular aching chills, vomiting, generalized convulsions, pulmonary oedema and renal failure which leads to coma and death as it disrupts the blood supply to vital organs. Fatality of *P. falciparum* malaria may occur if there is a delay beyond 24 hours after the onset of clinical symptoms. Immuno-suppressed individuals, pregnant women, young and elderly travellers are at a high risk to contract malaria. Malaria in non-immune pregnant travellers increases the risk of miscarriage, stillbirth, neonatal and maternal death (WHO, 2015).

1.1 Life cycle of the malaria parasite

There are two malaria parasite hosts, namely the mosquito and human (Figure 1.1). During a blood meal, a malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host (Figure 1.1. #1). Sporozoites infect liver cells #2 and mature into schizonts #3,

which rupture and release merozoites #4. After this initial replication in the liver (exo-erythrocytic schizogony A), the parasites undergo asexual multiplication in the erythrocytes B. Merozoites infect the RBCs #5. The ring stage trophozoites mature into schizonts, which rupture releasing merozoites. Some parasites differentiate into sexual erythrocytic stages (gametocytes) #7. Blood stage parasites are responsible for the clinical manifestations of the disease (Centres for Disease Control and Prevention (CDC), 2016).

The gametocytes, male (microgametocytes) and female (macrogametocytes) are ingested by an *Anopheles* mosquito during a blood meal #8. The sporogonic cycle is when the parasites multiply in the mosquito C. While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes #9. The zygotes in turn become motile and elongated (ookinetes) #10 which invade the midgut wall of the mosquito where they develop into oocysts #11. The oocysts grow, rupture, and release sporozoites #12, which make their way to the mosquito's salivary glands. Inoculation of the sporozoites #1 into a new human host perpetuates the parasite life cycle. The mosquito acts as a vector, and unlike the human host, the mosquito vector does not suffer from the infection with the parasites (CDC, 2016).

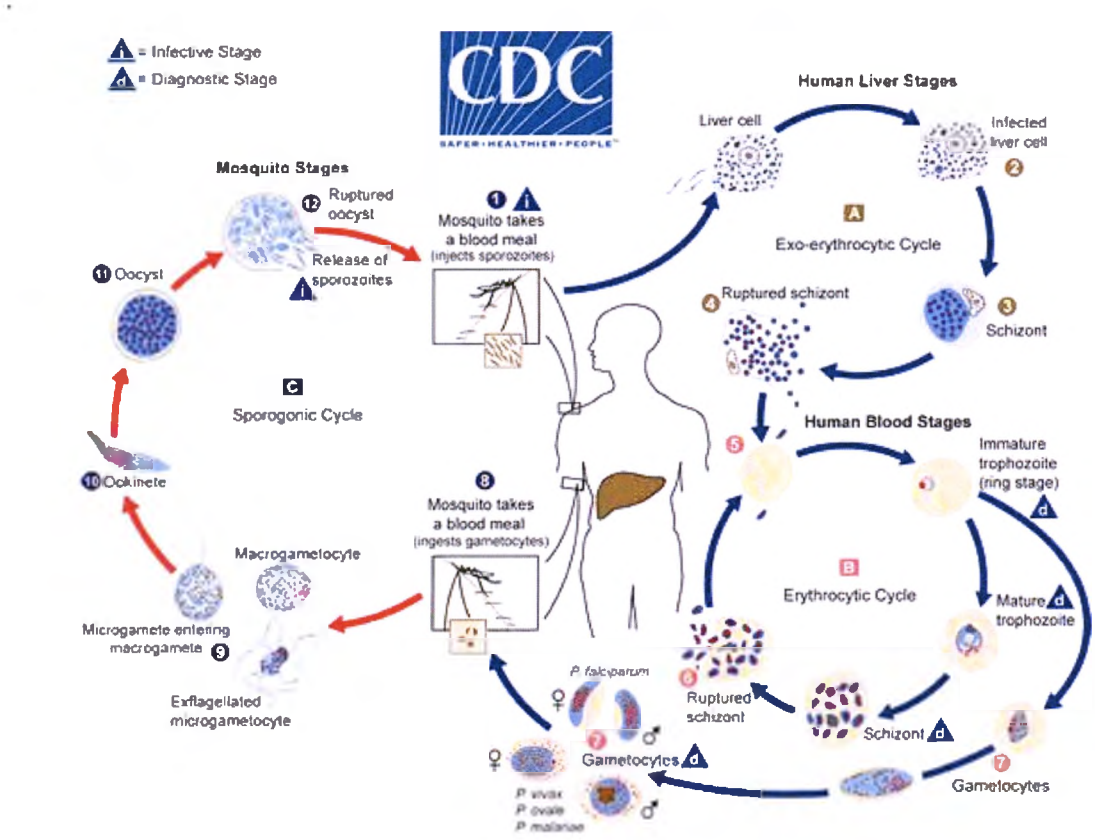

Figure 1.1: Life cycle of the *Plasmodium* parasite (CDC, 2016).

1.2 Life cycle of the mosquito

The mosquito goes through four distinct stages of its life cycle which includes egg, larva, pupa, and adult mosquito (Figure 1.2). The length of each stage depends on the temperature and species characteristics (American Mosquito Control Association®, 2016).

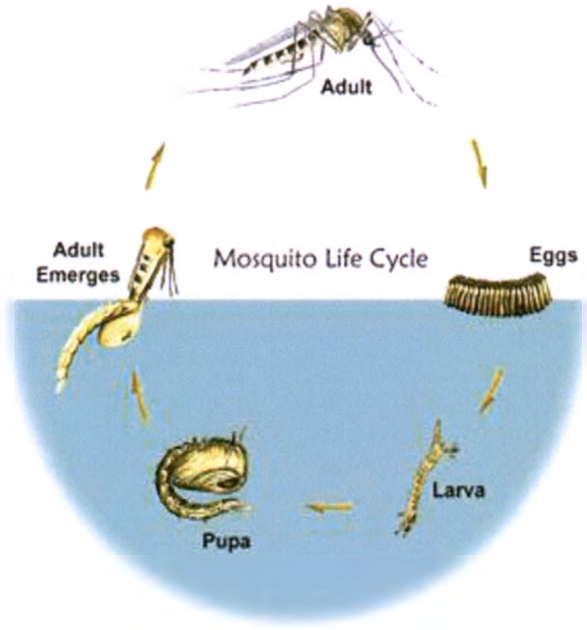

Figure 1.2: The mosquito life cycle including eggs, larva, pupae and adult mosquito (American Mosquito Control Association®, 2016).

The eggs are laid separately or attached together as rafts floating on the water surface. The eggs of *Culex* and *Culiseta* species are in rafts of up to 200. *Anopheles*, *Aedes* and *Ochlerotatus* as well as many other species lay their eggs separately. *Anopheles, Culex* and *Culiseta* lay their eggs on the water surface while *Aedes* and *Ochlerotatus* lay their eggs on damp soil that will be flooded by water. Most eggs hatch into larvae within 48 hours. The larvae live in the water and surface to breathe. Larvae moult four times and grow larger after each moult. Most larvae have siphon tubes for breathing and hang upside down from the water surface (Bosak and Crans, 2002; American Mosquito Control Association®, 2016). *Anopheles* larvae do not have a siphon on the rear end and lie parallel to the water surface to get a supply of oxygen through a breathing opening (Figure 1.3). *Coquillettidia* and *Mansonia* larvae attach to plants to obtain air supply. The larvae feed on microorganisms and organic matter in the water. During the fourth moult, the larvae changes into pupae. The pupal stage is a resting, non-feeding stage of development, but pupae are mobile, respond to light changes and move with a flip of their tails towards the bottom or protective areas. The pupal skin splits and the adult mosquito emerges. The newly emerged adult rests on the surface of the water for a short period to allow itself to dry and its body parts to harden. Blood

feeding and mating only occurs after a few days once the adult emerges (American Mosquito Control Association®, 2016).

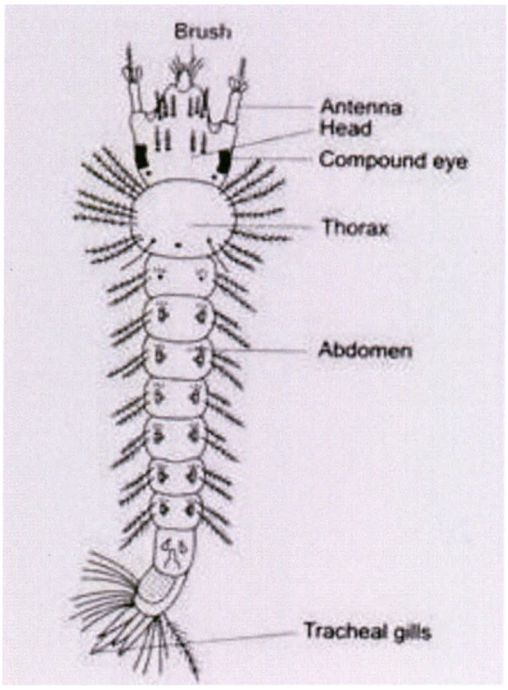

Figure 1.3: The *Anopheles* larva (Biology Discussion, 2016).

1.3 Diagnosis of malaria

Before antimalarial treatment is administered, WHO recommends one of the two prompt methods of diagnosis, namely microscopy or malaria rapid diagnostic test (RDT). Both microscopy and RDTs should be supported by a quality assurance programme. Early and accurate diagnosis is important to prevent morbidity or mortality. The RDT is beneficial to manage malaria in areas with a limited access to larger health care institutes whereby good quality microscopy services are available (WHO, 2015).

Suspected malaria is based clinically on the presence of fever or a history of fever. The focus of malaria diagnosis should be to identify patients who truly have malaria in order to guide rational use of antimalarial medication. Malaria is suspected in patients presenting with temperature ≥37.5°C or a history of fever with no other obvious cause in malaria-endemic areas. In areas where malaria transmission is stable, malaria should be suspected in children with a haemoglobin concentration of <8 g/dL or with palmar pallor. In areas with low malaria incidences, parasitological diagnosis of all cases of fever may result in considerable expenditure to detect only a few patients with malaria, and thus healthcare workers should

be trained to identify patients who may be exposed to malaria and have a fever or a history of fever with no obvious cause before parasitological tests are conducted. In all settings, parasitological tests should be conducted to confirm malaria. In children less than 5 years of age, case management provided by the WHO-United Nations Children's Fund strategy for Integrated Management of Childhood Illness should be used (WHO, 2015).

The two methods of parasitological diagnosis of malaria are light microscopy and immune-chromatographic RDTs. Rapid diagnostic tests detect parasite-specific antigens or enzymes that are either genus or species specific. It should be used if microscopy is not readily available (WHO, 2015).

1.4 Control and prevention of malaria

The three main strategies for malaria prevention and therapy include vector control, chemoprevention and case management. Vector control is an important way to reduce malaria transmission as it reduces transmission of malaria between human and mosquito by means of insecticide-treated mosquito nets (ITNs) and indoor residual spraying (IRS) (Fillinger and Lindsay, 2011; WHO, 2016). WHO-recommended preventive therapies include intermittent preventive treatment of pregnant women, intermittent preventive treatment of infants, and seasonal malaria chemoprevention. The objective of these interventions is to prevent malarial illness by maintaining therapeutic drug levels in the blood throughout the period of greatest malarial risk. Chemoprevention suppresses malaria infection in humans and case management allows for prompt detection, diagnoses and treatment of an infection (WHO, 2016).

It is imperative to take precaution in order to prevent mosquito bites. However, effective chemoprophylaxis should be taken when at the risk of acquiring malaria (Fischer and Bialek, 2002). Non-drug methods should be applied even when chemoprophylactic agents are used. Chemoprophylactic agents should be individualised taking into consideration the resistance pattern, intensity of malaria transmission, duration of stay in the malarious area, age and medical history. The chemoprohylactic drugs may inhibit liver-stage (pre-erythrocytic) development (causal prophylaxis) or may kill asexual blood stages (suppressive prophylaxis). Causal prophylaxis (primaquine, atovaquone and proguanil) can be stopped soon after leaving the endemic area, whereas suppressive prophylaxis should be continued for at least 4 weeks after leaving the area. This will ensure that asexual parasites emerging from the liver weeks after exposure are eliminated. Chemoprophylactic agents include mefloquine, doxycycline, atovaquone-proguanil and chloroquine. Mefloquine is a blood schizonticide which is active against the active stages of *P. vivax* and *P. falciparum*.

Doxycycline is a bacterio-static agent used against *P. falciparum* malaria and the preferred agent for short-term prophylaxis in multidrug-resistant areas. Atovaquone-proguanil is a fixed dose combination which interfers with plasmodial pyrimidine synthesis and is the prophylactic agent of choice (WHO, 2010).

The most appropriate chemoprophylactic antimalarial drugs in the correct dose should be prescribed for the appropriate destination. No antimalarial prophylactic regimen gives complete protection, but reduces the risk of fatal disease (WHO, 2015).

1.5 Treatment of malaria

While the prevention and control of malaria infection is avidly encouraged, antimalarial medication is available for such infections. It is necessary to taper the treatment of malaria as well according to an individual. The WHO (2015) prescribed for the treatment of uncomplicated *P. falciparum* one of the following combinations: artemether and lumefantrine, artesunate and amodiaquine, artesunate and mefloquine, dihydroartemisinin and piperaquine and artesunate and sulfadoxine-pyrimethamine.

Treatments of uncomplicated malaria caused by *P. vivax, P. ovale, P. malariae* or *P. knowlesi* in areas with chloroquine-susceptible infections include artemisinin-based combination therapy (ACT) or chloroquine. In areas with chloroquine-resistant infections, ACT is prescribed. Severe malaria in adults and children is treated with intravenous or intramuscular artesunate followed by ACT (WHO, 2015).

Malaria due to non-*falciparum* is usually uncomplicated. Chloroquine is effective for the use of treating an acute attack caused by *P. ovale, vivax* and *malariae*. *Plasmodium knowlesi* is treated as for *P. falciparum*. A 14-day course of oral primaquine is added to the treatment of *P. ovale* and *P. vivax*. Primaquine is a tissue schizonticide effective against the latent hepatic phase of *P. ovale* and *vivax*, as well as *P. falciparum* gametocytes. Artemether-lumefantrine has rapid schizonticidal activity and is effective even against multidrug-resistant strains of *P. falciparum*. Quinine is a rapidly acting blood schizonticide with limited gametocide activity (SAMF, 2014).

In South Africa, the basic management of malaria according to the Department of Health (2016) for the treatment of uncomplicated *P. falciparum* malaria includes a fixed dose artemisinin-based combination therapy (ACT), artemether-lumefantrine. When artemether-lumefantrine is not available or is contradicted, uncomplicated *P. falciparum* malaria can be treated with oral quinine plus either doxycycline or clindamycin. In South Africa, *P. ovale* is

the most common non-*falciparum* malaria. The preferred treatment in non-*falciparum* infections is artemether-lumefantrine as well, however; for *P. vivax* or *P. ovale,* a follow on treatment course of primaquine is essential. Management of severe malaria includes prompt treatment with intravenous (or intramuscular) artesunate or quinine. It is important to note that if the patient has taken chemoprophylaxis, the same medication should not be used for treatment; as well as individualised treatment should be prescribed for pregnant women, young children and immuno-suppressed patients (WHO, 2015).

1.6 Antimalarial drug resistance

Antimalarial drug resistance is defined as "the ability of a parasite strain to survive or multiply despite the proper administration and absorption of an antimalarial drug at a recommended dose" (WHO, 2015). In various parts of the world, the parasites have developed resistance to several of the antimalarial drugs, with the greatest problem attributing to *P. falciparum.* Chloroquine resistance in *P. falciparum* has spread across most of the world and appears to have arisen *de novo* (a rare event) and then spread on only a few occasions. Whereas, anti-folate drugs and atovaquone resistance arises frequently. The main consequence of antimalarial drug resistance is treatment failure, which is failure to clear malaria parasitaemia or the inability of correct dose of an antimalarial drug to resolve clinical symptoms. Antimalarial drug resistance may also be caused by poor compliance, poor drug quality, interactions with other drugs, comprised drug absorption, vomiting of medicine, unusual pharmacokinetics or misdiagnoses of the disease (White *et al.*, 2009; WHO, 2015). A resistant mutant due to genetic events is a factor which leads to resistance. Resistance of *P. falciparum* to chloroquine, sulfadoxine-pyrimethamine, mefloquine, atovaquone and artemisinins originated in South-East Asia. Chloroquine and pyrimethamine resistance spread to Africa (Roper *et al.*, 2004). Parasite resistance to the artemisinin derivatives has been detected in Thailand, Vietnam, Cambodia, the Lao People's Democratic Republic and Myanmar. This resistance has arisen due to artemisinin-based monotherapy. And thus, routine monitoring of antimalarial drug resistance is recommended by WHO (2015).

The WHO (2015) suggested the use of combination therapy to prevent resistance as it slows down the emergence of resistance. If two drugs with different modes of action are used in combination, the per-parasite probability of developing resistance to both drugs is the product of their individual per-parasite probabilities (White, 1997). Artemisinin derivatives are particularly effective in combinations with other antimalarial drugs due to their high killing rates, lack of adverse effects and the absence of significant resistance outside South-East Asia (WHO, 2015). Monitoring is required to determine geographical trends in susceptibility

and the emergence and spread of drug resistance to guide treatment as there are no tests to determine the susceptibility of malaria parasites to antimalarial medication (WHO, 2015).

1.7 Larval source management

The decrease in the global malaria burden is attributed through the scale-up of vector control interventions, particularly by the use of ITNs and IRS (WHO, 2016). While long-lasting ITNs and IRS remain the backbone of malaria vector control, larval source management targets the immature, aquatic stages of the mosquito, thereby reducing the abundance of adult vectors (Kitron 1989; Bockarie 1999; Fillinger and Lindsay, 2011). The use of larval source management has been stipulated by the WHO as a supplementary approach to vector control. By treating or eliminating all potential breeding sites (unlikely for most rural areas of sub-Saharan Africa), the number of infective bites per person per year could be reduced which reduces malaria transmission. There are four types of larval source management (Fillinger and Linsay, 2011; Rozendaal, 1997; WHO, 2016):

1. Habitat modification: a permanent alteration to the environment, e.g. land reclamation;
2. Habitat manipulation: a recurrent activity, e.g. flushing of streams;
3. Larviciding: the regular application of biological or chemical insecticides to water bodies;
4. Biological control: the introduction of natural predators into water bodies.

1.7.1 Larvicidal treatment

Larviciding is a useful method for complementing on-going malaria control programmes. The costs of larviciding may compare favourably with those for IRS and ITNs, especially where malaria transmission is moderate and where larval habitats are accessible and discrete. However, intensive surveillance and treatment programmes are required to maintain coverage of potential larval habitats (WHO, 2015). Larviciding is the regular application of biological or chemical insecticides to water bodies (Rozendaal, 1997). Larvicide treatment is effective in preventing the maturation of larvae into adult mosquitoes. The treatment of breeding habitats facilitates a decrease in the adult mosquito population in nearby areas (Tusting *et al.*, 2013). There are five main groups of larvicides namely; oils and surface agents, synthetic organic chemical, bacterial larvicides, spinosyns and insect growth regulators (Table 1.1) (Fillinger and Linsay, 2011; WHO, 2015).

Larvicides are present in liquid, tablet, pellet, granular and briquette formulations. Organophosphate insecticides affect the nervous system of the insect. These pesticides are applied in mosquito breeding areas including standing water, lakes, tidal waters, swamps

and marshes. Temephos is an organophosphate with significant larvicidal use by public health officials, personnel of mosquito abatement districts or similar agents (EPA, 2016).

Table 1.1: Classification of larvicidal agents (Fillinger and Linsay, 2011; Rozendaal, 1997; Soper and Wilson, 1943; WHO, 2015).

Larvicide	Mode of action	Example
Surface oils and films	Dispersed on the surface of the water causing the larvae and pupae to suffocate	Highly refined oils and biodegradable ethoxylated alcohol surfactants, or "monomolecular films" (MMF)
Synthetic organic chemicals	Interfers with the nervous system of immature larval stages	Organophosphates including temephos, fenthion, chlorpyrofos and pirimiphos-methyl
Bacteria	The bacteria produces insecticidal crystal proteins which, when ingested by larvae, attack the gut lining causing cessation of feeding and subsequent mortality	*Bacillus* species including *B. thuringiensis* subsp. *Israelensis* and *B. sphaericus*
Spinosyns	Act as nicotinic acetylcholine receptor (nAchR) allosteric activators and can cause mortality through both contact and ingestion	Includes metabolites extracted from the bacterium e.g. *Saccharopolyspora spinosa*
Insect growth regulators	Interferes with the metamorphoses of the insect and prevents emergence of adults from the pupae stage	Diflubenzuron, methoprene, novaluron and pyriproxyfen

The US Environmental Protection Agency (EPA) issued a cancellation of the use of temephos in 2011; however, distributors were allowed to sell products in stock until December 31, 2016. Oils and films are also used as larvicides which are dispersed on the surface of the water and cause the larvae and pupae to drown (EPA, 2016). Not all larvicides are safe for water consumption. The safety of methoprene, pyriproxyfen, temephos and

Bacillus thuringiensis israelensis for use in potable water has been assessed by the WHO Programme on Chemical Safety and are approved as safe for use in drinking water (Rozendaal, 1997; EPA, 2016).

1.8 Traditional phytotherapy

Plants have been traditionally used in many parts of the world to kill or repel mosquitoes (Seyoum *et al.*, 2003). Since plant products are easily bio-graded and are usually non-toxic, it is safer to use compared to synthetic compounds (Sharma *et al.,* 1993; Sharma and Ansari, 1994). Plant products are usually more accessible to certain communities making it cost effective (Seyoum *et al.*, 2002; Yarnell and Abascal, 2004). Natural treatment is commonly used in South Africa, with several plants used in the management of the symptoms associated with malaria and used as insecticidal repellents (Maharaj *et al.,* 2010) (Figure 1.4). Essential oils (EOs) are a complex mixture of terpenes/ essential oil constituents (EOCs) and to a lesser extent of non-terpenoid compounds. Widespread research has been done on determining antiplasmodial activities of the EOs extracted from a wide variety of plants (Durant *et al.*, 2014). Included in the latter group are *Cymbopogon citratus* (lemon grass), *Cymbopogon nardus* (citronella), *Artemesia afra* (African wormwood), *Lippia javanica* (fever tea) and *Ocimum basilicum* (basil) (Figure 1.4). C*ymbopogon citratus and C. nardus* has been shown to display insecticidal activity (Bossou *et al.,* 2013), and have anti-oxidant activity which modulate plasmodial infection (Tarkang *et al.,* 2013). *Artemisia afra* is a very common species found in South Africa extending to the tropical East Africa, as far as Ethiopia and is used in the treatment of malaria (Van Wyk *et al.*, 1997; Cumes *et al.,* 2008). *Lippia javanica* is distributed over large parts of South Africa extending north into tropical Africa (Van Wyk *et al.*, 1997). For the treatment of malaria, leaf infusions of *L. javanica* are used by the Vhavenda (Hutchings *et al.,* 1996), as well as the roots (Cumes *et al.,* 2008). The leaves of *O. basilicum* have larvicidal activity and are used as an insect repellent (Cumes *et al.,* 2008).

The main EOCs that are found in the above mentioned plants, based on previous studies, have capable *in vitro* activity. These plants have shown to be promising antimalarial with anti-pyretic, repellent and insecticidal activity, but have not been tested on the entire malaria life cycle (Nethengwe *et al.*, 2012). Both trees and shrubs are used with leaves being the most common part of the plant used to repel mosquitoes. Plant seeds, roots and barks are also used with a majority of plant parts being dried before use. A common method of repelling mosquitoes is burning the plant material to make smoke. The burning of some herbs such as *Artemisia* (Asteraceae) species in rural areas in China is used to repel mosquitoes and protect cattle from blood sucking insects (Hwang *et al.*, 1985). Hanging plants inside the

house or sprinkling leaves on the floor is another method used (Sangat-Roemantyo, 1990). In East Africa, Lua communities lay the branches of *O. basilicum* (Labiatae) inside the houses to drive away mosquitoes (Kokwaro, 1976). Although there is a widespread use of plants in the treatment of malaria, much more scientific research is required (Mavundza *et al.,* 2013).

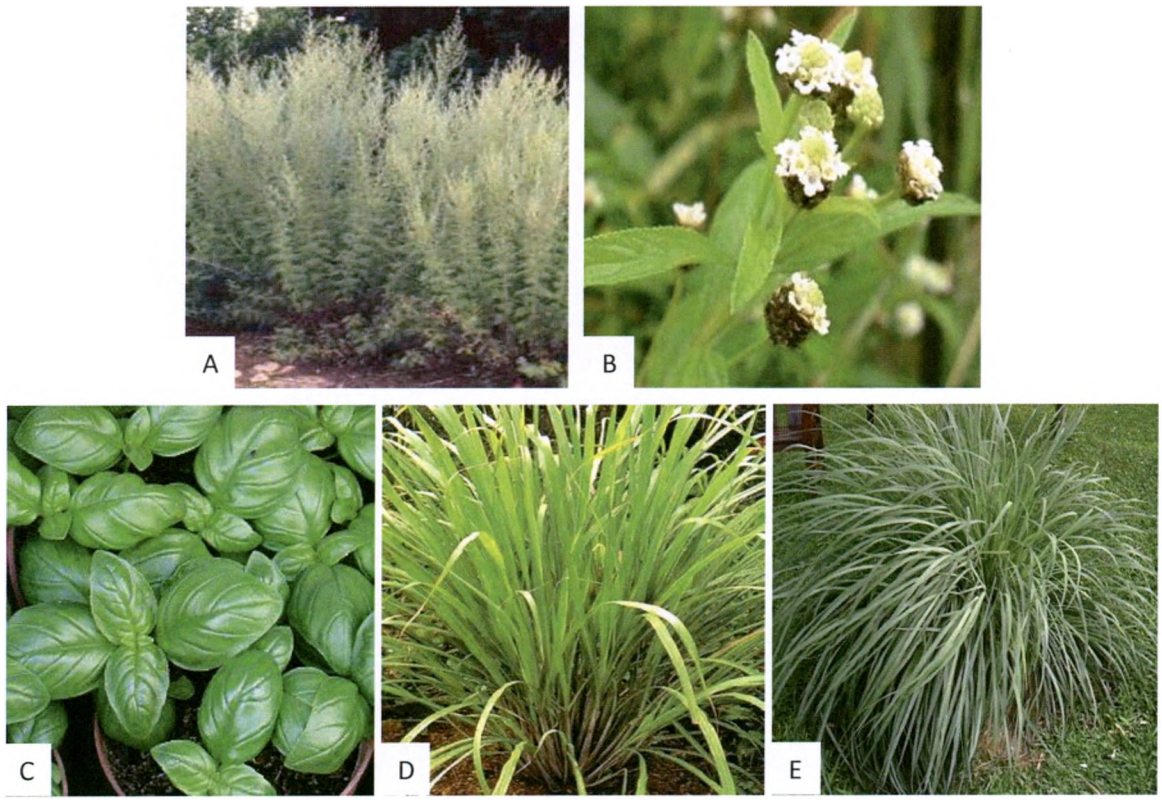

Figure 1.4: Common plants used in various parts of the world in the management of malaria, due to their repellent, insecticidal and larvicidal properties. The plants include African traditional medicinal plants *A. afra* (A) and *L. javanica* (B), as well as *O. basilicum* (C), *C. citratus* (D) and *C. nardus* (E) (PlantsZAfrica, 2015).

1.8.1 Plants used in the management of malaria

1.8.1.1 *Lippia javanica*

Lippia javanica (Burm. f.) Spreng, also known as fever tea, is an erect aromatic woody shrub belonging to the Verbenaceae family. It is found throughout Southern Africa and abundant in the north of South Africa and Swaziland. The shrub grows up to 5 meters in height (Olivier *et al.*, 2010). It is not endemic to South Africa but its distribution within South Africa includes Eastern Cape, Free State, Gauteng, KwaZulu-Natal, Limpopo, Mpumalanga and North West

(Figure 1.5). Common names other then fever tea, includes Beukesbos and Bokhukhuwane (SANBI, 2015).

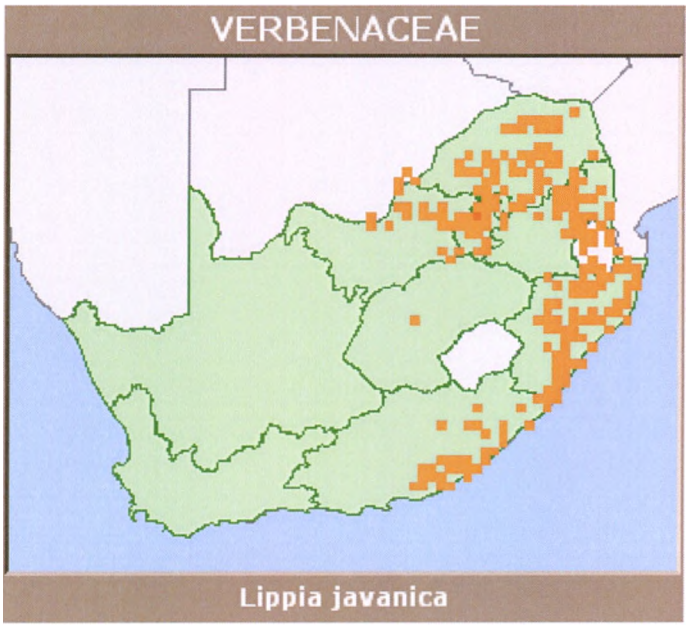

Figure 1.5: The geographical distribution of *L. javanica* in South Africa (SANBI, 2015).

Lippia javanica has been reported to have anti-pyretic and analgesic properties and its infusion is used for a variety of ailments ranging from skin disorders to bronchial infections. *Lippia javanica* together with *A. afra* is prescribed by traditional practitioners as a prophylaxis against dysentery and to treat fever, influenza and malaria (Olivier *et al.*, 2010). It is commonly used in South Africa to treat measles, chest ailments, influenza, skin disorders, stomach problems and headaches. The EO which contains up to 75% piperitenone has good insect repellent, antibacterial and antiplasmodial activity (Ludere *et al.*, 2013). The EO of *L. javanica* and five other plants were previously screened for their repellent activity against *Anopheles gambiae*. *Lippia javanica* showed the third most repellent activity following the oils of *Conyza newii* (Compositeae) and *Plectranthus marrubioides* (Labiateae) (Omolo *et al.*, 2004). The leaves of *L. javanica* are used in its fresh state as a mosquito repellent (Mavundza *et al.*, 2013). The leaves are also dried and burnt as the smoke has repellent properties. Infusions are commonly used as a tea in Africa (Karunamoorthi *et al.*, 2009). Lukwa (1994) determined *L. javanica* to have larvicidal activity against *An. gambiae* larvae.

Essential oils obtained from plants have various extents of toxicological properties. Toxic effects of EOs include topical effects affecting the skin, eye irritation, phototoxicity, vomiting, diarrhea, epigastric pain, mucosal irritation, renal failure, convulsions and central nervous system depression (Lane *et al.*, 1991; Riodan *et al.*, 2002). There are numerous beneficial

properties of the EO *L. javanica*, however, it is known to cause liver damage and photosensitisation in livestock, resulting in stock losses. Triterpenoids isolated from the genus *Lippia* are icterogenic and cause jaundice as a result of liver damage. In view of the known toxicity of *Lippia* species, the prolonged use of high doses of *L. javanica* is potentially harmful (van Wyk *et al.*, 2009). The EOCs may contribute to the toxic properties exerted by *L. javanica*, as determined by Seatlholo (2008), whereby; (±)-linalool, (+)-α-pinene and *p*-cymene displayed cytotoxic properties on human kidney epithelial (HEK-293) cells with IC_{50} values of 882.70, 172.20 and 673.6 µM, respectively. Linalool, which is a major EOC of *L. javanica* as well as *O. basilicum*, has shown to be cytotoxic to human skin cells (Prashar *et al.*, 2004).

1.8.1.2 *Artemisia afra*

Artemisia afra (Jacq. ex Wild), belonging to the Asteraceae family, is a multi-stemmed perennial woody shrub which grows up to 2 meters in height. The plant occurs in Kenya, Uganda, Tanzania and Ethiopia, and is widely distributed in Southern Africa including South Africa, Zimbabwe and Namibia. In South Africa, it is found in Limpopo, Gauteng, Western Cape and KwaZulu-Natal (Figure 1.6) (PlantzAfrica, 2015). *Artemisia afra* is also known as African wormwood and is traditionally used for treating ailments such as colds, coughs, chills, headaches, asthma, influenza, croup, convulsions, dyspepsia and malaria amongst others. Its uses indicate that it contains antibacterial, antiviral and anti-inflammatory properties as well as anti-oxidant, antimalarial, antinemodal, cardiovascular, cytotoxic and sedative effects (Watt and Breyer-Brandwijk, 1962).

Artemisia afra is used in many forms including syrups, infusions, leaves and twigs (Liu *et al.*, 2010). To treat a blocked nose and headache, the leaves are inhaled or the tip of a fresh leaf is inserted into the nostril. Fresh twigs may also be inserted into a hollow tooth to relieve toothache. The plant is usually burned and used as an inhalant for ailments of the bronchial passages. The oils of the plant impart a bitter taste and the presence of the EOC, thujone, makes this plant toxic if consumed in sufficient quantities (Hutchings *et al.*, 1996). *Artemisia afra* has also been used for the treatment of ailments associated with the topical inflammation, gastrointestinal tract and fever (Scott *et al.*, 2004). Reports have shown that the combination of *A. afra* with *Agrimonia bracteata, O. asteriscoides, L. javanica,* and *Tetradenia riparia* treats respiratory disorders (Hutchings *et al.*, 1996).

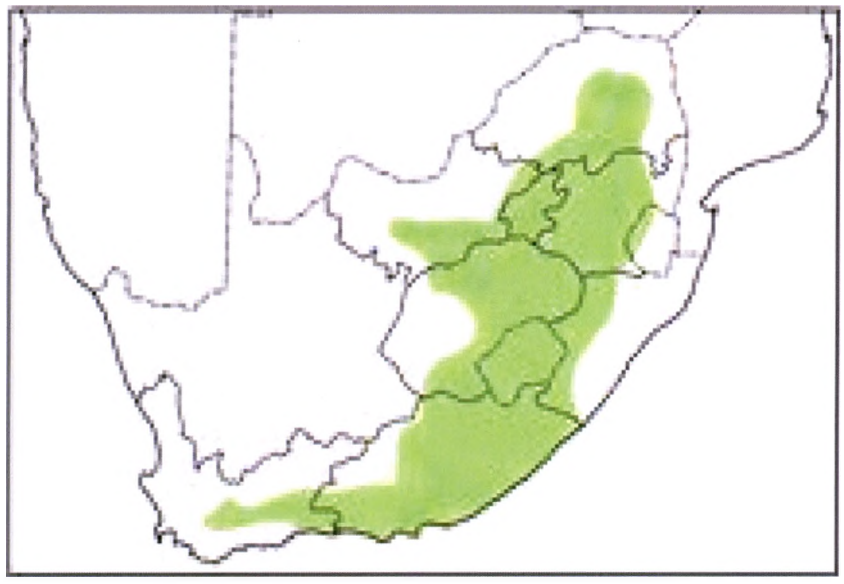

Figure 1.6: The geographical distribution of *A. afra* in South Africa (PlantsZAfrica, 2015).

Although *A. afra* has been used in the treatment of numerous ailments, there are certain deleterious effects of EOs and EOCs on all living systems. Such as drugs, plant EOs and EOCs may possess unfavourable topical and systemic effects including skin irritation, phototoxicity, mutagenicity, hepatotoxicity, carcinogenicity and cardiotoxicity (Yoo *et al.*, 2005). The plant itself is not patented for it being used tradionally for a number of ailments (Calixto, 2000). Thujone, an EOC, which is often found of some chemotypes of *A. afra,* is well reported for its toxicity and has thus made the safety of *A. afra* a controversial issue (Oyedeji *et al.*, 2009; Viljoen, 2007). Due to the thujone content, The Directorate Agricultural Information Services instructs the users that *A. afra* should not be taken longer than a period of 7 to 10 days as it can cause headaches and shaking. The EO should not be used internally, and should be used with caution during pregnancy and in epilepsy (Directorate Agricultural Information Services, South Africa, 2009).

Oyedeji *et al.* (2009) studied the α-thujone content isolated from the EO of fresh and dried twigs of *A. afra* plants obtained from different locations in the Eastern Cape, Free State and KwaZulu-Natal. The GC-MS analysis obtained revealed the presence of the EOCs of α- and β-thujone, eucalyptol and camphor. Mukinda and Syce (2007) investigated the safety of *A. afra* aqueous extract (mimicking the traditional decoction dosage form) by determining its pharmaco-toxicological effects after acute and chronic administration in mice and rats, respectively. They concluded that the *A. afra* extract is non-toxic when given acutely and has low chronic toxicity potential; in high doses it may have a hepatoprotective effect (Mukinda and Syce, 2007).

1.8.1.3 *Ocimum basilicum*

Ocimum basilicum (L) popularly known as sweet basil belongs to the Lamiaceae family. It is an erect branching herb which can grow up to 0.9 meters in height. The leaves secrete a strongly scented volatile oil. It originates in Asia and Africa and is widely cultivated around the world. The crop is frost sensitive, as thus production areas in South Africa during winter includes Mpumalanga lowveld, KwaZulu-Natal coastal areas and Limpopo. In summer, production occurs in Mpumalanga, high-lying regions of Gauteng and Free State (South African Department of Agriculture, Forestry and Fisheries, 2012) (Figure 1.7). Basil has been used from the age of Dioscorides as a scorpion sting antidote. It is widely used as traditional medicine including Ayuverda and Chinese medicine (Bilal *et al.*, 2012).

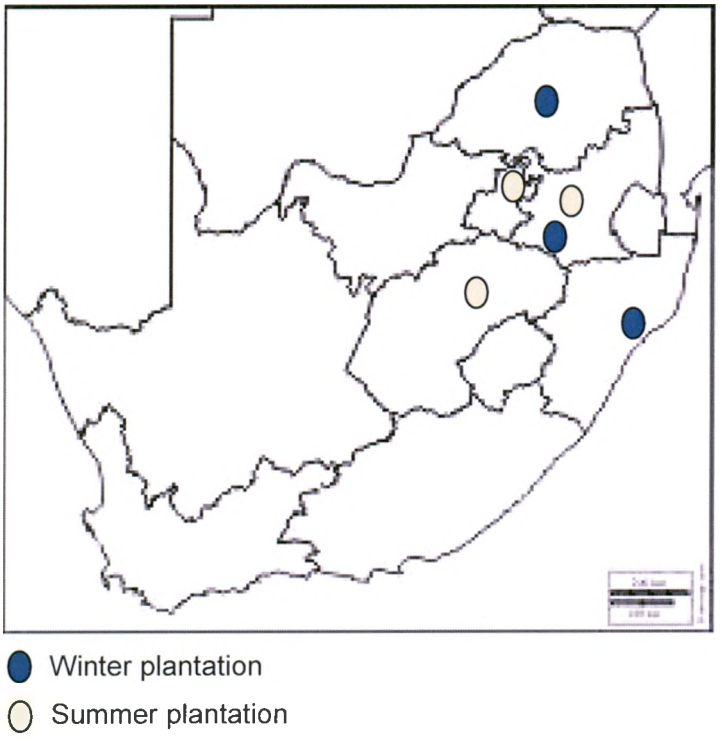

Figure 1.7: The geographical distribution of *O. basilicum* in South Africa (D-maps 2016; adapted from the South African Department of Agriculture, Forestry and Fisheries, 2012).

Studies have shown that *O. basilicum* possessed anti-inflammatory, analgesic, antimicrobial, anti-oxidant, anti-ulcerogenic, cardiac stimulant, chemo-modulatory, central nervous system depressant, immunomodulator, hepatoprotective, hypoglycemic, hypolipidemic and larvicidal activities. Traditional use of basil tea is drunk hot to treat flatulence, dysentery and nausea. The plant alleviates mental fatigue, cold and spasms (Bilal *et al.*, 2012).

The leaves of *O. basilicum* are used to treat malaria by being boiled with other plants including *C. citratus* and *Citrus limonum* (Asase and Asafo-Agyei, 2011). Manzoor *et al.* (2013) determined that *O. basilicum* possessed potent larvicidal properties against *Ae. aegypti* and *Cx. quinquefasciatus* mosquito larvae. The EOCs have shown to possess insecticidal activity, as the leaves are used to protect crops from beetle damage (Khare, 2008). Toxicological effects of *O. basilicum* are present on the central nervous system which could warrant disadvantageous after long exposure (Mohamed, 2015). Eugenol, linalool and eucalyptol were major EOCs present in the *O. basilicum* EO. Eugenol has been reported to be safe for public consumption. This was supported by a study conducted by Seatlholo (2008), whereby a cytotoxic IC_{50} value of 1358.40 µM was observed on human kidney epithelial cells (HEK-293). It was mentioned that eugenol, a main EOC of *O. basilicum* and *C. nardus* is hepatotoxic (Duke and Beckstrom-Sternberg, 1994)

1.8.1.4 *Cymbopogon citratus*

Cymbopogon citratus (DC.) Stapf (Poaceae) is indigenous in tropical and semi-tropical areas of Asia. It is cultivated in Africa, South and Central America amongst other tropical countries. It can be characterized as a tufted perennial grass with a short rhizomatous rootstock from which numerous stiff stems arise. It is commonly used as a food flavourant due to its citrus flavour. *Cymbopogon citratus* can grow up to 1.8 meters in height and 1.2 meters in width. It is noted that this plant produces an EO rich in citral (Shah *et al.*, 2011). *Cymbopogon citratus* is mainly grown in the lowveld of Mpumalanga, KwaZulu-Natal and Limpopo province. Limited planting occurs in North West, Gauteng, Western and Eastern Cape (Figure 1.8) (South African Department of Agriculture, Forestry and Fishery, 2012).

The extracts vary in accordance to the geographical origin, genetic differences, part of the plant used, method of extraction, age/stage of maturity, and season of harvest (Ekpenyong *et al.*, 2014). It has a wide range of applications as it has been used to treat diabetes, anxiety, gastrointestinal disorders, malaria, fever and pneumonia, as *C. citratus* possess antiprotozoal, anti-carcinogenic, antibacterial, anti-inflammatory, antifungal, anti-oxidant, anti-rheumatic, cardioprotective, antitussive, and antiseptic properties (Ekpenyong *et al.*, 2014).

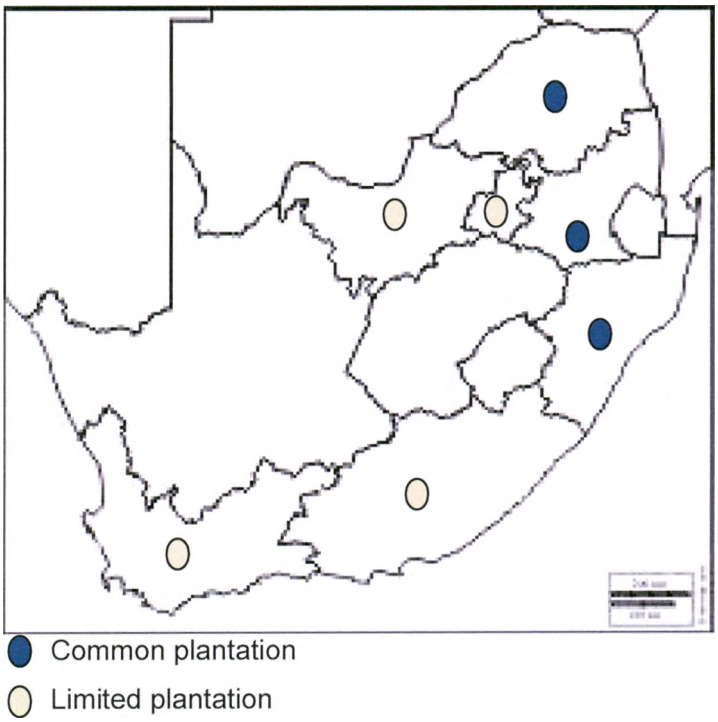

Figure 1.8: Geographical distribution of *C. citratus* in South Africa (D-maps, 2016; adapted from the South African Department of Agriculture, Forestry and Fishery, 2012).

The stalk decoction reduces faecal output in a dose-dependent manner; whereas the fresh leaves are used against *Setaria digitata* due to its antifilarial activity. The oil is applied to topical fungal infections and is used as an insecticide and herbicide as well. The oil works effectively as a remedy against bacteria, flu and colds (Shah *et al.*, 2011). It induces perspiration, cools the body and reduces fever (South African Department of Agriculture, Forestry and Fishery, 2012). The leaves of the plant are used in Nigeria for the treatment of diabetes, inflammation and nerve disorders (Aibinu, 2007). The leaves are also used specifically in Cuba and Argentina to treat colds and flu, digestive ailments, and as a decoction with other plants for malaria (Valdés *et al.*, 2010).

Although *C. citratus* may have beneficial properties, toxicological effects may be harmful on living organisms. *C. citratus* has been reported to be neurotoxic, teratogenic, nephrotoxic, hepatotoxic and have dermatological affects (Ekpenyong *et al.*, 2014). Toxicity may be due to the active EOCs present in the plant oil, such as *trans*-geraniol and geranyl acetate which have displayed cytotoxic properties as observed by Seatlholo (2008) on human kidney epithelial (HEK-293) cells with IC_{50} values of 128.50 and 796.40 µM, respectively. The EOC, citral (neral + geranial), has shown to induce skin irritation in humans as determined by Motoyoshi *et al.*, (1979). Fifty make volunteers were dermally exposed to 32% citral mixed

with acetone for 48 hours and positive skin reactions including the presence of erythema, papules and bullous reaction were observed. Citral has also shown to induce hepatic cytochrome P450 activity, with potential consequences for interaction with other drugs (Chen *et al.*, 2013). Caution should be exercised when administering high doses over a long period, especially in individuals with indices of compromised hepatic and renal function (Ekpenyong *et al.*, 2014).

1.8.1.5 *Cymbopogon nardus*

Cymbopogon nardus (L.) Rendle also belongs to the Poaceae family and is commonly known as citronella. It is a perennial grass cultivated in Southeast Asia (Nakahara *et al.*, 2003). It is not endemic to South Africa, however, it is distributed through Eastern Cape, Gauteng, Limpopo, KwaZulu-Natal, North West and Mpumalanga (Fish and Victor, 2005) (Figure 1.9). It can be described as a tall tufted long-lived (perennial) grass with narrow leaf-blades. It has narrow panicle (flower cluster), 15-30 cm long with racemes 8-10 mm long (Harrington and Pratchett, 1974). The leaves of the plant are used by the Chinese for rheumatism, the treatment of fever, intestinal parasites, digestive and menstrual ailments (Awuah, 1999). The leaves in the India region are used as insect repellents and as perfumes (Noor *et al.*, 2012.)

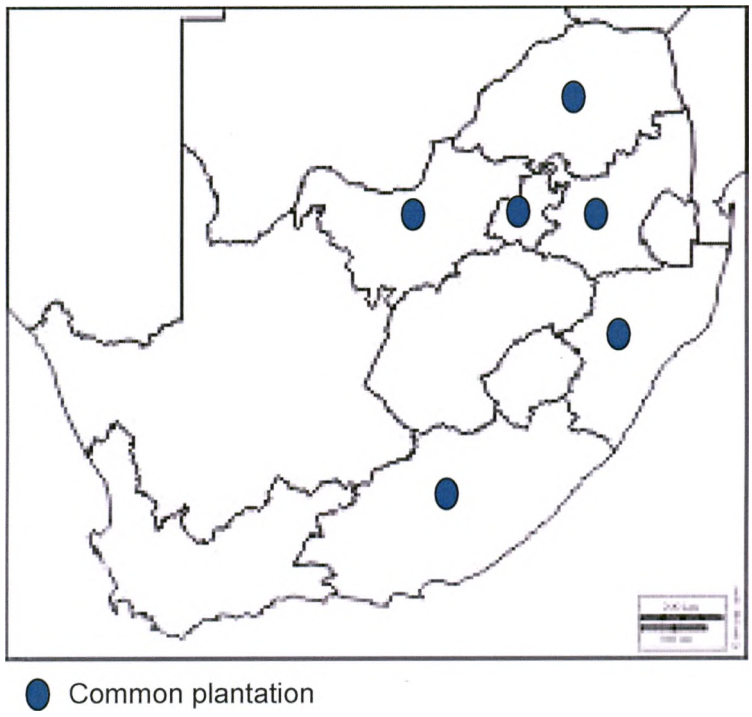

Figure 1.9: Geographical distribution of *C. nardus* in South Africa (D-maps, 2016; adapted from Fish and Victor, 2005).

The oil produced by *C. nardus* is burnt for use as a mosquito repellent as well (Chomchalow, 1993). The oil has also shown good adulticidal activity and excellent larvicidal and repellent activity against *An. arabiensis* mosquitoes (Naidoo, 2007). *Cymbopogon* is considered a 'hot' oil and should be diluted before topical application as an adverse effect (Pisseri *et al.,* 2008). The toxicological effects of *C. nardus* may be contributed by its main EOCs, as Seatlholo (2008) determined the cytotoxicity of *trans*-geraniol, (-)-citronellal, geranyl acetate and eugenol on HEK-293 cells with IC_{50} values 128.50, 238.30, 796.40 and 1358.40 µM, respectively. The cytotoxicity of the EO, *Daucus carota*, which contained a majority of geranyl acetate (29.00%) was screened in several mammalian cells lines including human hepatocellular carcinoma cell line HepG2, human alveolar epithelial cell line A549, mouse leukaemic monocyte macrophage cell and human keratinocyte cell line HaCaT in order to evaluate a potential pharmacological application. It was inferred that the concentration of 0.64 µL/mL induced different cell viability results among all the cell lines studied, with macrophages being the most resilient (92.83% cell viability) and hepatocytes the most susceptible (60.73% cell viability). It was mentioned that geranyl acetate had very detrimental cytotoxic effects (Alves-Silver *et al.*, 2016).

1.9 Terpenes

Comprising of at least 30,000 compounds, terpenes are the largest group of plant natural products with the widest assortment of structural types. There are numerous monoterpene, sesquiterpene, diterpene, triterpene and tetraterpene carbon skeletons known (Connolly and Hill, 1991; Connolly and Hill, 2005). Monoterpenes and sesquiterpenes are the main constituents of EOs (Mbaveng *et al.,* 2014).

The basic unit of a secondary plant metabolite consists of a simple hydrocarbon molecule, isoprene (C5) (Figure 1.10), which includes terpenes. A terpene refers to a hydrocarbon molecule and terpenoid refers to a modified terpene. These two terms are often used interchangeably. The isoprene unit is a five-carbon molecule and thus represents the most basic class of terpenes, the hemiterpenes. Monoterpenes (C10) is a two bonded isoprene units. Sesquiterpenes contain three isoprene units (C15), diterpenes (C20) and triterpenes (C30) contain two and three terpene units, respectively. Tetraterpenes consist of four terpene units and polyterpenes contain more than four terpene unit (Zwenger, 2008).

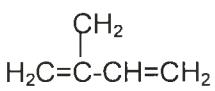

Figure 1.10: Structure of an isoprene (Mann *et al.,* 1994).

Many terpenes have biological antimalarial, anticancer, anti-inflammatory, anti-allergen, antispasmodic, antihyperglycemic, antimodulatory activities and may affect infectious diseases. Terpenoids can be used as protective substances in storing agriculture products as they have insecticidal properties. Artemisinin is one of the most renowned terpene-based drugs (Mbaveng *et al.*, 2014). Artemisinin (*qinghaosu*) was identified as an active ingredient in *Artemisia annua* in the early 1970's as an unstable *in vivo* antimalarial (Ashley, 2004). However, centuries before that, Chinese medicinal healers used *A. annua* in the treatment of fevers (Burns, 2008). Bioactive terpenoids were identified in African medicine with both organ-protective and non-beneficial properties in humans. Certain terpenoids found in plants have the ability to interfere with the physiology and biochemistry of herbivores, bacteria, viruses and fungi. Certain terpenoids are toxic and affect the nervous system, cell functioning and metabolic poisoning and can therefore affect the heart, kidneys, liver, respiration, muscles and reproductive system (Mbaveng *et al.*, 2014).

Nearly a half of a century ago, low-temperature chromatography was the technique used to separate plant terpenes (Clements, 1958). Techniques such as exposing plants to pyrolysis and gas chromatography lead to the identification of different volatile organic compounds (Greenberg *et al.*, 2006). Other methods used for the extraction of terpenes include the use of ultra pure water including subcritical water, column chromatography liquid-liquid extraction, two dimensional gas chromatography time-of-flight mass spectrometry (GCx GC-TOFMS) and gas chromatography mass spectrometry (GC-MS) (Zwenger, 2008).

1.10 Essential oils

1.10.1 Essential oil isolation

Essential oils are obtained by hydro-distillation, steam distillation, cold pressing, microwave irradiation and mechanical and thermo-chemical reaction (Lahlou, 2004). In hydro-distillation, indirect steam from outside the still is applied to the plant material in water two to three times its weight. During steam distillation, production occurs in the still whereby the plant material is through direct steam or produced outside the still by indirect steam and thereafter fed into the still (Buchbauer, 2000). Cold pressing is a special method of the fresh or dried material for *Citrus* (Rutaceae) peel oil extraction (Baaliouamer *et al.*, 1992; Dellacassa *et al.*, 1992). In microwave irradiation, the microwaves cause the rupturing of plant cells thereby releasing the EOs present in the extracellular plant tissue (Bélanger *et al.*, 1991).

1.10.2 Application of essential oils

The route of administration of EOs is important to note as the oils are fat soluble and highly volatile making it easily absorbed through the skin (Pisseri *et al.*, 2008). The volatile parts of aromatic plants were inhaled, applied topically or ingested according to ancient practices (Tseng, 2005).

The French model of aromatherapy involves three methods of EO use: inhalation, topical, and oral use. There are certain "hot" oils that always must be diluted before skin application: *C. nardus*, cinnamon bark, and cassia, for example. But use of other oils "neat" or at 100% has been safely documented in many studies (Johnson and Boren, 2013). It has been reported that the naturally occurring whole plant is safer to use as a reduced amount of side effects are experienced. The isolation of the compounds from plants removes safety checks and balances innately developed in the plant, having pharmacological effects that differ significantly from the whole plant (Williamson, 2001).

However, EOs which are properly distilled to preserve the optimal compound profile of naturally occurring constituents verified through GC-MS analysis, offer comparable benefits (Onawunmi *et al.*, 1984; Hyldgaard *et al.*, 2012; Veras *et al.*, 2012).

1. 11 Aim and objectives

Due to the increase in resistance to antimalarial medication and insecticides, the treatment and prevention of malaria is at a shortfall. Phytochemicals have been used for centuries as traditional medicine, as such novel compounds are required for the management of malaria (Seyoum *et al.*, 2003). Thus the aim of the study was to determine the effects of the EOs and EOCs on the human and vector stages of the *P. falciparum* malaria parasite and to elucidate a preliminary mechanism of action and toxicological profile.

To achieve this aim, the objectives of the study were to:

- Determine the *in vitro* antimalarial activity of the EO/ EOCs in the asexual stages of the chloroquine-sensitive *P. falciparum* parasite.
- Assess the effects of the EO/EOCs on the malaria vector larvae.
- Evaluate possible drug interactions for the combinations of the two most active EO/ EOCs.
- Determine the effect on the RBCs and lipid peroxidation inhibition ability of the EO/EOCs.
- Investigate a preliminary toxicological profile of the EO/EOCs on the *Artemia franciscana* nauplii.

- Elucidate the cytotoxic properties of the EO/EOCs against human kidney epithelial (HEK-293) cells.

CHAPTER TWO – METHODS AND MATERIALS

2.1 Materials used

Twenty two EOCs and chemicals of the highest quality were purchased from Sigma-Aldrich® (USA) and Fluka (Switzerland) (Table 2.1). Five EOs were purchased/obtained from Burgess & Finch and Prof. S van Vuuren from the University of the Witwatersrand (Table 2.2).

Table 2.1: Properties of the selected EOCs and their corresponding structural formulas (Figure 2.1) (Sigma- Aldrich®, 2016).

#	Essential oil constituent	Empirical formula	Molecular weight (g/mol)	Density (g/mL)	Synonyms	Source
1	(+)-β-Pinene	$C_{10}H_{16}$	136.23	0.872	(1*R*,5*R*)-2(10)-Pinene, (1*R*,5*R*)-6,6-Dimethyl-2-methylenebicyclo[3.1.1]heptane	Fluka
2	(-)-β-Pinene	$C_{10}H_{16}$	136.23	0.858	(1*S*,5*S*)-2(10)-Pinene, (1*S*,5*S*)-6,6-Dimethyl-2-methylenebicyclo[3.1.1]heptane	Fluka
3	(+)-α-Pinene	$C_{10}H_{16}$	136.23	0.858	(1*R*,5*R*)-2-Pinene, (1*R*,5*R*)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene	Fluka
4	(-)-α-Pinene	$C_{10}H_{16}$	136.23	0.858	(1*S*,5*S*)-2-Pinene, (1*S*,5*S*)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene	Fluka
5	(*R*)-(+)-Citronellal	$C_{10}H_{18}O$	154.25	0.851	(+)-Citronellal, (3*R*)-3,7-Dimethyl-6-octenal	Sigma-Aldrich®
6	(*S*)-(-)-Citronellal	$C_{10}H_{18}O$	154.25	0.851	(−)-Citronellal, (3*S*)-3,7-Dimethyl-6-octenal	Sigma-Aldrich®
7	(±)-Linalool	$C_{10}H_{18}O$	154.25	0.870	Linalool, (±)-3,7-Dimethyl-1,6-octadien-3-ol, (±)-3,7-Dimethyl-3-hydroxy-1,6-octadiene	Sigma-Aldrich®
8	(-)-Linalool	$C_{10}H_{18}O$	154.25	0.862	(*R*)-(−)-3,7-Dimethyl-1,6-octadien-3-ol	Sigma-Aldrich®
9	(-)-α-Thujone	$C_{10}H_{16}O$	152.23	0.914	(1*S*,4*R*)-1-Isopropyl-4-methylbicyclo[3.1.0]hexan-3-one	Fluka

Table 2.1 (Continued; Properties of the selected EOCs and their corresponding structural formulas (Figure 2.1) (Sigma- Aldrich®, 2016).

10	α-β-Thujone	$C_{10}H_{16}O$	152.23	0.925	(−)-1-Isopropyl-4-methylbicyclo[3.1.0]hexan-3-one	Fluka
11	*cis*-Geraniol	$C_{10}H_{18}O$	154.25	0.876	Nerol, *cis*-3,7-Dimethyl-2,6-octadien-1-ol	Sigma-Aldrich®
12	*trans*-Geraniol	$C_{10}H_{18}O$	154.25	0.879	*trans*-3,7-Dimethyl-2,6-octadien-1-ol	Sigma-Aldrich®
13	*cis*-Nerolidol	$C_{15}H_{26}O$	222.37	0.876	*cis*-3,7,11-Trimethyl-1,6,10-dodecatrien-3-ol	Fluka
14	*trans*-Nerolidol	$C_{15}H_{26}O$	222.27	0.876	(*E*)-3,7,11-Trimethyl-1,6,10-dodecatrien-3-ol, *trans*-3,7,11-Trimethyl-1,6,10-dodecatrien-3-ol	Fluka
15	Geranyl acetate	$C_{12}H_{20}O_2$	196.29	0.916	*trans*-3,7-Dimethyl-2,6-octadien-1-yl acetate, *trans*-3,7-Dimethyl-2,6-octadienyl acetate	Sigma-Aldrich®
16	Citral (neral + geranial)	$C_{10}H_{16}O$	152.23	0.888	3,7-Dimethyl-2,6-octadienal, Geranial and neral mixture	Fluka
17	Eugenol	$C_{10}H_{12}O_2$	164.20	1.067	2-Methoxy-4-(2 propenyl) phenol, 4-Allyl-2 methoxyphenol, 4 Allylguaiacol	Fluka
18	*p*-Cymene	$C_{10}H_{14}$	134.22	0.860	1-Isopropyl-4methylbenzene, 4 Isopropyltoluene	Sigma-Aldrich®
19	Eucalyptol	$C_{10}H_{16}O$	154.25	0.921	1,8-Cineole, 1,3,3-Trimethyl-2-oxabicyclo[2.2.2]octane, 1,8-Epoxy-*p*-menthane	Fluka
20	*Artemisia* ketone	$C_{10}H_{16}O$	152.23	0.87	3,3,6-Trimethylhepta-1,5-dien-4-one; 1,5-Heptadien-4-one, 3,3,6-trimethyl-	Fluka
21	Ocimene	$C_{10}H_{16}$	136.23	0.818	3,7-Dimethyl-1,3,6-octatrien, 3,7-Dimethyl-1,3,6-octatriene	Fluka
22	(-)-*trans*-Caryophyllene	$C_{15}H_{24}$	204.35	0.902	β-Caryophyllene, *trans*-(1*R*,9*S*)-8-Methylene-4,11,11-trimethyl bicyclo[7.2.0]undec-4-ene	Sigma-Aldrich®

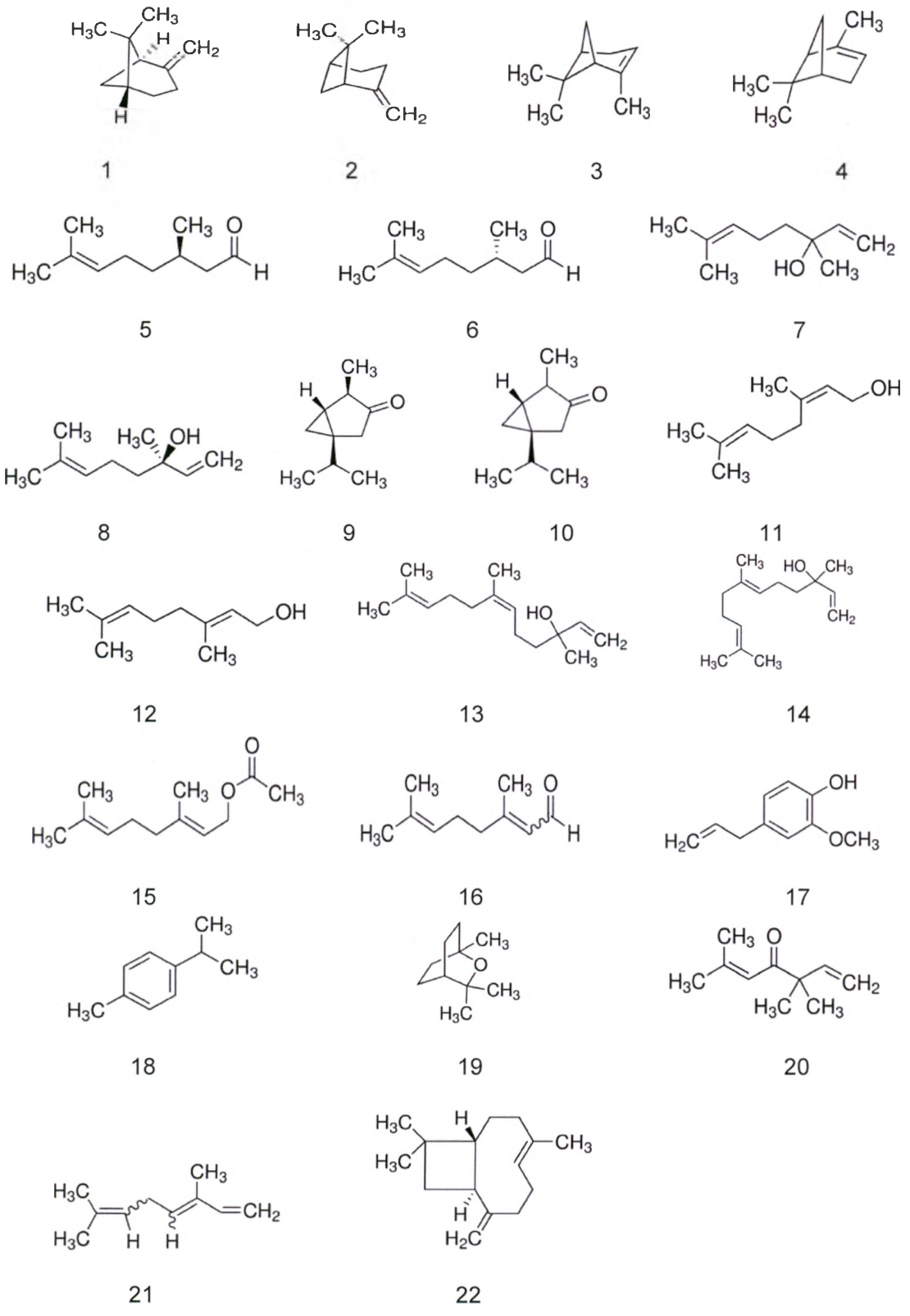

Figure 2.1: Structural formulas of the main EOCs derived from the plants used in the management of malaria, whereby the figure number corresponds to Table 2.1 (Sigma-Aldrich®, 2016).

Table 2.2: Properties of the selected EOs (PlantZAfrica, 2015).

Essential oil	Binomial name	Family	Synonyms	Source
Cymbopogon citratus	*C. citratus* (DC.) Stapf	Poaceae	Lemongrass, oil grass	Burgess & Finch
Cymbopogon nardus	*C. nardus* (L.) Rendle	Poaceae	Citronella grass	Burgess & Finch
Ocimum basilicum	*O. basilicum* L.	Lamiaceae	Sweet basil,	Burgess & Finch
Artemisia afra	*Artemisia afra* Jacq. *ex* Wild	Asteraceae	*African worm-wood, lengana,* wilde-als, umhlonyane	935, Klipriviersberg (Prof Van Vuuren)
Lippia javanica	*Lippia javanica* (Burm. f.) Spreng	Verbenaceae	Fever tea, Koorbossie, umSutane, bokhukhwane	797b, Fairlands (Prof Van Vuuren)

Table 2.3: Properties of the controls used in various assays with their corresponding structures in Figure 2.2 (Sigma- Aldrich®, 2016).

	Compound	Empirical formula	Molecular weight (g/mol)	Density (g/mL)	Source
1	Quinine hydrochloride dehydrate	$C_{20}H_{24}N_2O_2 \cdot HCl \cdot 2H_2O$	396.91	-	Sigma-Aldrich®
2	Chloroquine diphosphate	$C_{18}H_{26}ClN_3 \cdot 2H_3PO_4$	515.86	-	Sigma-Aldrich®
3	Dihydroartemisinin	$C_{15}H_{24}O_5$	284.35	-	Sigma-Aldrich®
4	4,4'-DDT	$C_{14}H_9Cl_5$	354.49	1.56	Sigma-Aldrich®
5	Trolox	$C_{14}H_{18}O_4$	250.29	-	Sigma-Aldrich®
6	Camptothecin	$C_{20}H_{16}N_2O_4$	348.36	-	Sigma-Aldrich®
7	Potassium dichromate	$K_2Cr_2O_7$	294.18	2.68	Sigma-Aldrich®
8	Triton X-100™	$(C_2H_4O)nC_{14}H_{22}O$	647	1.07	Sigma-Aldrich®

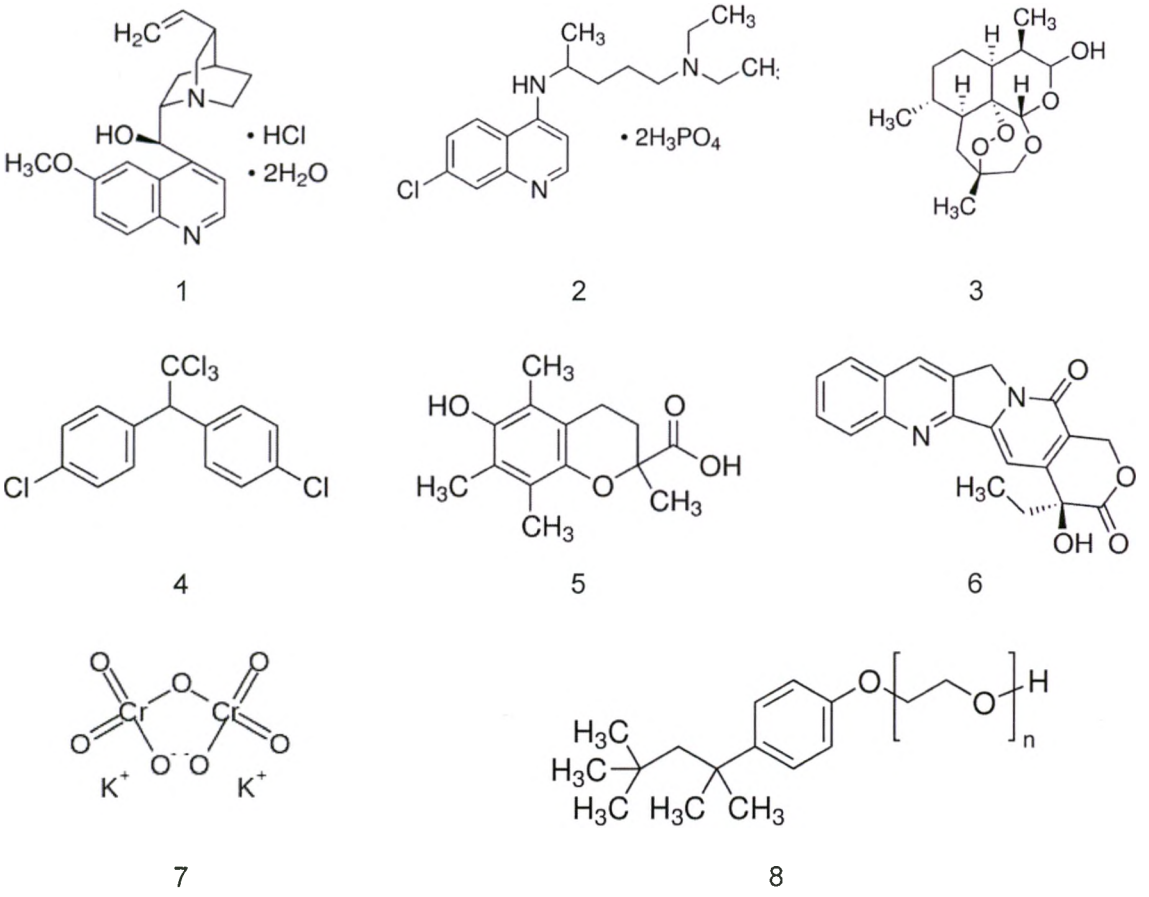

Figure 2.2: Structural formulas of the controls used in various assays, whereby the figure number corresponds to Table 2.3 (Sigma- Aldrich®, 2016).

2.2 Gas chromatography mass spectrometry analysis of the essential oils

The GC-MS analysis of the EOs of *A. afra* and *L. javanica* were done by Prof. S van Vuuren from the University of the Witwatersrand (2007) and that of *C. citratus*, *C. nardus* and *O. basilicum* by Dr. GPP Kamatou from the Tshwane University of Technology (2014) (Appendix A).

2.3 Malaria parasite maintenance

2.3.1 Culturing and maintenance of parasite culture

The chloroquine-sensitive strain of *P. falciparum* (NF-54) was obtained from the Department of Molecular Medicine and Haematology, University of the Witwatersrand, South Africa, and was maintained *in vitro* according to Freese *et al.* (1988) and Jensen and Trager (1977) methodology and departmental protocol (Van Zyl *et al.*, 2010). Biosafety ethics clearance (protocol number: 20090503; Appendix B) was obtained from the Biosafety Committee of the University of the Witwatersrand which allowed for the *in vitro* malaria parasite culturing and

experimentation and Human Ethics clearance (waiver certificate number: W-CJ-131030-1; Appendix C) for the utilisation of human RBCs and human plasma. In order to prevent contamination all procedures were conducted in Class II sterile biosafety units with 70% ethanol.

The parasites were maintained in 75 cm^2 tissue culture flasks (Corning®, Sigma-Aldrich®) at between 5-10% parasitaemia and a 5% haematocrit in RBCs. The parasites were examined daily by making a Giemsa-stained thin blood smear (Section 2.3.6) and viewed microscopically 1000x magnification under oil immersion. Complete culture media (Section 2.3.3) was replaced daily and if >10% parasitaemia (trophozoite and/or schizont stages) was present; approximately half the culture was discarded to allow for asynchrony of the culture, removal of excess metabolites and prevent parasite death. The culture was centrifuged at 400*g* for 5 minutes using the Sorvall® T-6000 centrifuge, culture was discarded, freshly washed RBCs (Section 2.3.5) was added and resuspended in 20 mL of complete culture media. The culture was then flushed with a gas mixture of 92% N_2, 5% CO_2 and 3% O_2 and re-incubated at 37°C (Jensen and Trager, 1977). Early to mid-ring stage parasite culture was synchronised (Section 2.3.7) to ensure the continuation of a synchronised culture for experiments and optimal growth.

2.3.2 Preparation of incomplete culture medium

The incomplete culture medium was prepared with 10.4 g Roswell Park Memorial Institution (RPMI)-1640 (Sigma-Aldrich®), 5.9 g (24.76 mM) N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (HEPES) buffer (Sigma-Aldrich®), 4 g (21.15 mM) *D*-glucose monohydrate (Merck), 44 mg (323.27 µM) hypoxanthine (Sigma-Aldrich®) and 50 mg (104.70 µM) gentamicin (Sigma-Aldrich®), dissolved in 1 litre autoclaved Milli-Q® (Millipore®) water. The incomplete culture medium was sterilised through a Sterivex™-GS 0.22 µm filter and stored at 4°C until used.

Hypoxanthine and gentamicin were omitted from the above preparation for incomplete experimental medium.

2.3.3 Preparation of complete culture medium

In order to prepare the complete culture medium for the *P. falciparum* NF-54 culturing, the incomplete culture medium (Section 2.3.2) was supplemented with 10% (v/v) lipid enriched bovine serum Albumax (Section 2.3.4) and 4.2 mL sodium bicarbonate ($NaHCO_3$). The 5% (w/v) $NaHCO_3$ was prepared in Milli-Q® water and sterilised through a 0.22 µm Sterivex™-GS filter unit and stored at 4°C. The complete experimental medium was prepared by the

addition of 10% (v/v) Albumax and 5% (w/v) (595.17 mM) $NaHCO_3$ to the incomplete experimental medium.

2.3.4 Preparation of the lipid rich bovine serum albumax

A stock concentration of 5% (w/v) Albumax was prepared with AlbuMAX™ II (Gibco®) (5 g/100 mL) in incomplete culture medium (Section 2.3.2) by being stirred on a hot plate at 37°C. It was sterilised through a 0.22 µm Sterivex™-GS filter unit and stored at -20°C until required.

2.3.5 Preparation of uninfected red blood cells

Human whole blood was obtained from healthy volunteers (clearance certificate number: M140669, Appendix D), ensuring that the volunteers did not consume aspirin, heparin, warfarin, antibacterial, antifungal, antimalarial or antiviral medication for at least 3 weeks before donating blood, and/or visited a malaria area one month prior to donating blood. Whole blood was collected in 6 mL blood tubes containing 1 mL of Acid Citrate Dextrose Solution B (ACD-B) anticoagulant (Lasec) and centrifuged at 400*g* for 5 minutes. The plasma and buffy coat was discarded and the remaining RBCs were washed thrice with equal volume of phosphate-buffered saline (PBS) (Sigma-Aldrich®), solution by centrifuging the resuspended RBCs at 400*g* for 5 minutes. The remaining pellet was resuspended in equal volumes of incomplete experimental medium (50% haematocrit) and stored at 4°C for no longer than a week.

The PBS (pH 7.4) was prepared with 136.89 mM NaCl (Merck), 4.1 mM di-sodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$; Riedel-de Haën®), 4.02 mM potassium chloride (KCl; Sigma-Aldrich®), and 1.47 mM potassium dihydrogen phosphate (KH_2PO_4; Fluka) in Milli-Q® water (pH 7.2), autoclaved (121°C at 1.2 kg/cm^2 for 20 minutes) and stored at 4°C.

2.3.6 Giemsa stain

Thin blood smears of the malaria culture were prepared daily on glass slides which were cleaned with ethanol and air dried. The smears were fixed with 100% methanol (Sigma-Aldrich®), air dried and then stained with 10% (v/v) Giemsa stain for 20 minutes. The Giemsa stain consisted of: Commercial Giemsa stain solution (Merck) diluted 5-fold with a Giemsa buffer. The latter was prepared by dissolving 25.72 M KH_2PO_4 and 40.49 mM sodium dihydrogen phosphate dodecahydrate ($Na_2HPO_4{\cdot}12H_2O$; Sigma-Aldrich®) in Milli-Q® water (pH 7.2). The buffer was autoclaved and stored at 4°C. The slides were stained for 20 minutes, rinsed with water and dried before viewing. An alternative and quick staining procedure composed of using three prepared solutions, Rapi-Diff Stain Set, was also used.

Solution 1 fixed the slide, solution 2 stained the RBCs and solution 3 stained the parasites. Five dips of 1 second each in sequential order was sufficient to optimally observe the parasites after washing the slide with water and then drying. The slide was microscopically examined at 1000x magnification under oil immersion. The stage, morphology of the parasite and the percentage parasitaemia (Equation 2.1) were determined after viewing and counting at least 10 fields of the thin blood smear before the appropriate action was taken for the optimal maintenance of *P. falciparum* culture (Section 2.3.1).

$$\% \text{ Parasitaemia} = \frac{\text{Total number of parasitized RBCs} \times 100}{\text{Total number of uninfected RBCs} + \text{Parasitised RBCs}}$$

Equation 2.1: The formula used to calculate the percentage parasitaemia from a stained thin blood smear.

2.3.7 Parasite culture synchronization

The parasite cultures were synchronised every alternate day to ensure the cultures were in the ring stage for experimental purposes (Lambros and Vanderberg, 1979). The 5% (w/v) D-sorbitol (Sigma-Aldrich®) lysed all the parasites except those in the ring stage. The parasite culture was centrifuged at 400*g* for 5 minutes, supernatant discarded and parasite pellet resuspended in 20 mL of 5% (w/v) D-sorbitol for 15-20 minutes at room temperature. D-sorbitol (274.47 mM) was prepared in Milli-Q® water, filtered with a 0.22 µm filter and stored at 4°C. The culture was centrifuged at 400*g* for 5 minutes and the supernatant was discarded. The remaining pellet was resuspended in complete culture media (Section 2.3.3), flushed with a gas mixture of 92% N_2, 5% CO_2 and 3% O_2 and re-incubated at 37°C in an optimal atmosphere.

2.4 Parasite lactate dehydrogenase assay

This assay was based on the detection of the enzyme, lactate dehydrogenase (LDH), which is found in the *P. falciparum* parasite. The method as described by Makler and Hinrichs (1993) detects *Plasmodium* parasitaemia as a measurement of pLDH. The parasites were sorbitolled to ensure a synchronised ring-stage culture was plated, to allow the development of a synchronized trophozoites stage to which the EO/EOCs and controls were added. The effect of the EO/EOCs and controls were determined after a 48 hour period during the trophozoites stage once again which contained the maximum level of pLDH enzyme level. LDH enzyme uses 3-acetyl-pyridine adenine dinucleotide (APAD) as a coenzyme in the formation of pyruvate from lactate. Parasite LDH in an infected lysed blood sample oxidised the lactate to pyruvate while reducing cofactor $APAD^+$ to APADH. The APADH then reduced

a yellow tetrazolium dye, nitroblue tetrazolium (NBT), to a blue diformazan compound with the assistance of phenazine ethosulfate (PES) (Figure 2.3).

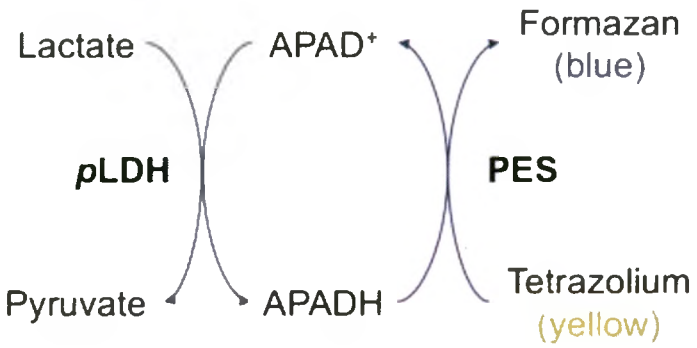

Figure 2.3: The Malstate assay for the detection of pLDH (Markwalter *et al.*, 2016).

2.4.1 Preparation of the uninfected red blood cells and parasites

The malaria parasite culture was pelleted by centrifugation at 400*g* for 5 minutes and washed three times with complete culture media (Section 2.3.3) before a thin blood smear was prepared to determine the percentage parasitaemia (Section 2.3.6). The experimental parasitaemia was adjusted to 2% with the addition of washed uninfected RBCs (Section 2.3.5) such that a final 2% haematocrit was prepared in complete experimental medium (Section 2.6.3).

2.4.2 Preparation of the EO/EOCs and control drugs

The EOCs and control drug, quinine hydrochloride dihydrate, were prepared taking into account the molecular weight and density of each compound (Section 2.1 and 2.2). A stock concentration of 500 µM of each EOC in dimethyl sulfoxide (DMSO) (Merck) was freshly prepared before each experiment. It has been a departmental standard that EOCs were screened at 50 µM, and since the EOCs made up 22 of the 27 compounds screened, the 50 µM concentration was kept standard. However; the EOs did not have molecular weights and densities, and therefore a 0.05% (v/v) concentration was used to screen. All further EO/EOC dilutions (1:10) were prepared in DMSO with a dilution factor of 112.5x taken into account when 1 µL EO/EOC with 11.5 µL of incomplete experimental medium was added to 100 µL 2% parasitized blood (Section 2.4.1). The control, 0.1 mM quinine hydrochloride dihydrate stock solution, was prepared in autoclaved Milli-Q® water and sterilised through a Sterivex™-GS 0.22 µm filter. Further dilutions (1:10) of quinine hydrochloride dihydrate were prepared in incomplete experimental medium taking the dilution factor of 9x into account where 12.5 µL quinine hydrochloride dihydrate was added to 100 µL 2% parasitized blood.

2.4.3 Preparation of reagents

A 54.48 mM Tris base (Sigma-Aldrich®) was prepared in autoclaved Milli-Q® water with a pH 9.1 and stored at 4°C until required. Malstat was prepared by dissolving 200 µL Triton X-100 (Sigma-Aldrich®), 183.30 mM calcium L-lactate (Sigma-Aldrich®), and 0.38 mM APAD (Sigma-Aldrich®) into 100 mL of the 54.48 mM Tris base.

A 0.299 mM PES (Sigma-Aldrich®) and 2.45 mM NBT (Sigma-Aldrich®) reagents were prepared in autoclaved Milli-Q® water. The solutions were filtered through a Sterivex™-GS 0.22 µm filter and stored in a dark container (to prevent light entering) at 4°C until required.

2.4.4 Preparation of microtitre plates

A 2% parasitaemia and 2% haematocrit of parasites in their early ring stage (100 µL) were plated into the wells, except the blank RBC control wells of B10 and B11 (Figure 2.4) of a 96-well sterile plate. To the blank controls, 100 µL of a 2% uninfected RBCs was added. The plates were incubated in an air-tight humidified glass desiccator with an optimal gaseous environment at 37°C for 24 hours. Thereafter the EO/EOCs and controls were plated in duplicate to wells B2 - G2 (Figure 2.4), except the control row, to which 12.5 µL incomplete experimental medium was added. As indicated in Figure 2.4, drug-free parasitised RBC and uninfected RBC controls were included. The plates were incubated for a further 48 hours under the same conditions (until the mid to late trophozoite stage), thereafter frozen in a -70°C freezer for 1 hour and then thawed out at 37°C. The plates were shaken at 1200 rpm for 2 minutes with an orbital shaker (MRC Thermo-Shaker™ MB100-4P) and 25 µL of lysate from each well was correspondingly transferred to a another sterile plate. Thereafter, 100 µL Malstat was added along with 20 µL (0.24 mM) NBT and (0.03 mM) PES in a 1:1 ratio. The plates were shaken at 1200 rpm for 30 seconds and incubated at 37°C for 40 minutes. A 5% (v/v) acetic acid (50 µL) was added to each well.

2.4.5 Data analysis

The plates were read at an absorbance wavelength of 620 nm using the UV-Vis spectrophotometer (Labsystems iEMS Reader MF) Ascent™ software version 2.6. The percentage parasite growth was determined using Equation 2.2. Log sigmoidal dose response curves were drawn using GraphPad Prism® (GraphPad Software, Inc, USA) version 5.00 and the half maximal inhibitory concentration (IC_{50}) values were determined.

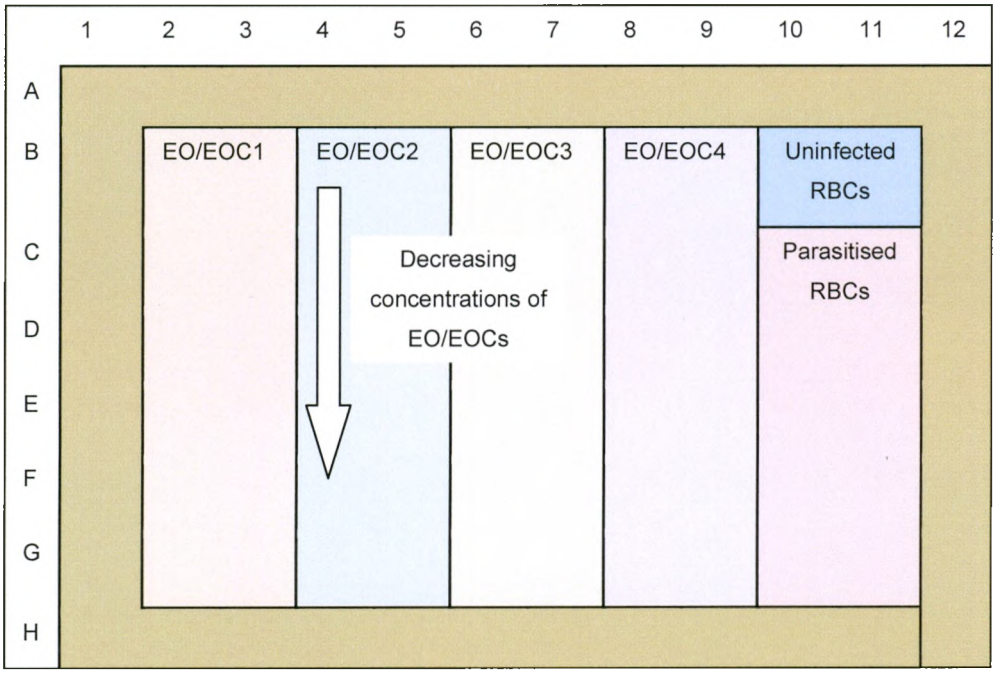

Figure 2.4: A schematic representation of the 96-well microtitre plate used in the pLDH assay with appropriate controls.

$$\% \text{ Parasite growth} = \frac{(\text{absorbance}_{test} - \text{average absorbance}_{RBC\ control}) \times 100}{(\text{average absorbance}_{parasite\ control} - \text{average absorbance}_{RBC\ blank})}$$

Equation 2.2: Percentage parasite growth taking into account the absorbances at 620 nm.

2.5 Toxicity studies

The assays, namely the haemolysis assay (Section 2.5.1), lipid peroxidation inhibition assay (Section 2.5.2), the 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyl-tetrazolium bromide (MTT) cell proliferation assay (Section 2.5.3) and the brine-shrimp lethality assay (BSLA) (Section 2.5.4), were employed in order to determine possible toxicity of the EO/EOCs. Performing the toxicity assays allowed for a comparison, to determine whether toxicity results were consistent over all the assays.

2.5.1 Haemolysis assay

The haemolysis assay was performed according to Hayat *et al.* (2011) which determined the extent of RBC lysis by release of the oxygen-carrying protein, haemoglobin, in the plasma as a result of exposure to the EO/EOCs and control compounds. Haemolysis occurred due to the RBCs destruction which resulted from lysis of membrane lipid bilayer. Many plants

contain chemical substances that might have a haemolytic or anti-haemolytic effect on human erythrocytes. Several reports indicate that the membranes of human erythrocytes from blood types have varying stability as determined from the mean corpuscular fragility (Manthey *et al.,* 2001). EO/EOCs may have serious adverse effects, which include induction of haemolytic anaemia (Freitas *et al.*, 2008).

2.5.1.1 Preparation of uninfected red blood cells

The uninfected RBCs were prepared (Section 2.3.5) by being washed thrice with PBS solution (Section 2.3.5) and then suspended at a 1% (v/v) haematocrit in complete experimental media (Section 2.3.3).

2.5.1.2 Preparation of EO/EOCs and control drugs

The EOCs and control drugs (chloroquine and quinine) were prepared in DMSO taking into account the molecular weight and density of each compound (Section 2.1 and 2.2). A 50 µM concentration of the EOCs and control drug, quinine hydrochloride dihydrate, were prepared taking into account the molecular weight and density of each compound (Section 2.2). As the EOs did not have molecular weights and densities; a 0.05% (v/v) concentration was used to screen. Further 1:10 dilutions were prepared in DMSO with a dilution factor of 10x taken into account.

2.5.1.3 Preparation of microtitre plates

A volume of 25 µL of the EO/EOCs were plated out in triplicate in a sterile 96-well plate except the control, to which 25 µL of 2% (v/v) Triton X-100 (Sigma-Aldrich®) was added to the 100 % lysis positive control and 25 µL of complete culture media was added to the 0 % lysis negative control. A volume of 200 µL of 1% haematocrit was added to all wells. The plates were incubated in an oxygen free environment at 37°C for 48 hours. After incubation, the plates were gently shaken with an orbital shaker (MRC Thermo-Shaker™ MB100-4P) at 1200 rpm for 2 minutes and then centrifuged at 400*g* for 10 minutes. A volume of 50 µL supernatant was transferred from each well to its corresponding well in another sterile plate to which 150 µL Milli-Q® water was added.

2.5.1.4 Data analysis

The plates were read at an absorbance wavelength of 414 and 540 nm using the UV-Vis spectrophotometer (Labsystems iEMS Reader MF) Ascent™ software version 2.6. If the EO/EOCs or control compounds displayed more than 60% haemolytic activity, IC_{50} values were determined from log sigmoidal dose response curves generated by using GraphPad

Prism® (GraphPad Software, Inc, USA) version 5.00. The percentage haemolysis was calculated with the appropriate controls taken into account according to Equation 2.3.

$$\% \text{ Haemolysis} = \frac{(\text{absorbance}_{test} - \text{average absorbance}_{0\% \text{ lysis}}) \times 100}{(\text{average absorbance}_{100\% \text{ lysis}} - 0\% \text{ lysis})}$$

Equation 2.3: Percentage haemolysis

2.5.2 Lipid peroxidation inhibition assay

Lipid peroxidation is the process by which carbon-carbon double bond(s) are attacked by oxidants. The method was carried out according to Westerlund (1996), to assess the ability of EO/EOCs to prevent the oxidative degeneration of lipids which result in cell membrane damage.

2.5.2.1 Preparation of ammonium thiocynate and ferrous chloride

A 3.94 M stock of ammonium thiocynate (Merck) was prepared in Milli-Q® water. The 20 mM stock of ferrous chloride (Fluka) was prepared in 3.5% (v/v) HCl (Sigma-Aldrich®). A working solution of 5 mM was prepared fresh for each experiment.

2.5.2.2 Preparation of linoleic acid emulsion

Linoleic acid (Fluka) (51.2 µL) and Tween-20 (Sigma-Aldrich®) (51.2 µL) were mixed and the volume made up to 10 mL with PBS solution (Section 2.3.5). This emulsion was prepared fresh for each experiment.

2.5.2.3 Preparation of the EO/EOCs and control drugs

The EOCs and controls (TROLOX™, chloroquine diphosphate, quinine hydrochloride dihydrate and dihydroartemisinin) were prepared in DMSO taking into account their molecular weight, density and weight (Section 2.1 and 2.2). A dilution factor of 10x was taken into account, a stock concentration of 500 µM was prepared for the EOCs and controls and further 1:10 dilutions were prepared in DMSO. As the compounds were normally screened at 50 µM in the department, and 22 out of 27 compounds were EOCs, the screening concentration for the EOCs was such. However, the EOs did not have molecular weights and densities, and thus a 0.05% (v/v) concentration was used as a screening concentration for the EOs.

2.5.2.4 Preparation of microtitre plates

The EO/EOCs (25 µL) were plated in triplicate in a 96-well sterile plate, except in the control wells, to which 25 µL DMSO was added. This was reacted with 125 µL linoleic acid emulsion (Section 2.5.2.2) and 100 µL PBS solution (Section 2.3.5) except in the blank control well to which 125 µL PBS solution was added. The mixture was shaken using the Thermo-Shaker™ for 1 minute at 1000 rpm and incubated in a humidified environment for 24 hours at 37°C. The plates were then shaken for 1 minute at 1200 rpm and 25 µL of the reaction solution was reacted with 150 µL of 70% (v/v) ethanol, 40 µL of 3.94 M ammonium thiocynate and 40 µL of 5 mM ferrous chloride.

2.5.2.5 Data analysis

The absorbance was read at 492 nm using the UV-Vis spectrophotometer (Labsystems iEMS Reader MF) Ascent® software version 2.6. If the EO/EOCs and/or controls showed more than 60% lipid peroxidation inhibition activity, IC_{50} values were determined by using log sigmoidal dose response curves using GraphPad Prism® version 5.00. The percentage inhibition of lipid peroxidation was calculated using Equation 2.4.

$$\% \text{ Inhibition} = \frac{(A_0 - A_1) \times 100}{(A_0)}$$

Equation 2.4: Percentage lipid peroxidation inhibition, whereby; A_0 = absorbance of control; A_1 = absorbance of test compound or standard

2.5.3 MTT cytotoxicity assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was conducted to determine cellular viability on a transformed human line cell, human kidney epithelial (Graham, HEK-293), according to the method described by Mosmann (1983) and Van Zyl *et al.* (2010) to investigate the cytotoxicity of the EO/EOCs and controls. The assay involved the cleavage of the MTT by succinate dehydrogenase found in the mitochondria of living cells. The yellow water soluble dye changed to a purple water-insoluble formazan crystal in viable cells due to the tetrazolium ring being cleaved. This cellular reduction involved the pyridine nucleotide co-factors NADH and NADPH (Figure 2.5) (Riss *et al.*, 2015). A clearance certificate (reference number: W-CJ-161129-2) was obtained from the Human Research Ethics Committee (Medical) of the University of the Witwatersrand (Appendix E), permitting the use of commercial human kidney epithelial (Graham, HEK-293) cell line for *in vitro* experimental purposes.

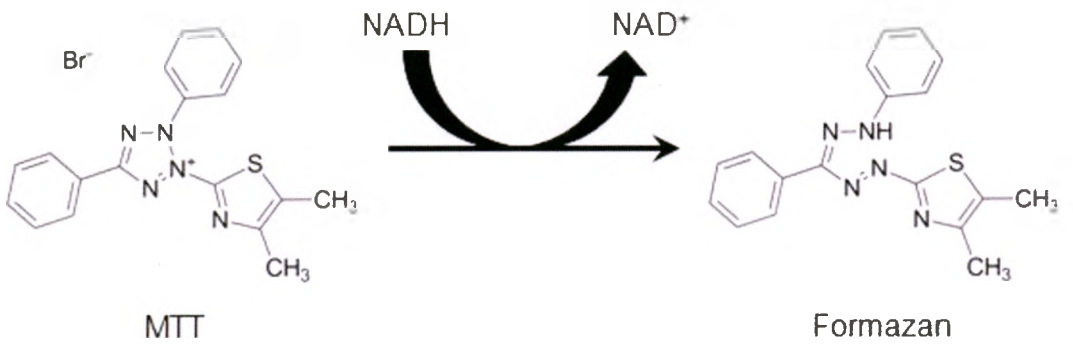

Figure 2.5: The metabolization of MTT to a formazan salt by viable cells (Riss *et al.*, 2015).

2.5.3.1 Maintenance of cell culture

The cells (transformed human kidney epithelial, Graham) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich®) culture medium (Section 2.5.3.3), which was replaced twice weekly, in a 37°C humidified environment with 5% CO_2 (Afrox incubator, Forma Scientific Water-Jacketed Incubator). The cells were trypsinized weekly to subculture and thrice weekly maintained with complete culture media (Section 2.5.3.3). Trypsinization was performed to optimise growing conditions by adjusting the number of adherent proliferating cells in the flask. The cell growth/ confluency was determined microscopically at 40x magnification. When the cells were 90% confluent, the medium was discarded and the cells were rinsed with 10 mL PBS solution (Section 2.3.5); followed by incubating the cells with 3 mL of 0.25% (v/v) trypsin-0.1% Verseneethylenediaminetetraacetic acid (Versene-EDTA; Sigma-Aldrich®) at room temperature for 3 minutes allowing the cells to detach from the surface of the flask. Thereafter, 3 mL complete culture media was added to ensure that the foetal bovine serum (FBS) (Section 2.5.3.4) inactivated the trypsin. To subculture, 1 mL of the cells was returned to the flask with 19 mL complete culture media before being incubate at 37°C in 5% CO_2 for the cells to re-adhere an grow. The remaining cells were discarded.

For the MTT assay, the cells were trypsinized as above, and then 7 mL complete culture media was added to 3 mL of cells and resuspended. To determine the cell density, 50 µL resuspended cells was stained with 50 µL of 0.2% (w/v) trypan blue solution (Sigma-Aldrich®) in PBS solution, of which 15 µL was placed on the haemocytometer. The number of viable

clear/ transparent cells were counted using a microscope at 100x magnification with the requirement of 10 000 cells per well for a 48 hour experiment incubation period (Equation 2.5). To determine the percentage cell viability of the assay, total cells were counted. The dead or unhealthy cells were stained blue, as the stain traversed the compromised cell membrane. Viable cells were not stained blue, as the healthy membrane integrity prevented the uptake of trypan blue. A minimum of 95% cell viability was required for the assay.

Cells/mL = (number of viable cells/ 4) X dilution factor (2x)

Equation 2.5: Determination of the concentration of cells when counted on the haemocytometer at a 100x magnification.

2.5.3.2 Preparation of incomplete culture medium

The incomplete culture medium was prepared with 13.5 g of DMEM supplemented with 3.7 g (44.04 mM) $NaHCO_3$ (Merck) prepared in one litre Milli-Q® water and sterilised through a 0.22 µm Sterivex™-GS filter and stored at 4°C until required.

2.5.3.3 Preparation of complete culture medium

The complete culture medium was prepared with 10% (v/v) FBS, 1 mL of 200 mM L-glutamine (Sigma-Aldrich®), 1ml penicillin-streptomycin solution (containing 10 000 units penicillin and 10 mg streptomycin per mL; Sigma-Aldrich®) and 1 mL of 10 mM sodium pyruvate (Sigma-Aldrich®) in 100 mL DMEM culture medium.

The complete experimental medium consisted of 10% (v/v) FBS in 100 mL DMEM culture medium (Section 2.5.3.2), with the L-glutamine and sodium pyruvate, ensuring no antibiotics were added. The culture medium was filtered through *a* 0.22 µm Sterivex™-GS filter unit and stored at 4°C.

2.5.3.4 Preparation of foetal bovine serum

The FBS was thawed at 4°C, brought to room temperature and then heat activated at 56°C for 45 minutes before being aliquoted out and stored at -20°C until required.

2.5.3.5 Preparation of MTT

A stock solution of 0.05 mM MTT (USB) was prepared using PBS solution (Section 2.3.5) and sterile filtered using a 0.22 µm Sterivex™-GS filter unit and stored at 4°C in a dark environment.

2.5.3.6 Preparation of the EO/EOCs and control drugs

A screening concentration of 50 µM of the EOCs were prepared, taking into account the molecular weight and density of each compound, in DMSO; where a dilution factor of 200x was taken into account. As the EOs did not have molecular weights and densities; a 0.05% (v/v) concentration was prepared to screen. The controls, 50 µM camptothecin and quinine were prepared in DMSO with a dilution factor of 10x taken into account. If the EO/EOCs displayed less than 60% viability, further 1:10 dilutions were prepared with DMSO, to determine the IC_{50} values.

2.5.3.7 Preparation of microtitre plates

The required volume of cells (10 000 cells/ 180 µL complete culture medium for a 48 hour experiment) were suspended in complete culture medium (Section 2.5.3.3), with 180 µL of cells added to each well (except for the cell-free control wells) and incubated for 24 hours at 37°C in a humidified 5% CO_2 environment. To the cell-free control wells, 180 µL complete culture media was added. After the 24 hour incubation (37°C under humidified conditions in 5% CO_2), 2 µL of the EO/EOCs plus 18 µL incomplete experimental DMEM medium was added to the 180 µL cells (except for control wells) and 20 µL complete culture media to the control wells before being further incubated for 48 hours. After the incubation, 20 µL of 12.01 µM MTT (sterilised through a 0.22 µm Sterivex™-GS filter unit and stored in the dark place at 4°C until required) was added to the wells and incubated for 4 hours at 37°C in 5% CO_2 under humidified conditions. The plates were centrifuged at 400*g* for 5 minutes and 180 µL supernatant was discarded. A 100 µL of DMSO was added to the wells to stop the reaction and dissolve the purple formazan crystals, the plate was shaken with an orbital shaker (MRC Thermo-Shaker™ MB100-4P) 1200 rpm for 2 minutes and was left to stand for 10 minutes in a 25°C dark environment to ensure the complete dissolution of the crystals.

2.5.3.8 Data analysis

The absorbances of the plates were read at a test wavelength of 540 nm (purple formazan colour) and reference wavelength of 690 nm (unreacted MTT colour) using the UV-Vis spectrophotometer (Labsystems iEMS Reader MF) Ascent™ software version 2.6. The data was analysed with Equation 2.6 to determine the percentage cell viability. The IC_{50} values were calculated from log sigmoid dose response curved generated by the computer software GraphPad Prism® (GraphPad Software, Inc., USA) for the EO/EOCs and/or controls that presented with less than 60% cell viability at the screening concentrations. The results were reported as mean ± s.d. of three or more experiments.

$$\% \text{ Cell viability} = \frac{[\text{absorbance of drug (540 nm)} - \text{absorbance of drug (690 nm)}] \times 100}{\text{mean absorbance of drug free control (540 nm)} - \text{mean absorbance of drug free control (690 nm)}}$$

Equation 2.6: Percentage cell viability taking both the test and the reference wavelengths into account.

2.5.4 Brine shrimp lethality assay

Toxicological properties of the EO/EOCs were determined using the BSLA. The BSLA is a toxicity test of bioactive chemicals based on the killing ability of test compounds on a simple zoological organism-brine shrimp. This BSLA was first proposed by Michael *et al.* (1956) and further developed by several groups (Van Walbeek *et al.*, 1971; Vanhaecke *et al.*, 1981). The BSLA is widely used in the evaluation of toxicity of heavy metals, pesticides, medicines especially natural plant extracts The method described by Ruebhart *et al.* (2009) was undertaken using *Artemia franciscana* nauplii (brine shrimp) (Ocean Nutrition™).

2.5.4.1 Hatching of eggs

Artemia franciscana nauplii eggs (0.5 g) were placed in a plastic container containing 500 mL sea water (38 g/L Tropic Marine® sea salt in deionised water). A rotary pump piping was placed into the water to allow for aeration and dispersion of eggs. The container was exposed to bright light (2000 Lux), in a 25°C environment for 18 hours until the eggs hatched.

2.5.4.2 Preparation of the EO/EOCs and control drugs

An initial concentration of 0.01% (v/v) EO/EOCs were made up in DMSO. The 0.01% (v/v) concentration was used as comparison to the larvicidal activity (Section 2.6), as that was the concentration mentioned in the WHO (2005) protocol. However, the percentage concentrations for the EOCs were also converted to molarity concentration for a unanimous discussion of the EOCs (Equation 2.7).

$$\text{Molarity (M)} = \frac{\text{concentration (\%)} \times \text{density}}{100\% \times \text{molecular weight}}$$

Equation 2.7: Conversion of percentage concentration to molarity.

If the EO/EOCs displayed more than 60% mortality (Equation 2.8), further 1:2 dilutions were prepared in DMSO to determine the LC_{50} values. A dilution factor of 100x was taken into

account. Potassium phosphate was used as a positive control. The synthetic salt water (Section 2.5.4.1) was added to the wells as a negative control.

$$\text{Mortality (\%)} = [(X - Y) / Y] \times 100$$

Equation 2.8: Abbot's formula, where X = percentage survival in untreated control; Y =percentage survival in the treated sample (WHO, 2005).

2.5.4.3 Preparation of microtitre plates

In each well of a 48 well microtitre plate, 197.5 µL salt water, 50 µL *Artemia* nauplii (containing ± 30 nauplii) and 2.5 µL EO/EOCs or controls was added in triplicate. The plates were incubated at 25 ± 1°C for 24 hours.

2.5.4.4 Data analysis

The number of dead *Artemia* nauplii were counted after 24 hours of treatment, thereafter, all the nauplii were sacrificed with the addition of 250 µL of 50% (v/v) acetic acid to count the total number of nauplii per well. This allowed the comparison of the percentage nauplii killed by the EO/EOC to the 100% kill of potassium dichromate. If the EO/EOCs at 0.01% (v/v) concentration caused more than 60 % nauplii mortality, nauplii were treated in a dose dependent manner to elucidate the concentration of the EO/EOCs that would cause 50% mortality (LC_{50} value). The % mortality and LC_{50} were determined from the counts using the Probit analysis method (IBM SPSS Statistictical version 22.0 Software Package, IBM Corp.) as described by Finney (1971).

2.6 Larvicidal assay

The larvicidal assay was conducted according to Mavundza *et al.* (2013) and the WHO (2005) protocol in order to assess the larvicidal activity of the EO/EOCs on an *Anopheles arabiensis* (*Kanyemba Gambiae* Species B (KGB)) mosquito strain. The insecticide-susceptible larvae were originally colonised from Kanyemba, Zimbabwe in the 1970's and thus obtained from the National Institute for Communicable Diseases (NICD), Sandringham, Johannesburg, South Africa. A clearance certificate (W-CJ-150911-1) was obtained from the Human Ethics Committee (Medical) of the University of the Witwatersrand (Appendix F), permitting the use of mosquitoes and mosquito parasites for experimental purposes.

2.6.1 Preparation of EO/EOC solutions

The EO/EOCs were made up to a 0.01% (v/v) concentration in acetone (Sigma-Aldrich®) taking the 100x dilution factor into account as mentioned in the WHO (Guidelines for lab and field testing of mosquito larvicides) protocol (2005). The larvae were initially exposed to this screening concentration to assess if the EO/EOCs had larvicidal activity. If the EO/EOCs displayed more than 60% mortality, serial dilutions (1:2) were prepared. The Equation 2.7 was also used to represent the concentration in molarity for the EOCs to standardised results.

2.6.2 Standard larval food

Standard larval food was obtained from the NICD which was prepared according to Hunt *et al.* (2005), whereby 500 g dog biscuits (West's Beeno brand only) and 100 g Brewer's yeast were pulverised to a fine powder consistency.

2.6.3 Larval toxicity bioassay

Deionised water (25 mL) was placed into plastic cups where the water depth was greater than 1 cm. Batches of twenty 3rd or 4th instar larvae were placed into the cups along with 250 µL EO/EOC or control. Four or more replicates were set up with controls, where the organochloride, dichlorodiphenyltrichloroethane (DDT) (Sigma-Aldrich®), was used as the positive 100% mortality control, and acetone and deionised water were the negative controls. Standard larval food was added to each cup to allow for an even spread over the surface of the water. The assay was kept in an environment at 25-29°C for 24 hours, after which larval mortality was recorded.

The larvicidal assay was conducted by evaluating each EO/EOC and/or combination of EOCs against at least 100 larvae, and thus each test was run five times on different days. After 24 hours of treatment, the number of dead larvae was counted and the percentage mortality calculated. Larvae were considered dead if they did not move when nudged with a toothpick in the siphon or cervical region. Moribund larvae were counted and added to dead larvae for calculating the percentage mortality. The average larval mortality was corrected for control mortality using Abbot's formula (Abbott, 1925) (Equation 2.8).

The EO/EOCs that displayed more than 60% mortality at a 0.01% (v/v) concentration were considered significant for further investigation. Thus, with regard to the EO/EOCs that exhibited notable mortality, the larvae were treated in a dose dependent manner to elucidate the LC_{50} values.

2.6.4 Combined effect of major essential oil constituents of the crude essential oils

The major EOCs of each active EO, namely; *C. citratus, C. nardus. O. basilicum* and *L. javanica* were combined per oil as reconstituted 'crude' oils in order to replicate the original crude EO. To determine whether these previously tested major EOCs (Section 2.6.1) on their own would elucidate the same larvicidal effects as the original EO, the EOCs were added in proportion according to their percentage composition of the original EO (Appendix A). This was determined based on the GC-MS analysis data performed by Dr. Kamatou (2014) and Prof. van Vuuren (2007) (Appendix A). In order to attain the correct reconstituted 'crude' EO percentage, the remaining volume was made up with acetone. The combined EOCs were incubated with the *An. arabiensis* larvae as per Section 2.6.3. The mortality (%) was obtained using Equation 2.8. The percentage mortality of the reconstituted 'crude' EO and original EO were then compared.

2.6.5 Combination study

A combined study of the two most active EOCs was carried out using the larvicidal method as described in Section 2.6.3, with modifications to accommodate the addition of two EOCs together. The two EOCs were added in proportion (Table 2.4.) to obtain the required concentrations from which five 1:2 serial dilutions were prepared taking the 100x dilution factor into account.

Table 2.4: The concentrations of the five combination ratios used to treat *An. Arabiensis* larvae.

Combination 1		Combination 2		Combination 3		Combination 4		Combination 5	
X	Y	X	Y	X	Y	X	Y	X	Y
0.0%	0.1%	0.025%	0.075%	0.05%	0.05%	0.075%	0.025%	0.1%	0.0%

In order to establish the combination effect, the LC_{50} values of the EOC combinations were determined to calculate the relative ratios (Equation 2.9) from which an isobologram was constructed (Figure 2.6).

$$(X{:}Y) = \frac{LC_{50} \text{ of compound X in combination}}{LC_{50} \text{ of compound X alone}} : \frac{LC_{50} \text{ of compound Y in combination}}{LC_{50} \text{ of compound Y alone}}$$

Equation 2.9: Relative ratios of compounds X: Y. The ratios were calculated using the LC_{50} value of combined ratio of compounds X and Y over the individual LC_{50} values of compounds X and Y, respectively.

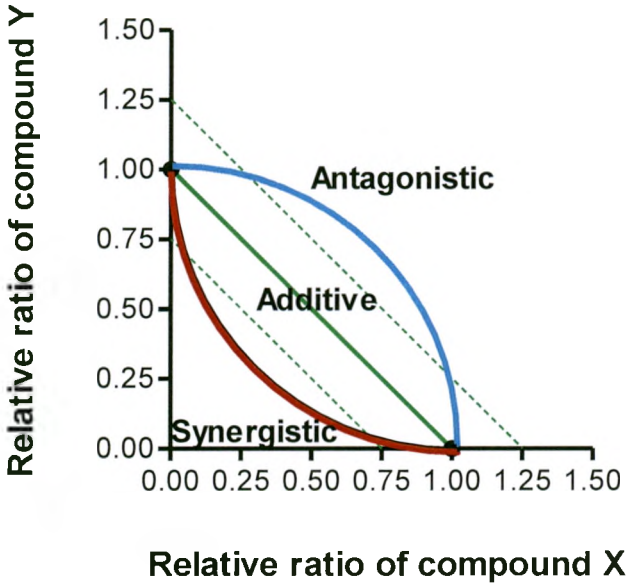

Figure 2.6: Three possible pharmacological effects between compound X and compound Y depicted using an isobologram (Berenbaum, 1978).

The EOCs were combined in order to determine one of three possible effects (Figure 2.6). An additive effect, when the resulting effect was the same as the sum of effects that each EOC produced separately. Synergistic effect, when the resulting effect was greater than the sum of the separate EOC's effects. Lastly, antagonistic effects, when the resulting effect was lower than the sum of the separate EOC's effects (Berenbaum, 1978).

To determine the strength of the interaction read from the isobologram, the sum of the fractional inhibitory concentration (ΣFIC) reflected the combined interactions of compound X and Y of the combinations according to Equation 2.10.

$$\Sigma FIC = \frac{LC_{50} \text{ of compound X in combination}}{LC_{50} \text{ of compound X alone}} + \frac{LC_{50} \text{ of compound Y in combination}}{LC_{50} \text{ of compound Y alone}}$$

Equation 2.10: The ΣFIC value; calculated using the LC_{50} values of the combined and individual LC_{50} values of compound X and Y (Berenbaum, 1978), where X and Y were the two EOCs used in combination.

In this study, the calculation and interpretation of the ΣFIC was interpreted as follows: the ΣFIC values <0.75 was synergistic, 0.75 - 1.25 was additive and >1.25 was antagonistic (Van Zyl *et al.*, 2010).

2.7 Safety index

The selective activity of the compounds against the *P. falciparum* parasites compared to their cytotoxicity; and the *An. arabiensis* larvae against the *Artemia* nauplii toxicity; was determined by calculating the safety index (SI; Equation 2.11). All compounds with S.I. values of >10 were normally regarded as non-toxic (Pink *et al.*, 2005; Bhat *et al.*, 2008). As the SI for quinine was observed as >1000 (cytotoxic SI for quinine was 3358.76 as determined by Chen (2015), an internal calibration of a SI >100 has been used as non-toxic.

$$\text{(a) Safety index} = \frac{\text{Cytotoxicity } (IC_{50})}{\text{Antimalarial activity } (IC_{50})}$$

$$\text{(b) Safety index} = \frac{\text{Nauplii toxicity } (LC_{50})}{\text{Larvicidal activity } (LC_{50})}$$

Equation 2.11: Safety index for *P. falciparum* compared to human kidney epithelial (HEK-293) cells (a); and the safety index for *An. arabiensis* larvae compared to *A. franciscana* nauplii (b).

2.8 Statistical analysis

The absorbances of the plates in the pLDH, haemolysis, lipid peroxidation inhibition, and MTT assays were read at their respective wavelengths using an ultraviolet-visible (UV-Vis) spectrophotometer (Labsystems iEMS Reader MF) Ascent™ software version 2.6. For the assays which required IC_{50} value determination (pLDH, lipid peroxidation inhibition, and MTT assay), the values were calculated from log sigmoid dose response curved generated by GraphPad Prism® (GraphPad Software, Inc., USA). For the assay which required LC_{50} values (BSLA and larvicidal assay), Probit analysis were conducted using the IBM SPSS Statistical 22 Software Package (IBM Corp., USA). The Mann-Whitney U-test was performed to determine the statistical differences/similarities between the EO/EOCs and the positive controls, where a *p*-value of ≤0.05 was considered significant. The results were reported as mean ± s.d. of three or more experiments, except the larvicidal assay which required five or more experiments.

CHAPTER THREE – RESULTS

3.1 Antimalarial assay

The EOs and EOCs had varying ability to inhibit the intra-erythrocytic parasite, as the thirteen most active EOs and EOCs resulted in parasite viability within a range of 0.10 and 47.00% at their respective screening concentrations (Section 2.4.2), compared to quinine (5.98%) at a 50 µM concentration (Table 3.1). Three EOCs and one EO displayed promising activity with parasite viability lower than 10% in addition to no corresponding haemolysis on the uninfected RBCs (Table 3.1).

Table 3.1: Percentage parasite viability and haemolysis. After treatment with 0.05% (v/v) EOs, 50 µM EOCs and controls (50 µM quinine and 0.2% (v/v) Triton X-100™)(n= 3).

Compound	Parasite viability (%) ± s.d.	Haemolysis (%) ± s.d.
cis-Nerolidol	0.10 ± 0.01	0.10 ± 0.01
trans-Nerolidol	13.27 ± 1.58	0.58 ± 1.29
trans-Geraniol	88.39 ± 5.36	0.10 ± 0.01
cis- Geraniol	8.12 ± 1.00	0.10 ± 0.01
Citral	21.52 ± 4.09	3.10 ± 0.79
Geranyl acetate	100.00 ± 0.01	11.00 ± 1.27
(±)-Linalool	47.00 ± 4.58	4.13 ± 0.47
(-)-Linalool	97.71 ± 16.39	5.18 ± 1.93
Eugenol	38.59 ± 16..04	0.10 ± 0.01
p-Cymene	100.00 ± 0.1	0.10 ± 0.01
Eucalyptol	0.10 ± 0.01	2.97 ± 1.22
(+)-α-Pinene	90.38 ± 9.83	0.10 ± 0.01
(-)-α-Pinene	100.00 ± 0.01	4.11 ± 0.24
(+)-β-Pinene	87.28 ± 22.68	0.10 ± 0.01
(-)-β-Pinene	100.00 ± 0.01	3.22 ± 0.01
(-)-α-Thujone	99.33 ± 22.76	0.58 ± 0.01
α-β-Thujone	84.42 ± 6.47	0.10 ± 0.01
Artemisia ketone	79.02 ± 3.85	0.10 ± 0.01
Ocimene	72.35 ± 7.88	0.10 ± 0.01
(R)-(+)-Citronellal	81.60 ± 21.34	0.10 ± 0.01
(S)-(-)-Citronellal	14.38 ± 2.25	0.10 ± 0.01
(-)-*trans*-Caryophyllene	69.98 ± 6.61	0.10 ± 0.01
C. citratus	0.10 ± 0.01	0.10 ± 0.01
C. nardus	25.43 ± 4.88	0.10 ± 0.01
O. basilicum	43.85 ± 10.03	0.10 ± 0.01
A. afra	28.15 ± 5.54	0.10 ± 0.01
L. javanica	12.97 ± 1.68	0.10 ± 0.01
Quinine	5.98 ± 1.29	0.10 ± 0.01
Triton X-100™	n.d.	100 ± 0.01

EOC with antimalarial/ haemolytic activity

EO with antimalarial/ haemolytic activity

Positive control

n.d: not determined

☐ No notable activity

All five EOs namely; *C. citratus* (IC_{50} value: 2 $x10^{-5}$%), *C. nardus* (IC_{50} value: 5.12 $x10^{-4}$%), *O. basilicum* (IC_{50} value: 2.99 $x10^{-3}$%), *A. afra* (IC_{50} value: 4.12 $x10^{-4}$%) and *L. javanica* (IC_{50} value: 5.69 $x10^{-4}$%); as well as six EOCs inhibited the growth of the intra-erythrocytic *P. falciparum* parasite in a dose dependant manner (Figures 3.2 and 3.3; Table 3.2). Figure 3.1 displayed the dose dependant activity of an active EOC, citral, in comparison to quinine. Eugenol and (±)-linalool displayed the least EOC inhibitory effect with IC_{50} values of 34.62 µM (5.41$x10^{-4}$%) and 76.81 µM (1.36 $x10^{-3}$%), respectively (Figure 3.2). The EO, *C. citratus* (IC_{50} value: 2 $x10^{-5}$%) displayed the most potent antimalarial activity in comparison to the control, quinine (IC_{50} value: 1.71 $x10^{-5}$%; 0.18 ± 0.05 µM) (Figure 3.3). The antimalarial activity of the active EOs and EOCs were significantly different to the control, quinine. Such as, *C. citratus* (*p*-value: 0.0008), eucalyptol (*p*-value: 0.0002), as well as the other active EO/EOCs (*p*-values: <0.0001).

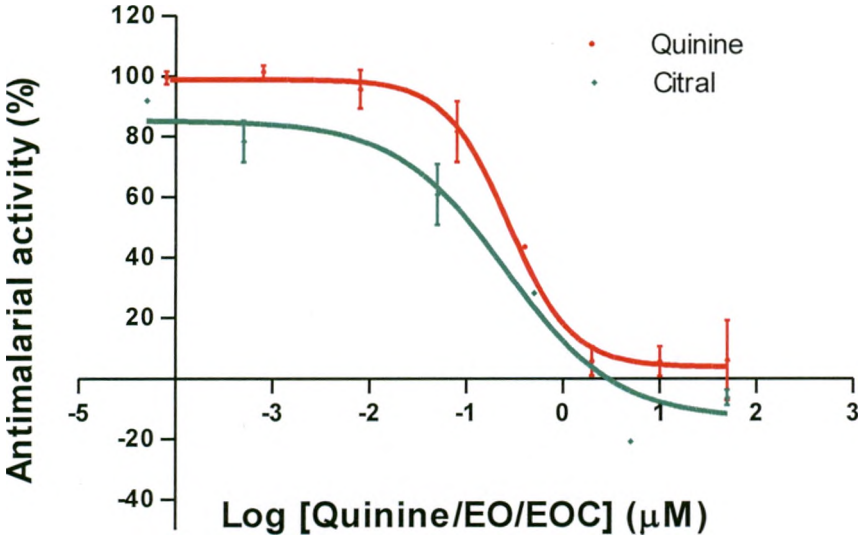

Figure 3.1: The dose dependant effect of quinine and citral on the *in vitro* growth of *P. falciparum*.

Table 3.2: The LC_{50} values of the EOCs and control presented in % concentration, corresponding to the micro molar (µM) concentration in Figure 3.2 (n = 3).

Compound	LC_{50} value (%)	Compound	LC_{50} value (%)
cis-Nerolidol	5.06 $x10^{-5}$	Eugenol	5.41$x10^{-4}$
trans-Nerolidol	1.55 $x10^{-4}$	Eucalyptol	6.19 $x10^{-6}$
cis-Geraniol	1.01 $x10^{-4}$	(S)-(-)-Citronellal	1.91 $x10^{-4}$
Citral	8.59 $x10^{-5}$	Quinine	1.70 $x10^{-5}$
(±)-Linalool	1.36 $x10^{-3}$		

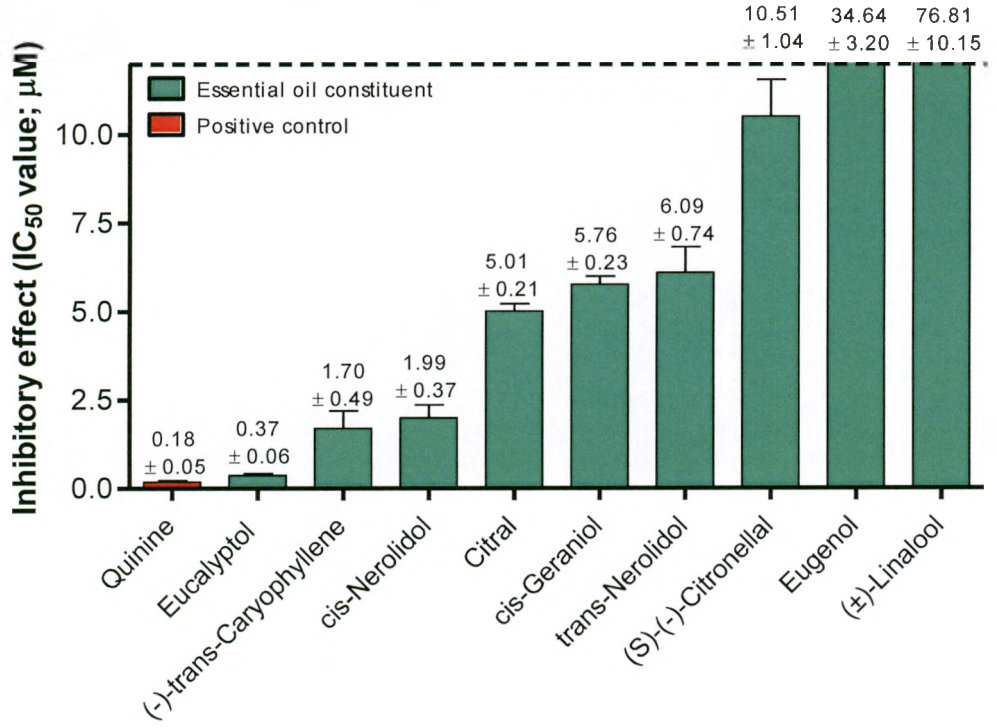

Figure 3.2: The antimalarial IC_{50} values of the EOCs on the *P. falciparum* parasite in comparison to the antimalarial agent, quinine.

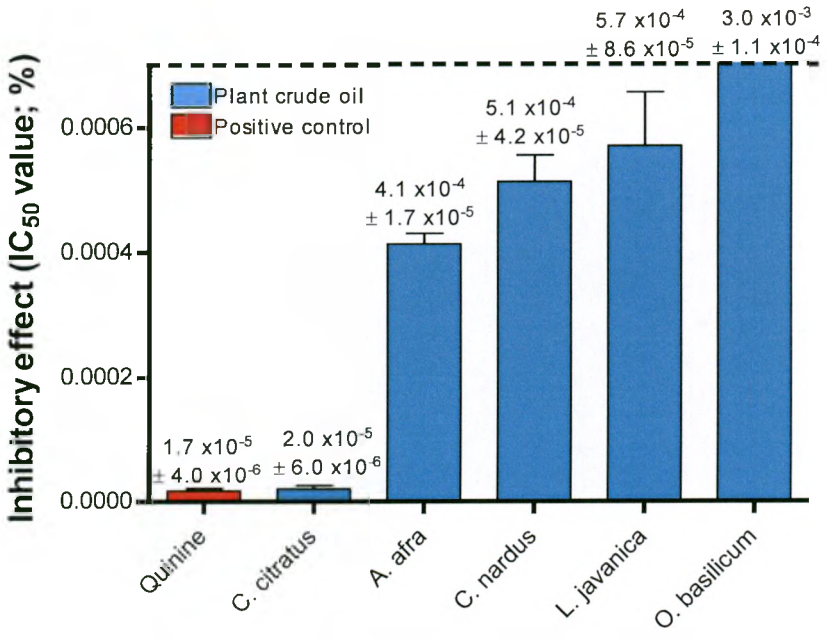

Figure 3.3: The antimalarial IC_{50} values of the EOs on the *P. falciparum* parasite in comparison to the antimalarial agent, quinine.

3.2 Haemolysis assay

None of the EOs (0.05%) and EOCs (50 µM) displayed significant haemolytic activity ranging from 0.10 to 11.00%. Quinine and chloroquine both induced 0.10 ± 0.01% haemolysis at 50 µM compared to the control, 3.31 nM (0.2%, v/v) Triton X-100™, which resulted in 100% haemolysis (Table 3.1).

3.3 Larval toxicity bioassay

3.3.1 Larvicidal toxicity bioassay of essential oils and constituents

When the EO/EOCs were screened for larvicidal activity against 3rd or 4th instar *An. arabiensis* larvae at a concentration of 0.01% (v/v) for 24 hours according to WHO protocol (2005), it was observed that the majority of the EO/EOCs exhibited high mortality rates, thus displaying good larvicidal properties (WHO, 2005). The EOs of *L. javanica*, *O. basilicum*, *C. citratus* and *C. nardus* resulted in 100, 85.4, 80.1 and 80.3% mortality, respectively (Figure 3.4). *Artemisia afra* was the only EO which did not result in significant mortality at 5.1%. Nine of the twenty-two EOCs displayed larvicidal activity within the range of 61 to 100%. With regard to the controls at 0.01% (v/v), the positive control (DDT) displayed 100% mortality, in contrast to 0% mortality for the negative control (acetone).

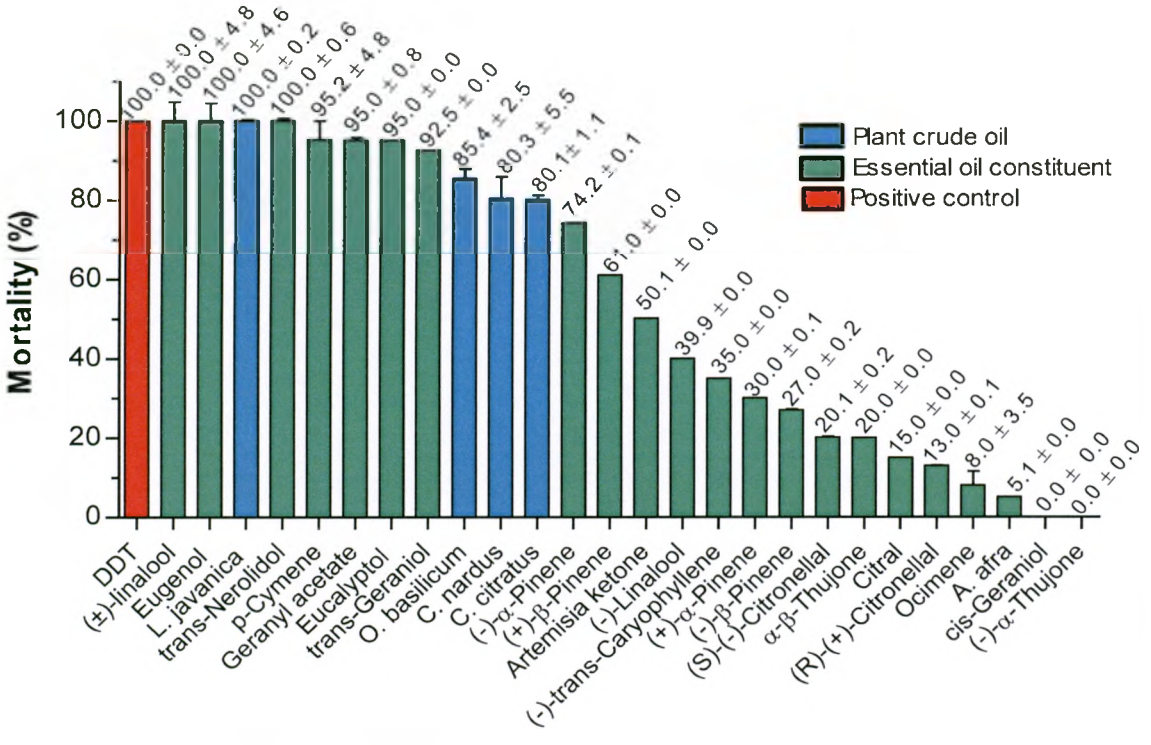

Figure 3.4: The percentage mortality of *An. arabiensis* larvae after 24 hours treatment with 0.01 % (v/v) EO/EOCs compared to the positive control, DDT.

The EO/EOCs that displayed potent mortality ranging between 61 and 100% (Figure 3.4) were found to inhibit *An. arabiensis* larvae in a dose-dependent manner. The EOs of *L. javanica, C. nardus, O. basilicum and C. citratus* yielded LC_{50} values of 0.010, 0.016, 0.017 and 0.019%, respectively. The EOCs which displayed the most larvicidal activity based on a percentage concentration included *cis*-nerolidol (LC_{50} value: 0.001%; 47.27 µM), *p*-cymene (LC_{50} value: 0.004%; 230.67 µM), *trans*-nerolidol (LC_{50} value: 0.006%; 248.29 µM), geranyl acetate (LC_{50} value: 0.007%, 335.99 µM), (+)-β-pinene (LC_{50} value: 0.007%; 467.27 µM) and (-)-α-pinene (LC_{50} value: 0.008%; 484.96 µM) compared to DDT (LC_{50} value: 2.47 x 10^{-10}%; 0.05 µM) (Figure 3.5). The other EOCs, *trans*-geraniol, eugenol, *artemisia* ketone, (±)-linalool and eucalyptol also displayed notable larvicidal activity (Figure 3.5). It was also noted that 0.01% (v/v) *cis*-nerolidol resulted in larvae lethality within the first minute of treatment. The lethality effects of the EO/EOCs compared to the control, DDT, were significantly different with *p*-values for the EOs <0.0001 and for the EOCs ranged between <0.0001 and 0.003.

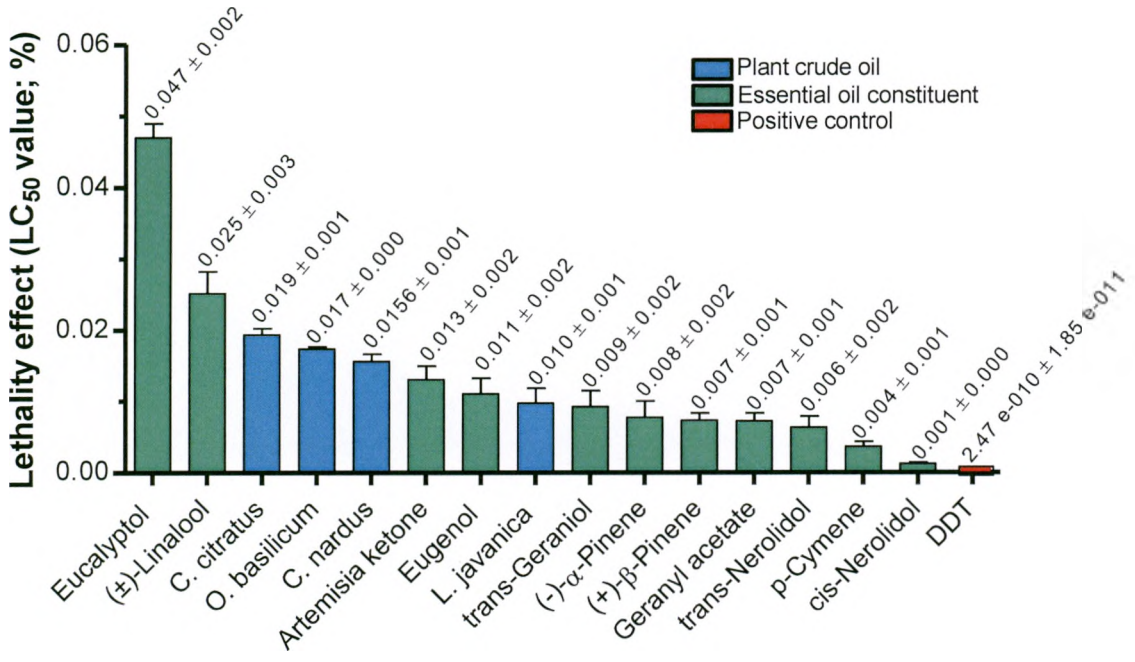

Figure 3.5: The lethality effect (LC_{50} values) of the EO/EOCs against the *An. arabiensis* larvae in comparison to the control, DDT after a 24 hour exposure.

3.3.2 Morphological effects of the EOs and EOCs on the *An. arabiensis* larvae

Morphological alterations were identified on certain *An. Arabiensis* larvae that were treated with 0.01% (v/v) of the EOs and EOCs after 24 hours incubation. It was noticeable that the treated larvae (Figures 3.6.A, C-I, K) showed a discoloured darkened abdominal section as

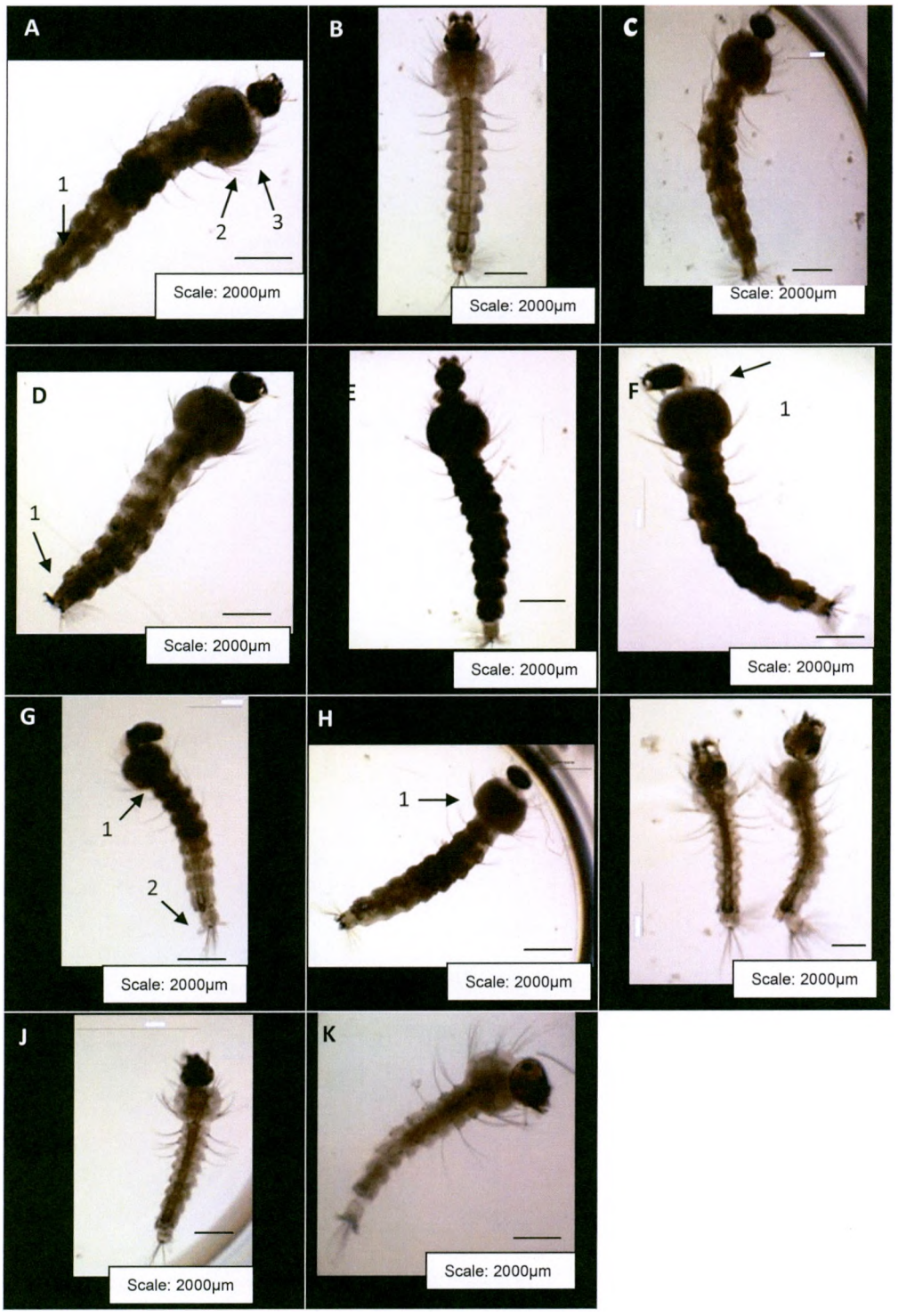

Figure 3.6: The morphological appearance of treated EO/EOCs (0.01% (v/v)) and untreated larvae after a 24 hour period.

The positive control, DDT (A), untreated larvae (B) and some of the active EO/EOCs tested included geranyl acetate (C), *p*-cymene (D), *trans*-nerolidol (E), *L. javanica* (F), (-)-α-pinene (G), *cis*-nerolidol (H) and *C. citratus* (I), (-)-linalool (J) and (+)-linalool (K) on *An. arabiensis* larvae.

compared to the normal untreated 3rd or 4th instar *An. arabiensis* larvae (Figure 3.6.B). Figure 3.6.H.1 showed a larva with an elongation of the neck region that died when treated with the most active EOC, *cis*-nerolidol. Figure 3.6.G showed that the (-)-α-pinene treated larva had an enlarged thorax (Figure 3.6.G.1) and loss of hair tufts in the posterior section (3.6.G.2). Figure 3.6.D.1 showed a lack in normal tracheal gills at the posterior area as well. Figure 3.6.F.1 (*L. javanica*) and 3.6.K.1 ((±)-linalool) showed disfigured heads in comparison to the untreated control. Following treatment with (±)-linalool, the larvae did not move and had to be probed in order for movement to be observed to determine if they were alive (Figure 3.6.K). This was due to the muscle relaxant effect caused by AChE inhibition possessed by (±)-linalool (Colovic *et al.*, 2013). However, (-)-linalool did not cause larval death nor any significant morphological abnormalities (Figure 3.6.J). The positive control, DDT showed an increased thorax to head ratio (Figure 3.6.A.1) as well as a lack of tracheal gills (Figures 3.6.A.2 and 3).

3.3.3 Combined effect of the two most active essential oil constituents

The EOCs, *cis*-nerolidol and *p*-cymene displayed the most potent larvicidal activity of all the EO/EOCs tested based on the % concentration, with the LC_{50} values of 0.002% (47.27 µM) and 0.003% (230.67 µM), respectively (Figure 3.4). Thus they were selected for further evaluation to observe their combined effect against the *An. arabiensis* larvae. As seen in Figure 3.7, the isobologram depicted an additive interaction between *cis*-nerolidol and *p*-cymene with an average ΣFIC of 0.94 ± 0.15.

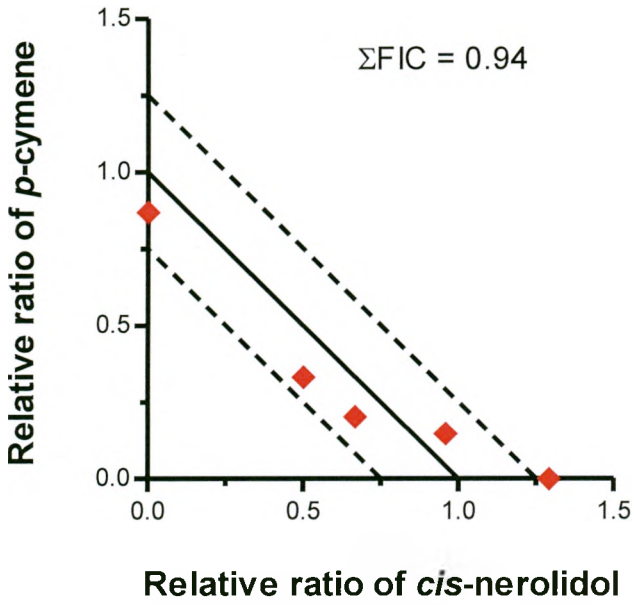

Figure 3.7: The additive interaction between *cis*-nerolidol combined with *p*-cymene when incubated with *An. arabiensis* larvae.

3.3.4 Combined effect of major essential oil constituents of the crude oil

In order to observe the contribution of the individual EOCs to the larvicidal activity of the crude EOs (Figure 3.5), the major EOCs, which could be purchased, were combined in proportion according to the EOs' percentage composition as determined by the GC-MS analysis (Table 3.3). In comparison the 80 to 100% mortality observed for the EOs, the reconstituted 'crude' EOs with the major EOCs only caused between 0.10 to 23% larvae mortality (Table 3.3). In contrast to the reconstituted 'crude' EOs, the majority of the individual EOCs were potent in inhibiting larvae viability.

Table 3.3: Comparison of the crude EO to the reconstituted 'crude' EOs with the major EOCs. The percentage mortality of *An. arabiensis* larvae after treatment with the crude EO in comparison to the combined effect of the major EOCs (which could be purchased) and the individual effect of the EOCs at a concentration of 0.01% (v/v) after 24 hours.

Crude EO	Mortality caused by 0.01% (v/v) crude EO (% ± s.d.)	Main EOCs present in the crude EO	GC-MS composition of EOCs in crude EO (%)	Mortality caused by the reconstituted 'crude' oil with the major EOCs (% ± s.d.)	Mortality caused by individual EOCs at 0.01% (v/v) (% ± s.d.)
C. citratus	80.0 ± 11.1	Citral	78.5	15 ± 4.2	15.0 ± 0.0
		Geraniol	6.2		92.0 ± 0.1
		Geranyl acetate	4.1		95.0 ± 0.8
C. nardus	80.0 ± 8.5	Geraniol	44.9	15.0 ± 0.0	92.0 ± 0.1
		Geranyl acetate	9.6		95.0 ± 0.8
		Eugenol	1.7		100.0 ± 0.1
O. basilicum	85.0 ± 2.5	Linalool	54.3	0.10 ± 0.01	100.0 ± 0.1
		Eugenol	13.9		100.0 ± 0.1
		Eucalyptol	5.3		95.0 ± 0.1
L. javanica	100.0 ± 0.2	Linalool	65.2	22.5 ± 9.11	100.0 ± 0.0
		Ocimene	19.2		7.5.0 ± 0.0
		trans-Caryophyllene	3.6		10.0 ± 0.0

3.4 Brine shrimp lethality assay

The brine shrimp lethality assay was conducted to determine the toxicity of the EO/EOCs on *A. franciscana* nauplii in comparison to the effects of the EO/EOCs to the larvae as a whole organism. After a 24 hour exposure, notable toxicity was observed by the EO/EOCs against the nauplii at a 0.01% (v/v) concentration. The EOs/EOCs, *cis*-nerolidol (98.77%), *trans*-nerolidol (100.00%), *cis*-geraniol (95.56%), *trans*-geraniol (99.28%), eugenol (100.00%), (+)-β-pinene (94.39%), ocimene (83.00%), *C. nardus* (97.00%) and *C. citratus* (99.00%) had similar mortality rates compared to the 100% mortality displayed by the positive control, potassium dichromate (Table 3.4).

Table 3.4: Percentage mortality of *A. franciscana* nauplii after 24 hours treatment with 0.01% (v/v) EO/EOCs and control (n = 3).

Compound	Nauplii mortality (%)	Compound	Nauplii mortality (%)
cis-Nerolidol	98.77 ± 2.14	(-)-β-Pinene	11.67 ± 3.06
trans-Nerolidol	100.00 ± 0.01	(-)-α-Thujone	6.50 ± 0.71
trans-Geraniol	99.28 ± 1.26	α-β-Thujone	2.31 ± 4.01
cis-Geraniol	95.56 ± 4.19	*Artemisia* ketone	1.39 ± 2.41
Citral	96.30 ± 6.42	Ocimene	83.00 ± 8.47
Geranyl acetate	66.72 ± 4.71	(-)-*trans*-Caryophyllene	3.97 ± 2.78
(±)-Linalool	6.84 ± 6.06	(*R*)-(+)-Citronellal	38.55 ± 3.15
(-)-Linalool	7.65 ± 2.17	(*S*)-(-)-Citronellal	32.46 ± 3.53
Eugenol	100.00 ± 0.01	*C. citratus*	99.00 ± 1.00
p-Cymene	3.12 ± 2.79	*C. nardus*	97.00 ± 5.20
Eucalyptol	3.13 ± 0.41	*O. basilicum*	47.78 ± 0.69
(+)-α-Pinene	9.67 ± 9.07	*A. afra*	24.00 ± 0.21
(-)-α-Pinene	23.67 ± 3.21	*L. javanica*	32.74 ± 4.11
(+)-β-Pinene	94.39 ± 6.04	Potassium dichromate	100.00 ± 0.01

EOC with cytotoxic activity ; EO with cytotoxic activity; Positive control;

No notable activity

The LC_{50} value of the positive control, potassium dichromate was determined to be 0.01% (95.99 µM) (Figure 3.8). The EOs, *C. citratus* and *C. nardus* displayed toxic properties with LC_{50} values of 0.007% (70.02 µM) and 0.003% (28.33 µM), respectively. The EOC, *cis*-

geraniol displayed the highest toxicity with an IC_{50} value of 5.87 $x10^{-7}$% (0.03 µM). The isomer, *trans*-geraniol (LC_{50} value: 0.001%; 34.38 µM) was the also toxic of the EOCs followed by *cis*-nerolidol (LC_{50} value: 0.003 $x10^{-2}$%; 1.31 µM). It is apparent that the isomers, *cis*-nerolidol (LC_{50} value: 0.003 $x10^{-2}$%; 1.31 µM) and *trans*-nerolidol (IC_{50} value: 0.002%; 59.84 µM), were very toxic. The (+)-β-pinene isomer was toxic (LC_{50} value: 0.002%; 113.30 µM), whereas the isomers, (-)-β-pinene and α-pinenes did not display toxicity properties. Both (±)- and (-)-linalool isomers, (-)-α- and α-β-thujone isomers, *artemisia* ketone, (-)-*trans*-caryophyllene isomers, *p*-cymene, eucalyptol, were part of the EOCs which did not display notable toxicity profiles with mortality ranging between 1.39 and 9.67% (Table 3.4). The IC_{50} values of *cis*-nerolidol, citral and *C. nardus* were not similar to potassium dichromate (*p*-values: 0.130, 0.069 and 0.054, respectively). Although the IC_{50} values were obtained for eugenol, citral, ocimene and geranyl acetate, the inhibitory effects were significantly less than potassium dichromate. The other toxic EOs and EOCs were significantly different with *p*-values ranging between 0.0005 and 0.0345.

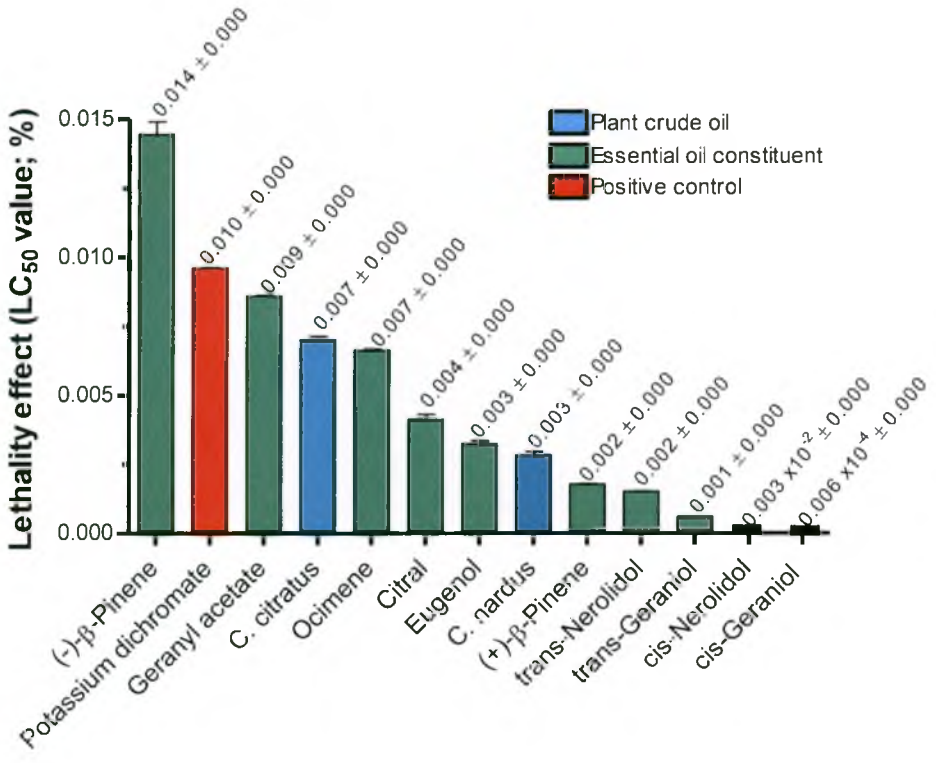

Figure 3.8: The lethality effects of the EO/EOCs on *A. franciscana* nauplii after 24 hours treatment in comparison to the positive control, potassium dichromate.

A LC_{50} value for geranyl acetate of 0.009% was obtained from the estimate concentration of the 95% confidence limit; and displayed in the graph obtained from the Probit transformed responses (Table 3.5; Figure 3.9).

Table 3.5: The 95% confidence limits displayed the LC_{50} value of geranyl acetate after a 24 hour treatment period (IBM SPSS Statistics, version 22.0).

Confidence Limits

	Probability	95% Confidence Limits for concentration		
		Estimate	Lower Bound	Upper Bound
PROBIT[a]	.010	.000	.000	.000
	.020	.000	.000	.000
	.030	.000	.000	.000
	.040	.000	.000	.000
	.050	.000	.000	.001
	.060	.000	.000	.001
	.070	.000	.000	.001
	.080	.000	.000	.001
	.090	.000	.000	.001
	.100	.000	.000	.001
	.150	.000	.000	.003
	.200	.000	.000	.006
	.250	.001	.000	.011
	.300	.001	.000	.022
	.350	.002	.000	.045
	.400	.004	.000	.102
	.450	.006	.000	.251
	.500	.009	.000	.695
	.550	.015	.001	2.172

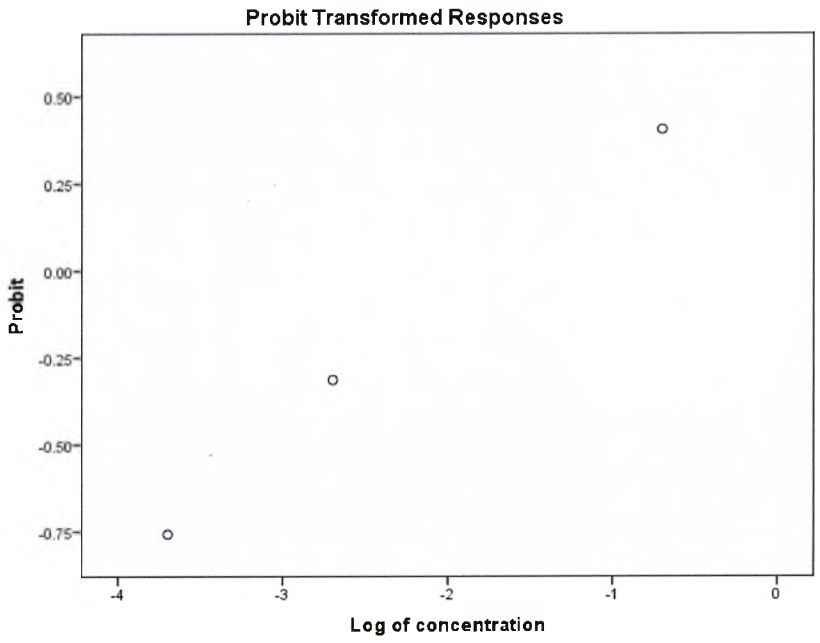

Figure 3.9: The lethality effect of geranyl acetate depicted on the Probit transformed graph after a 24 hour treatment period (IBM SPSS Statistics, version 22.0).

3.4.1 Morphological effects of the EOs and EOCs on *A. franciscana* nauplii

Untreated and 0.01% (v/v) EO/EOC treated *Artemia* nauplii were compared to the control, potassium dichromate. Morphological aberrations including alteration of nauplii size, length, pigmentation and external features were not observed at a concentration of 0.01% (v/v). However, it was observed that the nauplii swum by means of the movement of their appendages. As the live nauplii were swimming in Figure 3.10.A.1; the appendages were not clear due to its movement. However, in Figures 3.10.B.1 and 3.10.C.1, the appendages of the dead nauplii were clearly visible.

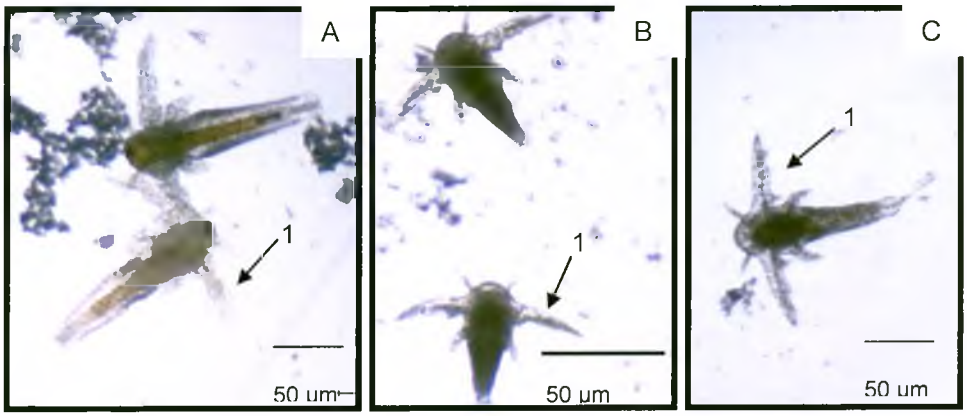

Figure 3.10: The morphological features of the untreated *A. franciscana* nauplii (A) compared to the positive control (potassium dichromate) (B) and *cis*-geraniol treated nauplii (C) after 24 hour treatment with 0.01% (v/v) EOC/control (100x magnification).

3.5 The tetrazolium cytotoxicity assay

At the screening concentration, all five EOs displayed cytotoxicity with cell viability ranging between 0.10 and 9.83%. Seventeen of the 22 EOCs displayed toxic properties with cell viability ranging between 0.01 and 58.75% (Table 3.6). One EO and two EOCs which were most toxic and inhibited 50% cell growth at the closest concentration to the control camptothecin (IC_{50} value: 0.11 µM; 9.6 $x10^{-5}$%), were (-)-linalool (IC_{50} value: 0.19 µM; 3.4 $x10^{-5}$%), (+)-β-pinene (IC_{50} value: 0.25 µM; 3.9 $x10^{-6}$%) and *O. basilicum* (IC_{50} value: 2.3 $x10^{-4}$%) (Figures 3.11 and 3.12). The IC_{50} values of the toxic EO/EOCs were significantly different to camptothecin, with *p*-values ranging from <0.0001 to 0.0033.

Table 3.6: Percentage viability of human kidney epithelial (HEK-293) cells after treatment with 0.05% (v/v) EO, 50 µM EOC and controls (n = 3).

Compound	Cell viability (%) ± s.d.	Compound	Cell viability (%) ± s.d.
cis-Nerolidol	0.10 ± 0.01	(-)-β-Pinene	0.10 ± 0.01
trans-Nerolidol	0.10 ± 0.01	(-)-α-Thujone	90.64 ± 2.18
trans-Geraniol	0.10 ± 0.01	α-β-Thujone	29.86 ± 8.55
cis-Geraniol	0.10 ± 0.01	*Artemisia* ketone	25.43 ± 2.45
Citral	73.83 ± 4.00	Ocimene	22.23 ± 9.13
Geranyl acetate	78.47 ± 6.31	(-)-*trans*-Caryophyllene	33.49 ± 3.56
(±)-Linalool	85.95 ± 3.34	(*R*)-(+)-Citronellal	0.10 ± 0.01
(-)-Linalool	37.02 ± 7.64	(*S*)-(-)-Citronellal	0.10 ± 0.01
Eugenol	58.75 ± 20.42	*C. citratus*	0.10 ± 0.01
p-Cymene	0.10 ± 0.01	*C. nardus*	0.10 ± 0.01
Eucalyptol	80.67 ± 17.88	*O. basilicum*	0.10 ± 0.01
(+)-α-Pinene	0.10 ± 0.01	*A. afra*	9.83 ± 0.11
(-)-α-Pinene	0.10 ± 0.01	*L. javanica*	0.10 ± 0.01
(+)-β-Pinene	0.10 ± 0.01	Camptothecin	23.24 ± 4.70

EOC with cytotoxic activity EO with cytotoxic activity Positive control No notable cytotoxic activity

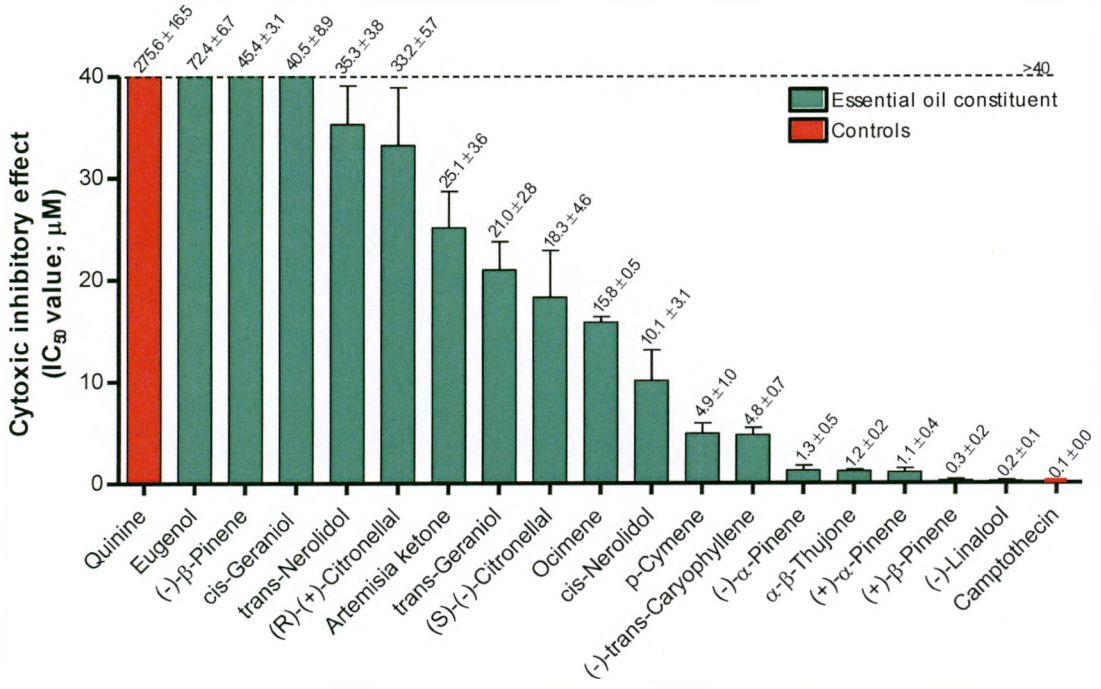

Figure 3.11: The cytotoxic effect of the EOCs on the human kidney epithelial (HEK-293) cells compared to the standard antimalarial, quinine (negative control), and cytotoxic control, camptothecin (positive control).

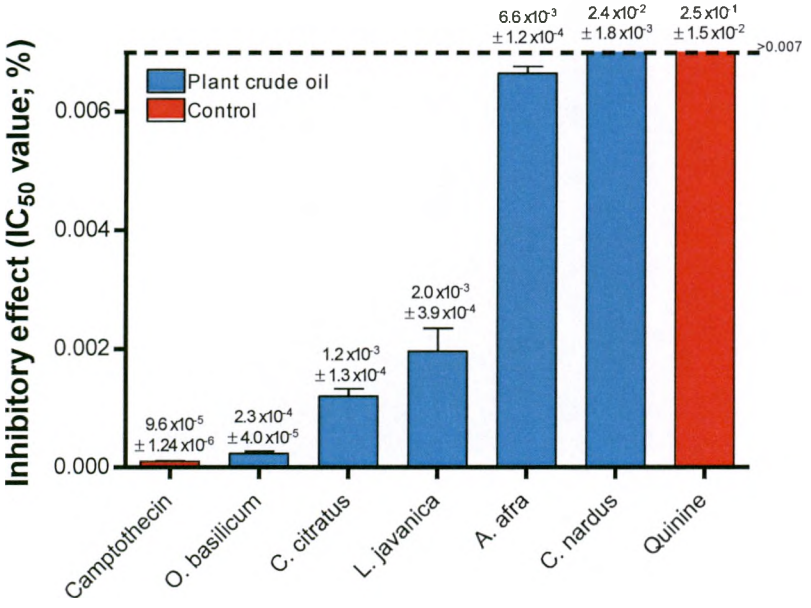

Figure 3.12: The cytotoxic effect of the EO on the human kidney epithelial (HEK-293) cells compared to the standard antimalarial, quinine (negative control), and cytotoxic control, camptothecin (positive control).

3.6 Lipid peroxidation inhibition assay

The EO/EOCs displayed a relatively low ability to inhibit lipid peroxidation within the range of 0.01 and 31.81% with the exception of *O. basilicum* and eugenol inhibiting 87.47 and 86.83%, at their respective screening concentrations (Table 3.7). Figure 3.14 showed the IC_{50} values of *O. basilicum* (IC_{50} value: 0.0018%) and Figure 3.13 showed that of eugenol (IC_{50} value: 18.90 µM; 0.18 $x10^{-1}$%) compared to the positive control, Trolox (IC_{50} value: 1.24 µM; 0.12 $x10^{-2}$%). As expected, quinine, chloroquine and dihydroartemisinin displayed insignificant inhibitory properties at 50 µM by inhibiting 18.81, 0.28 and 0.10% lipid peroxidation, respectively (Table 3.7). In comparison to the IC_{50} value of the control, Trolox, there was a significant difference between *O. basilicum* (*p*-value: 0.0003) and eugenol (*p*-value: 0.01).

Table 3.7: Percentage lipid peroxidation inhibition after treatment with 0.05% (v/v) EO, 50 µM EOCs and the control, Trolox (n = 3).

Compound	Lipid peroxidation inhibition (%) ± s.d.	Compound	Lipid peroxidation inhibition (%) ± s.d.
cis-Nerolidol	7.36 ± 5.98	α-β-Thujone	19.47 ± 2.31
trans-Nerolidol	0.10 ± 0.01	*Artemisia* ketone	11.66 ± 1.44
trans-Geraniol	0.10 ± 0.01	Ocimene	24.63 ± 6.76
cis-Geraniol	3.90 ± 1.90	(*R*)-(+)-citronellal	9.34 ± 6.00
Citral	0.10 ± 0.01	(*S*)-(-)-citronellal	0.10 ± 0.01
Geranyl acetate	0.10 ± 0.01	(-)-*trans*-Caryophyllene	14.33 ± 4.58
(±)-Linalool	0.10 ± 0.01	*C. citratus*	16.67 ± 1.66
(-)-Linalool	3.09 ± 2.44	*C. nardus*	22.15 ± 8.86
Eugenol	86.83 ± 9.54	*O. basilicum*	87.41 ± 8.28
p-Cymene	12.08 ± 2.22	*A. afra*	0.10 ± 0.01
Eucalyptol	8.07 ± 3.03	*L. javanica*	31.81 ± 3.49
(+)-α-Pinene	10.35 ± 8.43	Quinine	18.87 ± 3.96
(-)-α-Pinene	3.21 ± 1.83	Chloroquine	0.28 ± 0.26
(+)-β-Pinene	0.10 ± 0.01	Dihydroartemisinin	0.10 ± 0.01
(-)-β-Pinene	0.10 ± 0.01	Trolox	76.34 ± 2.09
(-)-α-Thujone	11.89 ± 2.20		

EOC with lipid peroxidation inhibitory activity;
EO with lipid peroxidation inhibitory activity;
Positive control; No notable activity

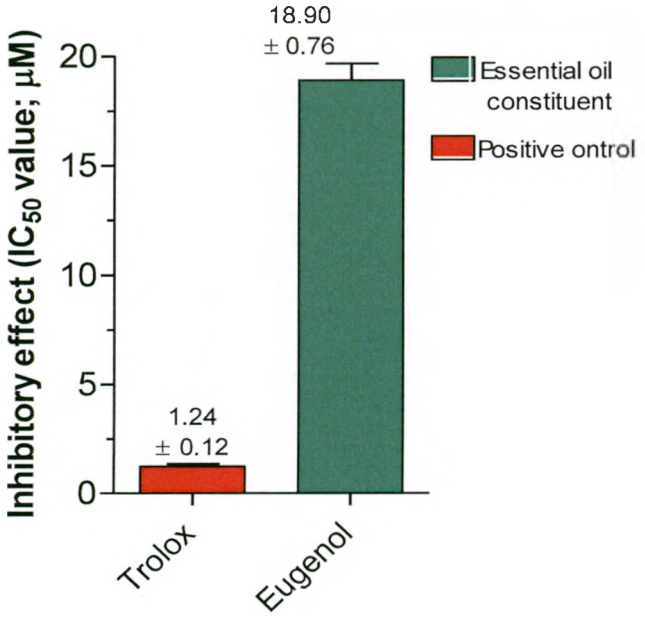

Figure 3.13: The lipid peroxidation inhibitory effect displayed by the EOC, eugenol after 24 hours treatment in comparison to the positive control, Trolox.

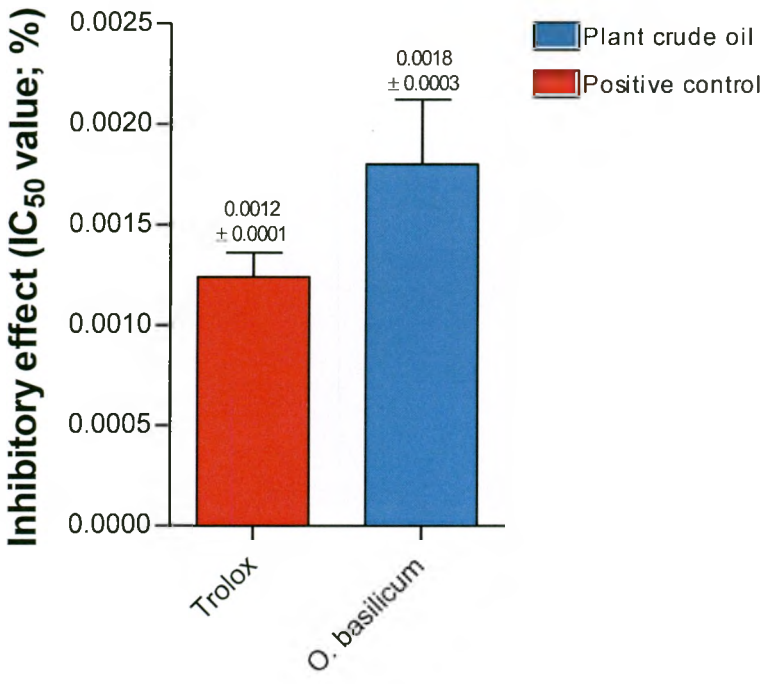

Figure 3.14: The lipid peroxidation inhibitory effect displayed by the EO, *O. basilicum* and eugenol after 24 hours treatment in comparison to the positive control, Trolox.

3.7 Safety index

3.7.1 The relationship between the EO/EOCs toxicity profiles and corresponding antimalarial and larvicidal activity

The safety indices of the 5 EOs and 22 EOCs were calculated to determine the selectivity for human kidney epithelial (HEK-293) cells or the malaria parasite (Table 3.8), as well as the larvae vector. Overall, the EOs and EOCs displayed general cytotoxicity rather than selectivity towards the malaria parasite shown by the low safety indices of the EOs and EOCs that ranged between 4 $x10^{-3}$ and 9.98 in comparison to quinine (1503.27). The only comparable safety index was that between the human kidney epithelial (HEK-293) cells and the malaria parasite of eucalyptol (135.21). All the EO/EOCs displayed toxicity towards the larvae shown by the low safety indices that ranged between 5.9 $x10^{-5}$ and 2.78.

Table 3.8: The relationship between the EO/EOCs toxicity profiles corresponding to their respective antimalarial and larvicidal activity.

Compound	Safety index		Compound	Safety index	
	Cytotoxic versus antimalarial activity	Nauplii toxicity versus larvicidal activity		Cytotoxic versus antimalarial activity	Nauplii toxicity versus larvicidal activity
cis-Nerolidol	5.05	0.03	α-β-Thujone	0.02*	1.00**
trans-Nerolidol	5.79	0.24	*Artemisia* ketone	0.50*	0.77**
trans-Geraniol	0.42*	0.07	Ocimene	0.32*	0.66**
cis-Geraniol	7.03	5.9 x10^{-5}**	(*R*)-(+)-Citronellal	0.66*	1.00**
Citral	9.98*	0.41**	(*S*)-(-)-Citronellal	1.74	1.00**
Geranyl acetate	0.03*	1.19	(-)-*trans*-Caryophyllene	0.01*	1.00**
(±)-Linalool	0.65*	0.40**	*C. citratus*	6.16	0.36
(-)-Linalool	4 x10^{-3}	1.00**	*C. nardus*	4.72	0.18
Eugenol	2.09*	0.29	*O. basilicum*	0.01	0.58**
p-Cymene	0.01*	2.78**	*A. afra*	1.66	1.00**
Eucalyptol	135.21*	0.21**	*L. javanica*	0.36	1.03**
(+)-α-Pinene	0.02*	1.00**	Quinine	1503.27	n.d.
(-)-α-Pinene	0.03*	1.30**	Camptothecin	n.d	n.d.
(+)-β-Pinene	0.01*	0.24	Potassium dichromate	n.d.	n.d.
(-)-β-Pinene	0.91*	1.44**	DDT	n.d.	n.d.
(-)-α-Thujone	1.00*	1.00**			

* Safety index calculated using 50 µM as cytotoxicity IC_{50}

** Safety index calculated using 0.01% (v/v) as nauplii toxicity IC_{50}

n.d. Not determined.

CHAPTER FOUR – DISCUSSION

Phytomedicine and herbal medicine are culturally accepted and have been used worldwide for centuries as therapeutic or prophylactic sources in the treatment of various diseases. The phytochemical constituents present in medicinal plants gives yield to the plant's physiological or pathological effects including its toxicological profiles (Ekpenyong *et al.*, 2014). Many researchers have reported that phytochemicals, other than managing symptoms related to malaria, may be alternative agents used for the control of the mosquito vector (Ansari *et al.*, 2000). Overall all five plant EOs evaluated in this study, namely; *L. javanica, A. afra, O. basilicum, C. citratus* and *C. nardus* have produced noteworthy antimalarial and larvicidal properties, however, there had been significant toxicity profiles reported as well. Based on the claims of numerous health benefits derived from these plants, they were prospective EOs to investigate their actions and elucidate their toxicological profile. The toxicological profile of the plants was also dependent on its phytochemistry and the prevailing micro-environmental physical or chemical stressors (Ekpenyong *et al.*, 2014). The various properties of the EOs including each EOC which was present in majority of the specific EO are described below.

4.1 *Lippia javanica*

Lippia javanica belonging to the Verbenaceae family has a long history of traditional uses, with the most important traditional applications as herbal tea and ethnomedicinal applications for colds, cough, fever or malaria, wounds, repelling mosquitoes, diarrhoea, chest pains, bronchitis, and asthma (Gelfland *et al.*, 1985; Samie *et al.*, 2005; Vhurumuku, 2015). *Lippia javanica* is rich in volatile oils, including its major EOCs; linalool, (-)-*trans*-caryophyllene, *trans*-nerolidol, α-pinene, (*Z*)-β-ocimene and (*E*)-β-ocimene (Table A.1).

Lippia javanica and its major EOCs displayed variable inhibitory properties including antimalarial, larvicidal and toxicity properties which has been collated in Figure 4.1 and will be discussed below.

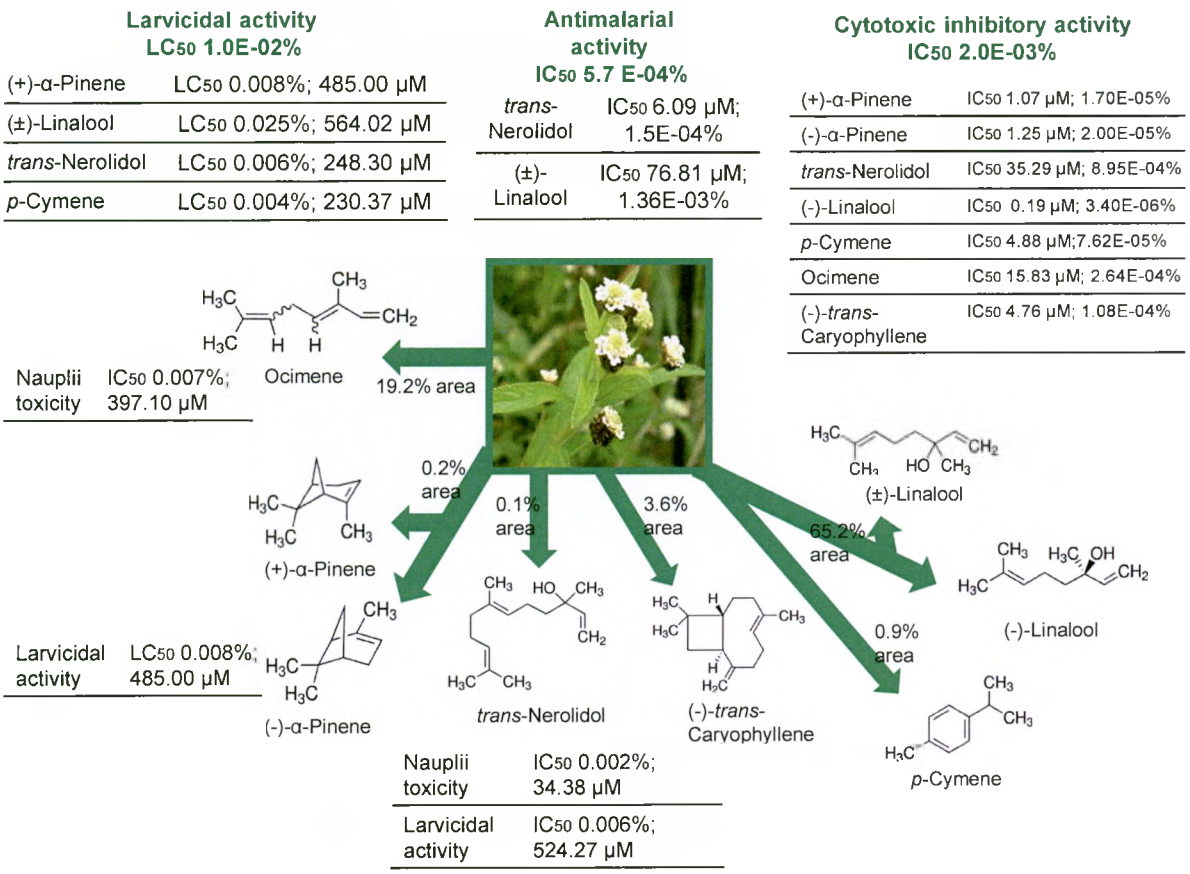

Figure 4.1: The various properties displayed by the EO, *L. javanica,* and its major EOCs.

Lippia javanica displayed antimalarial activity on the *P. falciparum* NF54 parasite strain with an IC_{50} value of 5.7 $x10^{-4}$% compared to the quinine (1.7 $x10^{-5}$%; 0.18 µM) (Figure 3.3). The major EOCs of the plant that displayed antimalarial activity included (±)-linalool and *trans*-nerolidol (Figure 3.2). Comparatively; the EO of *L. javanica* from South Africa was also evaluated by Manenzhe *et al.* (2004) for its antiplasmodial activity against the chloroquine-sensitive strain of *P. falciparum* (D10) and an IC_{50} value of 8.0 $x10^{-4}$% compared to chloroquine (IC_{50} value: 2.0$x10^{-6}$%) was obtained. In contrast, several studies have been undertaken on various extracts of this plant, where Prozesky *et al.* (2001) evaluated the leaf acetone extract against *P. falciparum* PfUP1 (chloroquine-resistant) strain by means of flow cytometry and found that the IC_{50} value for *L. javanica* was 4.4 $x10^{-4}$% compared to chloroquine (IC_{50} value: 4.3 $x10^{-6}$%). Whilst, Clarkson *et al.* (2004) evaluated the root and stem extracts against a chloroquine-sensitive stain of *P. falciparum* (D10) compared to chloroquine using the pLDH assay. The dichloromethane (DCM), DCM/ methanol (MeOH) (1:1), MeOH and water extracts of the root had IC_{50} values of 3.8 $x10^{-4}$, 2.7 $x10^{-3}$, 2.4 $x10^{-3}$ and >1 $x10^{-2}$%, respectively. The DCM, DCM/MeOH (1:1), MeOH and H_2O extracts of the stem had IC_{50} values of 4.5 $x10^{-4}$, 2.2 $x10^{-3}$, 3.0 $x10^{-3}$ and >1 $x10^{-2}$%, respectively. In

comparison to the MeOH extracts IC_{50} values, Ayuko *et al.* (2009) showed that *L. javanica* MeOH plant root extracts had antiplasmodial activity against chloroquine-sensitive from Sierra Leone (D-6) and chloroquine-resistant from Vietnam (W-2) *P. falciparum,* with IC_{50} values of 1.4 $x10^{-4}$ and 1.75 $x10^{-4}$%, respectively.This was in comparison to chloroquine with IC_{50} values of 2.4 $x10^{-6}$ and 3.4 $x10^{-6}$% for the D-6 and W-2 strain, respectively.

The antimalarial properties noted for the *L. javanica* and the other EOs and EOCs obtained were due to the direct inhibitory effect on the intra-erythrocytic parasite (Hayat *et al.,* 2011) and not due to the integrity of the RBC membrane being affected, as it was observed that none of the EO/EOCs displayed significant haemolytic activity compared to the 100% lysis control (Triton X-100™) (Table 3.1). The use of erythrocytes as a model system allowed the study of interaction of drugs with membranes (Sessa and Wesman, 1968). If the permeability of the RBC membrane to the EO/EOC was higher than that to the solutes comprising the cell cytosol, the rate of influx of material into the cell exceeded the rate of efflux, resulting in a net uptake of solute and water. This would cause the cell to swell and eventual haemolysis (Figure 4.2; Kirk, 2001).

It was mentioned that most EO/EOCs readily interact with biomembranes and membrane proteins. When the EO/EOCs get in contact with the cells, as seen in several monoterpenes and sesquiterpenes, they would bind to the lipophilic inner core of the membrane layer. If their concentration is high enough, this would increase the membrane fluidity and permeability which could lead to uncontrolled efflux of ions and metabolites leading to cell leakage and eventual apoptosis (Wink, 2007). Essential oils and their constituents can modulate the activity of ion channels and proteins. There has been a correlation to many lipophilic drugs having cytotoxic properties as well (Van Wyk and Wink, 2004; Van Wyk *et al.*, 2004). Essential oils and EOCs can enhance the permeation of lipophilic and hydrophilic drugs based on their degree of saturation, size and chirality, boiling point and lipophilicity (Aqil *et al.*, 2007). It has been mentioned that hydrocarbon, non-polar EOCs are more potent enhancers for lipophilic drugs than oxygen-containing polar EOCs. The monoterpene, thujone, contains a cyclopropane ring, which makes the molecule highly reactive and allowed it to alkylate proteins of the neuronal signal transduction, therefore causing neuronal death (Van Wyk and Wink, 2004; Van Wyk *et al.*, 2004; Teuscher and Lindequist, 2010). Linalool was reported to bind to sulfhydryl groups of proteins and change their conformation as well as being the best possible enhancer for transdermal delivery of haloperidol (Vaddi *et al.*, 2002; Van Wyk and Wink, 2004; Van Wyk *et al.*, 2004). Monoterpenes with aldehyde functions such as citronellal and citral can bind to proteins and are reactive against bacteria and fungi (Van Wyk and Wink, 2004). The sesquiterpenes displayed their activity by binding

to sulfhydral groups of proteins via 1 or 2 exocyclic methylene groups and the enon configuration in the furan ring (Van Wyk and Wink, 2004; Van Wyk *et al.*, 2004; Teuscher *et al.*, 2004). It was mentioned that artemisinin from *A. annua*, being a sesquiterpene has a reactive peroxide bridge, was developed into artesunate (antimalarial drug) against *P. falciparum* (Woldemichael and Wink, 2001). Nerolidol enhances hydrophilic drugs permeation more than lipophilic drugs (El-Kattan *et al.*, 2001). Essential oils or EOCs which possessed potent biological activity may not be useful in pharmacological preparation if they possessed haemolytic activity (Zohra and Fawzia, 2014). Haemolytic activity of EO/EOCs may lead to serious side effects including haemolytic anaemia (Freitas *et al.*, 2008). Furthermore, the haemolytic activity of each EO/EOC is related to the individual chemical composition of such. However, after application to the skin, EO/EOCs are rapidly metabolized, not accumulated in the organism and fast excreted what strongly suggest that they can be successfully used as safe penetration enhancers (Herman and Herman, 2014).

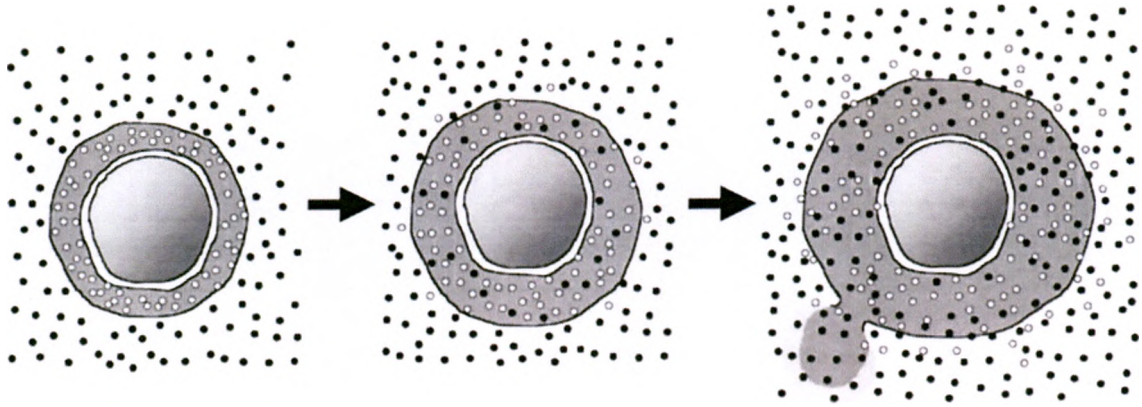

Figure 4.2: The process by which parasitised erythrocytes suspended in an isosmotic solution of a permeant solute underwent "isosmotic haemolysis". The influx of extracellular solutes (•) at a rate greater than the efflux of cytosolic solutes (◦) would give rise to a net uptake of solute and water, leading ultimately to haemolysis (Kirk, 2001).

In conjunction to the advantageous antimalarial properties of *L. javanica*, the EO also displayed notable larvicidal properties. *Lippia javanica* displayed the most potent larvicidal activity of the five EOs with a LC_{50} value of 0.78 ± 0.21% (Figure 3.9) with DDT as the control (LC_{50} value: 2.5 $x10^{-10}$%). However, Mavundza *et al.* (2013) determined 21% larvae mortality of *L. javanica* (0.05% (w/v) ethanolic leaf extracts from South Africa on 3rd instar *An. arabiensis* larvae as opposed to the 100% mortality in this study. Earlier research by Mwangi *et al.* (1992) in Kenya, indicated that the EOs of a variety of *Lippia* species demonstrated a larvicidal activity against *A. aegypti* larvae and a maize weevil (*Sitophilus zeamais* Motsch)

repellancy. The active species included: *Latana dauensis* (Chiov.) Chiov., *L. grandifolia* Hochst., *L. javanica* (Burm. f) Spreng., *L. somalensis* Vatke, *L. ukambensis* Vatke (the most active as repellant and with a low larvicidal activity) and *L. wilmsii* H. H. W. Pearson (the most active larvicide and with a low repellency). *p*-Cymene, (-)-α-pinene, (±)-linalool, (-)-*trans*-caryophyllene and *trans*-nerolidol were the major EOCs of *L. javanica* that displayed larvicidal activity, with *p*-cymene and *cis*-nerolidol the most active of all the EO and EOCs tested, and thus they were tested in combination against the larvae (Figure 3.5; Section 4.1.1.1).

Lippia javanica is known to cause liver damage and photosensitivity in livestock. It has been mentioned that the prolonged use of high doses of *L. javanica* was potentially harmful (Van Wyk *et al.*, 2002). Toxicity was only present in this study against the human kidney epithelial (HEK-293) cells with an IC_{50} value of 2.0 $x10^{-3}$% compared to camptothecin (IC_{50} value: 9.6 $x10^{-4}$%; 0.10 µM) (Figure 3.10). The major EOCs found in *L. javanica* which displayed cytotoxicity included (-)-linalool > (+)-α-pinene > (-)-α-pinene > (-)-*trans*-caryophyllene > *p*-cymene > ocimene > *trans*-nerolidol (Figure 4.1). No toxicity of the *Artemia* nauplii was observed in this study with a mortality of 32.74% after 24 hours treatment of 0.01% EO (Table 3.4). Toxicity of *L. javanica* against the *Artemia* nauplii was observed by Ayuko *et al.* (2009), whereby an LC_{50} value of 0.12% was obtained. Samie *et al.* (2009) demonstrated that a pure compound piperitenone isolated from *L. javanica* EO had low cytotoxicity activity against intestinal adenocarcinoma cells (HCT-8 monolayers, IC_{50} value: 0.03%). The major EOCs, ocimene and (-)-*trans*-caryophyllene, which constituted 19.2 and 3.6%, respectively, of *L. javanica* (Table A.1) were the only EOCs that displayed unfavourable toxicity profile with ocimene (IC_{50} value: 15.83 µM; 2.64 $x10^{-4}$%) and (-)-*trans*-caryophyllene (IC_{50} value: 4.67 µM; 1.08 $x10^{-4}$%) cytotoxic to human kidney epithelial (HEK-293) cells (Figure 3.10). Ocimene also displayed toxicity towards the *Artemia* nauplii (LC_{50} value: 0.007%; 397.10 µM) (Figure 3.8). The EOC, (-)-*trans*-caryophyllene is a natural bicyclic sesquiterpene found in a high concentration in *L. javanica* and *C. citratus* (Table A.1 and A.4). Incubation of human (MoFir) and mouse (BS-24-1) tumor cell lines for 2 hours with (-)-*trans*-caryophyllene (2.4 $x10^{-4}$ µM) resulted in the activation of the enzymatic activity of caspase-3 which led to cell death. (-)*trans*-Caryophyllene showed a concentration dependent inhibition of proliferation and induced 85–90% cell death in both cell lines at a concentration of 4.8 $x10^{-4}$ µM (Maroyi, 2017).

Phytochemical studies need to be conducted especially to determine the mechanism of action of *L. javanica* and its bioactive EOCs, as a basis to demonstrate the correlation between ethno-medicinal uses and pharmacological activities. There is a need for extensive

in vivo studies to validate existing pharmacological activities as well as toxicological properties of this plant (Maroyi, 2017).

4.1.1 Nerolidol

Nerolidol is a naturally occurring sesquiterpene alcohol existing in two geometric isomers, a *trans* and *cis* form. The presence of a double bond at the C-6 position and asymmetric centre at the C-3 position resulted in the existence of these isomeric forms (Chan, 2016). Only the *trans* isomer had been observed as a major EOC in *L. javanica* (Table A.1). However, both isomers, *trans*- and *cis*-nerolidol displayed antimalarial activity (IC_{50} value: 6.09 µM; 1.55 $x10^{-4}$% and 1.99 µM; 5.06 $x10^{-5}$%, respectively) and larvicidal properties (LC_{50} value: 0.006%; 248.30 µM and 0.001%; 47.27 µM, respectively) (Figures 3.2 and 3.5). Toxicity against the *Artemia* nauplii was displayed by both isomers (*trans*- and *cis*-nerolidol; LC_{50} value: 1.25 $x10^{-3}$%; 173.00 µM and 3.33 $x10^{-5}$%; 156.00 µM, respectively) (Figure 3.7); similarly, cytoxicity was also displayed by both isomers (IC_{50} value: 36.3 µM; 8.95 $x10^{-4}$% and 0.07 µM; 2.56 $x10^{-4}$%, respectively) (Figure 3.9). It has been noted that nerolidol was highly hydrophobic, allowing for easier penetration across the plasma membrane and interaction with intracellular proteins and intra-organelle sites (Park *et al.*, 2009). Although both *trans*- and *cis*-nerolidol displayed toxicity against the *Artemia* nauplii and human kidney epithelial (HEK-293) cells, its advantageous antimalarial and larvicidal properties could lead to further investigation. In the combat against malaria transmission and symptomatic treatment, it was also mentioned that nerolidol derived from various sources may have beneficial insect repellent, anti-oxidant and anti-inflammatory properties (Figure 4.3; Chan *et al.*, 2016).

Various research has been done with regard to the antimalarial properties of nerolidol. Both *trans*- and *cis*-nerolidol inhibited 100% of the development of young trophozoites to the schizont stage of *P. falciparum* after 48 hours of treatment by a 10% nerolidol concentration from the *Virola surinamensis* leaf extract (Lopes *et al.,* 1999). Nerolidol (23.7%), which was one of the major volatile components extracted from inflorescences oil of *Piper claussenianum* (Miq.) C. DC., demonstrated an antimalarial activity with an IC_{50} value of 1.1 $x10^{-3}$% (Marques *et al.*, 2011). Seatlholo (2008) performed the tritriated-hypoxanthine incorporation assay to determine the antimalarial activity of (*E*- and *Z*-)-(±)-nerolidol in comparison to the control quinine (IC_{50} value: 0.29 µM) on *P. falciparum* FCR-3 chloroquine-resistant strain. The racemic *cis/trans* mixture was found to be active yielding an IC_{50} value of 0.90 µM compared to this study, whereby; *trans*- and *cis*-nerolidol displayed antimalarial activity (IC_{50} value: 6.09 µM and 1.99 µM, respectively) (Figure 3.2). The antimalarial mechanism of nerolidol was proposed to involve nerolidol inhibiting the biosynthesis of the

isoprenoid chain attached to the benzoquinone ring in the intra-erythrocytic stages of the *P. falciparum* parasite (Section 4.1.3).

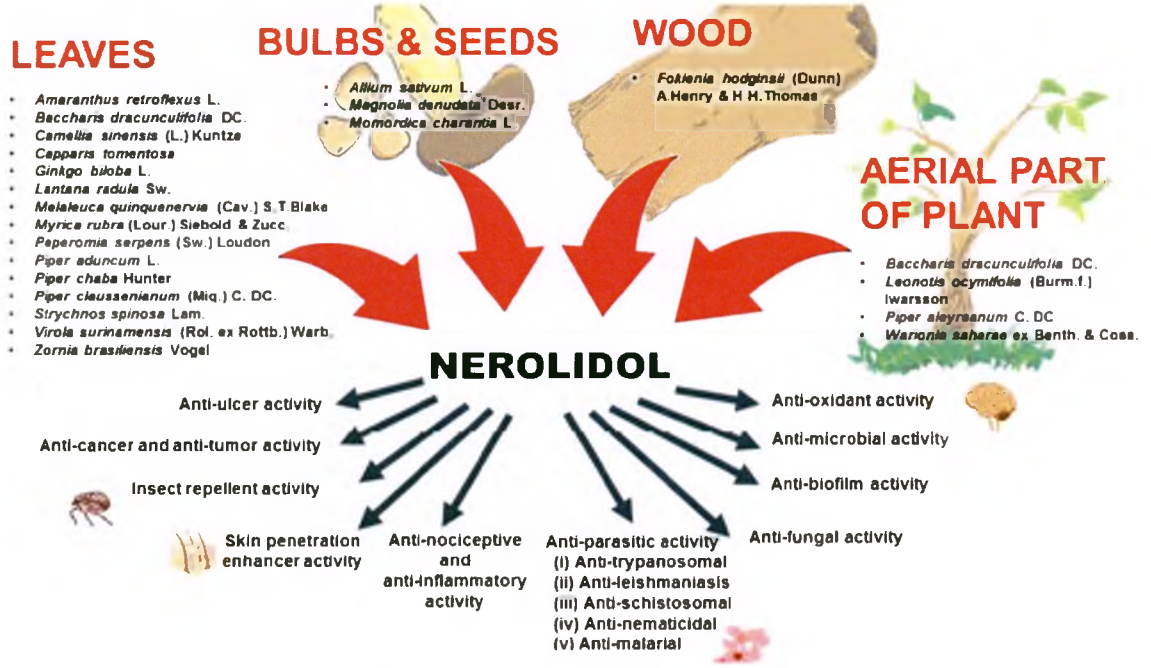

Figure 4.3: Common sources of extraction of nerolidol and an overview of the biological activities of nerolidol (Chan *et al.*, 2016).

Moreover, treatment with nerolidol at doses 2.2 times below the IC_{50} of 0.12 µM has been shown to inhibit the isoprenic chain production attached to coenzyme Q. The *cis*-, *trans*-nerolidol inhibited the isoprenylation of proteins which interfered with mitochondrial metabolic processes (De Macedo *et al.*, 2002). These findings indicated that nerolidol possessed strong antimalarial activity by inhibiting the development of the intra-erythrocytic stages of the parasites.

The EOC, cis-nerolidol showed greater toxicity than *trans*-nerolidol on the human kidney epithelial (HEK-293) cells and on the *Artemia* nauplii (Figures 3.11 and 3.8). Marques *et al.* (2011) also determined the toxicity of the nerolidol-rich EO of *Piper claussenianum;* in contrast, no toxicity was observed on either L929 fibroblast cells (mouse) nor Raw cells (mouse macrophages) at any concentration tested (4 to 0.056%; w/v). Arruda *et al.* (2005) determined the cell viability of nerolidol on *Leishmania amazonensis* promatigotes and amastigotes using the MTT assay and reported IC_{50} values of 85.22 ± 5.45 and 67.73 ± 3.79 µM, respectively. Seatlholo (2008) determined the effect of the racemic *cis/trans*-nerolidol mixture on human kidney epithelial (HEK-293) cells and obtained an IC_{50} value of 5.50 ± 1.2 µM. This is comparable to the toxicity obtained by *cis*-nerolidol in this research (IC_{50} 10.07 ± 3.05 µM) (Figure 3.9). With regard to dermatological safety levels of *cis*-nerolidol oil in

formulae that goes into fine fragrances, the maximum daily skin exposure of 1.38 $x10^{-6}$% for high end users of the product was reported (Lapczynski *et al.*, 2008).

With regard to the larvicidal assay, it was found that *cis*-nerolidol possessed more potent activity than *trans*-nerolidol (LC_{50} values 0.001 and 0.006%, respectively). Wang *et al.* (2015) reported that nerolidol had LC_{50} values of 0.0016 and 0.0021%, for *Ae. albopictus* and *An. sinensis* larvae, respectively. In the same study, Wang *et al.* (2015) reported LC_{50} values for *p*-cymene and (-)-*trans*-caryophyllene as 0.0035 and 0.0038% against *Ae. albopictus* and 53.14 and 0.006% against *An. sinesis* larvae. These findings are also very similar to that of *cis*-nerolidol reported in this study (Figure 3.5). Ali *et al.* (2013) performed the assay on *Ae. aegypti* larvae with *trans*-nerolidol and obtained a LD_{50} value of 0.0013%. These studies support the possibility that components present in EOs are responsible for the larvicidal activity (Silva *et al.*, 2008).

4.1.1.1 Combination of the two most active EOCs

It was observed that the major EOCs of *L. javanica* namely, *p*-cymene and the *cis*-nerolidol isomer, displayed the most active larvicidal properties amongst all tested EOs and EOCs with DDT as a control (LC_{50} value: 2.5 $x10^{-10}$%; Figure 3.5). The potent larvicidal activity of *cis*-nerolidol and *p*-cymene (Figures 3.5 and 3.7) was supported by the findings of the inhibitory effects against the *Ae. aegypti* species with LC_{50} values of 0.001 and 0.004% for *cis*-nerolidol and *p*-cymene, respectively (Wang *et al.*, 2016). Similarly, the LC_{50} value for *p*-cymene on *Ae. aegypti,* was reportedly within the range of 0.002 to 0.004% (Santos *et al.,* 2012). Due to their high larvicidal activity, *cis*-nerolidol and *p*-cymene were combined in a 1:1 ratio and determined to have an additive effect on larvicidal activity (Figure 3.5).

Individual phytochemical insecticides have been perceived as impractical and uneconomical for field application as they tend to be slow acting, time consuming and only active at high concentrations. Phytochemical-combined formulations are thus considered advantageous in vector control management as the combination effect improves activity and decreases the required dose volume. It is important to have a synergistic combination of plant extracts plus EOs as it would target different modes of action and therefore increase the efficacy (Intirach *et al.*, 2012). The present study has established the potential of EO/EOCs as larvicidal agents against *An. arabiensis* larvae. Hence, these results can be utilised for further research to develop safe and effective measures for controlling the malaria vector.

4.1.1.2 Combination of major EOC to replicate the crude EO

In order to observe the contribution of the individual EOCs to the larvicidal activity of the crude EOs, the major EOCs, were combined in proportion according to the EOs' percentage composition (Table 3.3). In comparison to the 80 to 100% mortality observed for the EOs, the reconstituted 'crude' EOs with the major EOCs only caused between 0.10 to 23% larvae mortality (Table 3.3). This aspect was interesting and supported by the statement that "even with the best human efforts, should you in a laboratory combine all the chemicals in the correct proportions, you would still not have the identical oil" (Esoteric Oils, 2017). Such a copy of the oil will not have the same therapeutic effect as the natural and pure essential oil. And though we pride ourselves on being a technology advanced society, modern science can still not unlock the secrets of EOs and why they can do what they do. It has been reported that if all the correct chemical components of lavender oil were combined, including lavandulol, borneol, terpineol, geraniol and linalool, the final product would not be as successful in treating burns the way that the whole lavender oil does (Cuttle *et al.*, 2009; Esoteric Oils, 2017). Not much research has been found to substantiate the fact that a reconstituted EO would have the same or different pharmacological effects as the original EO.

4.1.1.3 Potential larvicidal mechanism of action

The EO/EOCs potential mechanisms of larvicidal action are based on findings that plant secondary metabolites cause physiological disruptions in insect bodies. It has been observed that the metabolites disrupted nerve cell membrane action, hormonal balance, mitotic poisoning, morphogenesis, blockage of calcium and GABA-gated chloride channels, sodium and potassium ion-exchange disruption and specifically acetylcholinesterase (AChE) inhibition by EOs. The most important mechanism was the inhibition of AChE as a key enzyme of the termination of nerve impulses through the synaptic pathway resulting in paralysis, as seen by organophosphates (Rattan, 2010). Organophosphates are anti-esterase insecticides and exerted their acute effects by causing overstimulation at cholinergic nerve terminals. This process occurs in both insects and humans. Normally, AChE catalyses the degradation of the neurotransmitter acetylcholine in the synapse (Figure 4.4.B). Organophosphate pesticides phosphorylate acetylcholine, thereby reducing the ability of the enzyme to break down the neurotransmitter (Figure 4.4.C). This produced an accumulation of acetylcholine in the central and peripheral nervous systems, resulting in an acute cholinergic syndrome via continuous neurotransmission. The clinical onset of cholinergic over-stimulation can vary from almost instantaneous to several hours after exposure depending on the dose or agent exposed to (Department of Environmental and Occupational Health Sciences, 2007) (Figure 4.4).

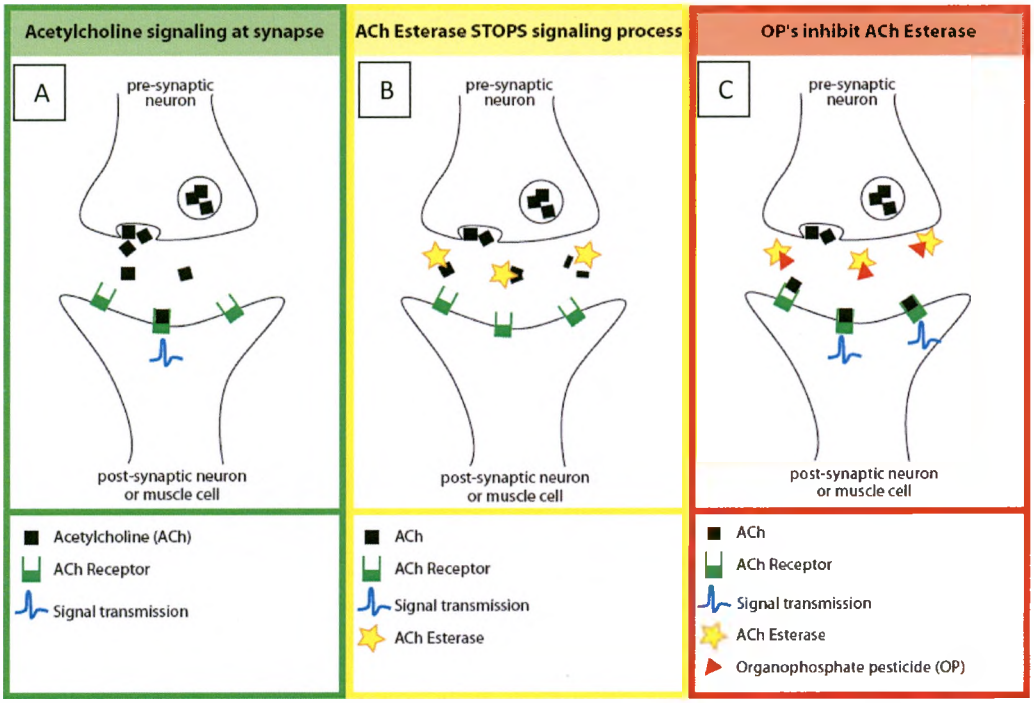

Figure 4.4: Mechanism of acetylcholinesterase action in neurotransmission (Department of Environmental and Occupational Health Sciences, 2007).

It had been observed that the EOs and EOCs affects AChE levels (Hematpoor *et al.*, 2016). The cholinergic toxidrome represented the acute phase of cholinesterase inhibitor poisoning as a result from the accumulation of excessive levels of acetylcholine in the synapses, glands, smooth muscles, and motor end plates where cholinergic receptors are found. As mentioned by Hematpoor *et al.* (2016), strong inhibition of AChE was displayed in various larvae species by plant extracts/ EOs. This muscle relaxant effect on the larvae may be the reason that treated larvae, such as that observed by (±)-linalool, did not moved and appeared dead; however they moved when probed (Section 3.4.1).

According to the mode of action, AChE inhibitors can be divided into two groups: irreversible and reversible. Reversible inhibitors, competitive or noncompetitive, mostly have therapeutic applications, while toxic effects are associated with irreversible AChE activity modulators (Colovic *et al.*, 2013). The pesticides, carbamates and organophophates are reversible and irreversible inhibitors of AChE, respectively (Aldridge and Reiner, 1969). The majority of organophosphates (Figure 4.5) had been used as nonspecific insecticides for over fifty years, to control a variety of insects in agriculture and the household environment. In the 1970's organochlorine insecticides (DDT, dieldrin and aheptachlor) were banned because of their accumulation in the environment, and were replaced by more degradable organophosphates.

Most of current insecticides such as organophosphorus are based on inhibiting AChE (Aldridge and Reiner, 1969). AChE catalyses the hydrolysis of acetylcholine, a neurotransmitter for cholinergic neurotransmission in insects. The neurotoxic compounds/EOs would hydrolyse the neurotransmitter acetylcholine to terminate neuronal excitement at the postsynaptic membrane (Delfino *et al.*, 2009). Supporting the inhibition of AChE by EO/EOCs, Hematpoor *et al.* (2016) determined the larvicidal activity on *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* larvae as well as the *in vivo* acetylcholine inhibition assay with constituents from *Piper sarmentosum* namely; asaricin, isoasarone and *trans*-asarone. High larvicidal potency of asaricin, isoasarone and mild activity of *trans*-asarone was observed along with strong inhibition on AChE. These findings coupled with the high AChE inhibition suggested that asaricin 1 and isoasarone 2 were neuro-toxic compounds towards the larvae.

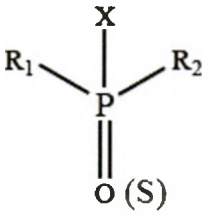

Figure 4.5: General structural formula of organophosphates (Colovic *et al.*, 2013).

4.1.1.4 Morphological aberrations observed on *An. arabiensis* larvae

Physical changes were microscopically observed between the treated and the untreated *An. arabiensis* larvae (Figure 3.6). The treated larvae were darkly discoloured and shrivelled in appearance. It was noted that the majority of the dead larvae sunk to the bottom of the cup; however, a few larvae were found floating on the edge. Morphological abnormalities such as lack of hair (Figure 3.6.G.2), enlarged thorax (Figure 3.6.G.1), elongation of the neck (Figure 3.6.H.1), and disfigured heads (Figures 3.6.F.1 and 3.6.K.1) were noted in the treated larvae. A finding was that the EO/EOCs namely; geranyl acetate, *p*-cymene, *trans*-nerolidol, *L. javanica*, (-)-α-pinene, *cis*-nerolidol, *C. citratus* and (±)-linalool, darkened the abdominal area of the larvae compared to the untreated larvae (Figures 3.6.A, C-I, K).

The darkening of the midgut and hindgut, as well as of their contents, may have many causes, and resulted from a number of mechanisms, including melanisation due to activation of phenoloxidase cascade (Shao *et al.*, 2012). In turn, this activation could be due to several factors such as microbial infection, presence of components of cell walls from microorganisms and algae, parasitoids, action of proteolytic enzymes and tissue damage (Ashida *et al.*, 1983; Rowley *et al.*, 1990).

There are very few studies on the morphological effects of EOs on mosquito larvae, but one done by Procópio *et al.* (2015) on *An. aegypti* larvae with leaf extracts to verify if the darkening of the midgut of mosquito larvae was due to melanisation was reported. Phenylthiourea, a strong phenoloxidase inhibitor, was added to a 1.0% (w/v) *Schinus terebinthifolius* leaf extract and the strong pigmentation of larval midgut was unaffected, indicating that the midgut darkening was not related to melanisation. Other possible mechanisms could include tissue injuries caused by the extract, or due to the accumulation of leaf extract into the larval midgut. It was, however, observed that the treated larvae had a shrunken and pigmented midgut compared to the control with no apparent alterations. Tomé *et al.* (2014) analyzed larvae to have a concentration-dependent decrease in swimming distance, increase in resting time and slower swimming abilities. Comparatively, the EO/EOCs including *O. basilicum*, *C. citratus*, *C. nardus,* geranyl acetate, *p*-cymene, eucalyptus, *trans*-geraniol, (-)-α-pinene, (-)-β-pinene, *artemisia* ketone, (-)-linalool and (-)-*trans*-caryophyllene decreased swimming abilities and increased resting time (Section 3.3.2).

Similarly to the morphological aberrations observed in this study (Section 3.3.2; Figure 3.6), Pratti *et al.* (2015) reported that the EO of *Schinus terebinthifolia* Raddi (major EOCs were δ-3-carene, α-pinene and sylvestrene) inhibited 3rd instar *Stegomyia aegypti* larvae in a dose-dependent manner with a LC_{50} value of 0.0476% after 24 hours of treatment. It was observed that there was a loss of tufts of bristles on the segments, reduced thickness of the exoskeleton, shrinkage, loss of definition in the internal organs and an overall loss of structural integrity of the larvae. The exoskeleton may be involved in toxicity caused by the EOCs. The reduced thickness of the exoskeleton may be due to a decreased chitin synthesis, which is a process sensitive to cellular adenosine triphosphate (ATP) concentrations. Internal organs including intestine, air siphon and Malphighian tubules are also depend on ATP supply for physiological functioning (Patti *et al.*, 2015). Soonwera and Phasomkusolsil (2016) evaluated the larvicidal activity of *C. citratus* and *Syzygium aromaticum* on 3rd / 4th instar *Ae. aegypti* and *An. dirus* larvae. It was noted that both EOs caused morphologically changes in *Ae. Aegypti,* including deformed larvae, elongated neck region, enlarged thorax, absent siphon tube, saddle and hair tufts which led to larval mortality. In *An. dirus*, morphological aberrations were rare and *S. aromaticum* caused a higher mortality than *C. citratus.* The abnormalities commonly observed were deformed, decolourised, and partially exuviated individuals attached to the moulting skin. The results indicated a metamorphosis-inhibiting effect of the EOCs. In the present study, it was determined that after the 24 hour treatment of *An. arabiensis* larvae, *C. citratus* resulted in a darkened abdominal region and disfigured head (Figure 3.6.I). These effects could be due to

the observations that phytochemicals produce morphological aberrations in the different developmental stages of mosquitoes (Soonwera and Phasomkusolsil, 2016). Life cycle abnormalities were observed in various experiments. Karmegam *et al.* (1997) observed that indigenous plant extracts prolonged the larval and pupal duration of *Cx. quinquefasciatus.* Saxena *et al.* (1993) observed that alkaloids isolated from *Annona squamosa* induced morphological abnormalities such as larval–pupal intermediates in *An. stephensi.* Saranya *et al.* (2013) observed that aqueous leaf extract of *Spathodea campanulata* affected *Ae. aegypti* larval morphology leading to dechitinised larvae (lack of the hard substance found in the larval exoskeleton) with damaged digestive tracts and exuvia of the proceeding instars attached to the dead. In the current study, some of the larvae exposed to *cis*-geraniol did progress to the pupal stage, however, no exuvia or adult mosquito was present after the 24 hour treatment.

The current study has shown that the EOs and EOCs produced significant larvicidal activity against 3rd or 4th instar larvae of *An. arabiensis*. However, these EOs/EOCs were toxic and would warrant further investigation to serve as suitable alternatives to synthetic insecticides, as they were relatively inexpensive and are readily available in many areas of the world (Soonwera and Phasomkusolsil, 2016).

4.1.2 *p*-Cymene

The EOC, *p*-cymene is a naturally occurring aromatic organic compound and is classified as an alkyl benzene related to a monoterpene, with its structure consists of a benzene ring *para*-substituted with a methyl group and an isopropyl group (Figure 2.1.18) (Favre and Powell, 2013). *p*-Cymene is an important intermediate used in pharmaceutical industries and for the production of fungicides, pesticides and flavouring agents (Selvaraj *et al.*, 2002). Toxicity was only observed against the human kidney epithelial (HEK-293) cells (IC_{50} value: 4.88 µM; 7.62 x10^{-5}%) (Figure 4.1). Kpoviessi *et al.* (2014) also determined cytotoxicity on the macrophage-like Chinese Hamster Ovary (CHO) and human non-cancer fibroblast (W138) cells yielding IC_{50} values >5 x10^{-3}%. *p*-Cymene has shown notable larvicidal properties with an IC_{50} value of 4.00 x10^{-3}% (230.37 µM) (Figure 3.5). This was comparable to the LC_{50} values of *p*-cymene (1.92 x10^{-3} and 4.67 x10^{-3}%) on *An. aegypti* and *An. albopictus* as determined by Cheng *et al.* (2009). As mentioned in Section 4.1.1.1 this potent activity of *p*-cymene was the reason for it being combined with *cis*-nerolidol and treating the *An. arabiensis* larvae. The observed additive interaction is an added advantage of possibly using *p*-cymene as a larvicide (ΣFIC of 0.94 ± 0.15; Figure 3.7).

4.1.3 Linalool

Linalool has been therapeutically used as an analgesic, antidepressant, anti-epileptic, anti-inflammatory, anti-psychotic, anxiolytic and a sedative agent (Kamatou and Viljoen, 2008). Linalool is a naturally occurring terpene alcohol with a stereogenic center at C3 which allows for the two stereoisomers (R)-(+)-linalool and (S)-(-)-linalool. It has been noted that pure enantiomers may have different activities from each other and that oils that contain the pure enantiomer can be used for isolation purposes (Ozek *et al.*, 2010); as such thesecommercially enantiomers, (±)-linalool and (-)-linalool (Figures 2.1.7 and 2.1.8), were purchased and separately evaluated. It was observed in the GC-MS analysis (Table A.2) that the 65.2% of linalool in *L. javanica* could be responsible for the crude EOs' antimalarial activity (Figure 3.3). Linalool is also a major EOC in *O. basilicum* and is present in a higher percentage yield (54.3%) than in *L. javanica* (Table A.3).

4.1.3.1 Potential antimalarial mechanism of action

The potential mechanism of *L. javanica* and *O. basilicum* antimalarial activity can be associated with the activity of their major EOCs, nerolidol (Section 4.1.1) and linalool (Section 4.3.1) (Appendix A.1 and A.3). The metabolic pathways in the parasite apicoplast are important as an antimalarial drug target, including doxylamine and artemisinin derivative production to overcome *P. falciparum* malaria (Odom, 2011; Jordão *et al.*, 2011). The biosynthesis of several isoprenoids in *P. falciparum*, as mentioned below, has also been observed as a possible mechanism of action (Goulart *et al.*, 2004). An apicoplast is a non-photosynthetic plastid-like organelle which contains metabolic pathways critical for liver-stage and blood-stage development of *Plasmodium*. Among several pathways in apicoplast, isoprenoid biosynthesis is one of the important pathways in parasites as its replication in human erythrocytes requires isoprenoids (Qidwai *et al.*, 2014). Two different routes have been identified to biosynthesise isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). The mevalonate pathway is present in most eukaryotes and the non-mevalonate pathway also known as the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway or 1-deoxy-D-xylulose-5-phosphate (DOXP) pathway is present in bacteria and plant plastids, especially in several pathogenic microorganisms (Lombard and Moreira, 2011). Malaria parasites produce their isoprenoids by means of the non-mevalonate pathway (Odom, 2011). The non-mevalonate pathway of isoprenoid biosynthesis is an alternative metabolic pathway leading to the formation of IPP and DMAPP which consists of 8 steps (Figure 4.6) (Eisenreich *et al.*, 2004). It plays an important role in protein prenylation, cell membrane maintenance, hormones, protein anchoring and *N*-glycosylation (Qidwai *et al.*, 2014).

Lopes *et al.* (1999) showed that 0.031 µM of *cis/trans*-nerolidol not only prevented the parasites from developing into schizonts, but it also prevented haemozoin formation. The mechanism of antiplasmodial action had been noted mainly in the ring and young trophozoite intra-erythrocytic stage by means of inhibiting glycoprotein biosynthesis by repressing the biosynthesis of *N*-glycoproteins as evaluated by following the incorporation of [$U^{14}C$]-glucose into the parasite (Lopes *et al.*, 1999). Goulart *et al.* (2004) noted that nerolidol inhibited the biosynthesis of the isoprenoid chain attached to the benzoquinone ring in the intra-erythrocytic stages of the *P. falciparum* parasite.

It was mentioned that hematophagous organisms which include *Plasmodium* do not contain any functional haem oxygenase activity. Instead, they must utilise a unique pathway to crystallize haem into a non-toxic biomaterial, known as haemozoin (Sigala *et al.*, 2012). During the trophozoite stage of the intraerythrocytic life cycle, *P. falciparum* ingests up to 80% of the host haemoglobin through a protozoan, phagocytic organelle known as the cytostome. Host haemoglobin is taken up by the parasite and transported to the digestive vacuole through the cytostome. In this acidic organelle the haemoglobin is digested into small peptides and four toxic haem units (ferriprotoporphyrin IX). Neutral lipid bodies mediate the detoxification of the haem byproduct through the formation of haemozoin (Figure 4.7; Goldberg *et al.*, 1990).

Moreover, exposure of the parasites with nerolidol at doses 2.2 times below the IC_{50} of 0.12 µM inhibited the production of the isoprenic chain attached to coenzyme Q at all intra-erythrocytic stages of *P. falciparum*. Both *cis*- and *trans*-nerolidol inhibited the isoprenylation of proteins which interfered with mitochondrial metabolic processes (De Macedo *et al.*, 2002). These findings indicated that nerolidol possessed strong antimalarial activity by inhibiting the development of the intra-erythrocytic stages of the parasites. It was found that *trans*-nerolidol, *cis*-nerolidol and (±)-linalool also displayed antimalarial activity against the *P. falciparum* NF54 strain with IC_{50} values of 6.09 ± 0.74, 1.99 ± 0.37 and 76.61 ± 10.15 µM, respectively, compared to the IC_{50} value of 0.120 µM of nerolidol obtained by De Macedo *et al.* (2002) on a chloroquine-sensitive *P. falciparum* isolate (S20). Seathlolo (2008) determined that the (±)-linalool antimalarial IC_{50} value was 254.40 µM on the chloroquine-sentitive *P. falciparum* FCR-3 strain.

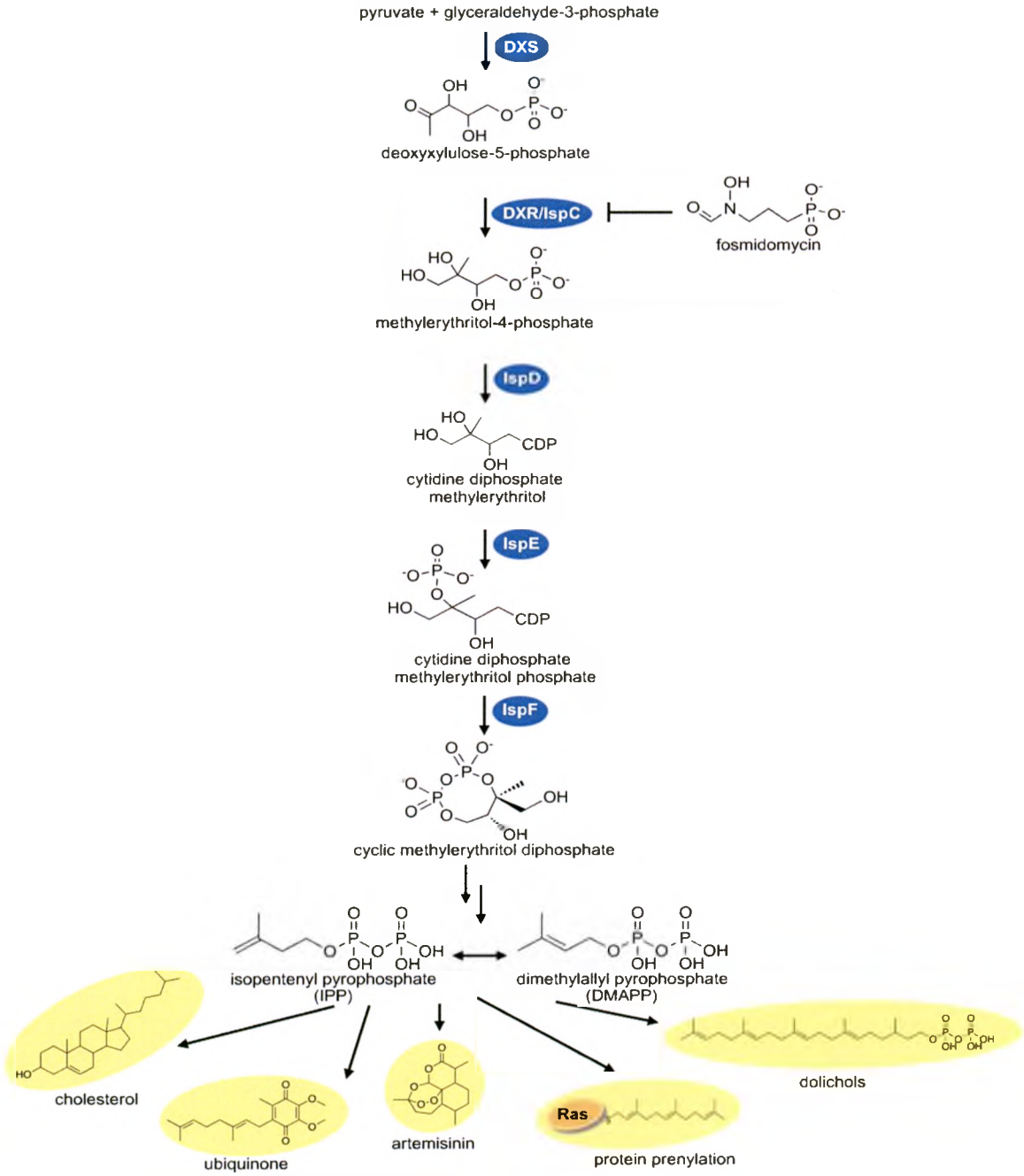

Figure 4.6: Synthesis of isoprenoid by the non-mevalonate pathway (Odom, 2011).

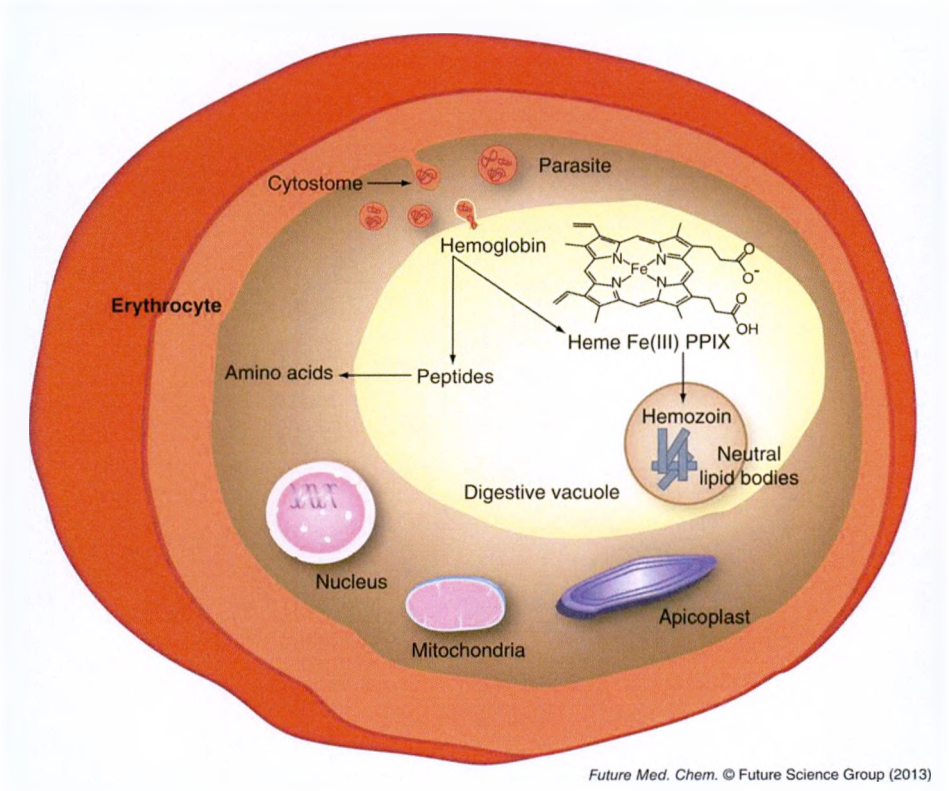

Figure 4.7: Haemozoin formation within the intraerythrocytic life cycle of *P. falciparum* (Goldberg *et al.*, 1990).

Goulart *et al.* (2004) evaluated the biosynthesis of several isoprenoids in *P. falciparum* and terpenes (molecules with a similar chemical structure to the intermediates of the isoprenoids pathway) as potential antimalarial drugs (Goulart *et al.*, 2004; Proteau, 2004; Steinbacher *et al.*, 2003). And thus the effects of different terpenes (farnesol, nerolidol, limonene and linalool) and S-farnesylthiosalicylic acid on the intra-erythrocytic stages of *P. falciparum* were determined. All the latter terpenes inhibited dolichol biosynthesis in the trophozoite and schizont stages. Farnesol, nerolidol, and linalool showed stronger inhibitory activity on the biosynthesis of the isoprenic side chain of the benzoquinone ring of ubiquinones in the schizont stage. The inhibitory effect of the terpenes and S-farnesylthiosalicylic acid on the biosynthesis of both dolichol, the isoprenic side chain of ubiquinones, and the isoprenylation of proteins in the intra-erythrocytic stages of *P. falciparum* appeared to be specific, because overall protein biosynthesis was not affected (Qidwai *et al.*, 2014) (Figure 4.8). These findings could not only result in the antimalarial properties of the terpenes and whole EOs, but also could lead to the possible larvicidal mechanism of action of *L. javanica* due to its high composition of linalool and nerolidol.

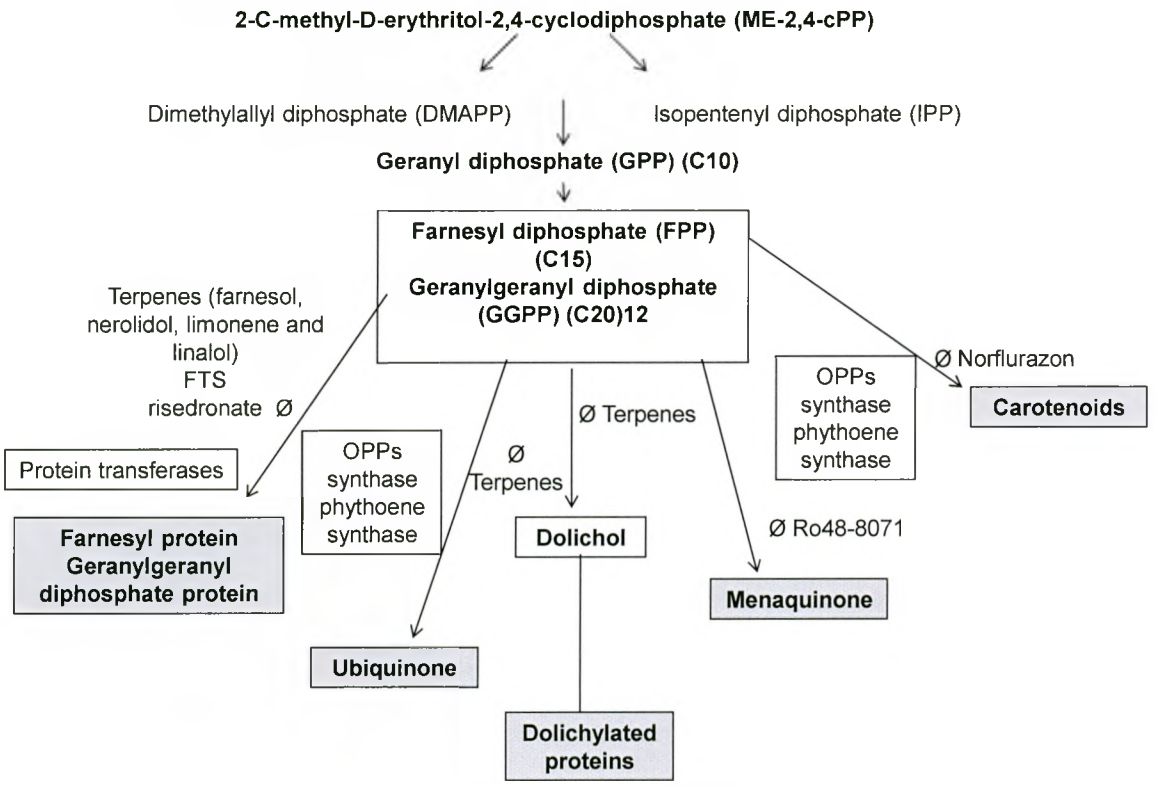

Figure 4.8: Biosynthesis of several isoprenoids by *P. falciparum*.

This figure is a continuation from cyclic methylerythritol diphosphate of Figure 4.6. IPP and DMAPP are biosynthesised by MEP. The enzymes of this pathway that were characterised in *P. falciparum* are represented in white boxes and the corresponding inhibitors are in circles. In subsequent steps, IPP and DMAPP are utilised by prenyltransferases to produce a variety of linear allylic prenyl diphosphates of increasing size. GPP C10, FPP C15 and GGPP C20 are intermediates for the synthesis of the wide range of end products derived from the isoprenoid pathway. The shaded boxes indicate the isoprenic compounds that have been characterised in *P. falciparum*. The inhibitor of each isoprenic product is indicated in circles. In the white box, the bi-functional enzyme octaprenyl pyrophosphate synthase (OPPs)/ phythoene synthase (PfB0130w) is represented (Jordão *et al.*, 2011).

Usta *et al.* (2009) assessed the cellular viability effect of the major EOCs of *C. sativum* (coriander) i.e. linalool on hepatocellular carcinoma cells (Hep-G2) and embryonic kidney cells (HEK-293) using the MTT assay. At a 2 µM concentration, significant cell death (100%) was obtained with a LD_{50} of 0.4 µM for the Hep-G2 cells. However, the viability of the HEK-293 cells was not affected by linalool. The same profile for the Hep-G2 cells was obtained using the trypan blue and neutral red viability assays. It was noted that linalool affected the Hep-G2 cell mitochondria by inhibiting the complex I (NADH oxidase and NADH-UP

reductase) and complex II (succinate dehydrogenase) activities. This led to a decrease in ATP production and an increase in reactive oxygen species generation which would consequently result in cell death. A decrease in glutathione level was also observed in Hep-G2 cells. A reduced glutathione level affects the defence mechanism in the cells against oxidative stress by decreasing the reduction of harmful peroxides and hydroperoxides (Usta *et al.*, 2009).

Goulart *et al.* (2004) reported on the antimalarial activity of (±)-linalool on a 3D7 chloroquine-sensitive strain with an IC_{50} value of 0.28 mM; where it was found to inhibit the isoprenoid pathway (Goulart *et al.*, 2004). It has been noted that (±)-linalool has a similar structure to that of *cis*- and *trans*-nerolidol with a hydroxyl group (Figures 2.1.13 and 2.1.14), however, the presence of a shorter chain reduced the antimalarial activity of (±)-linalool (Nakatsu *et al.*, 2000). The biological properties of linalool was determined and the enantiomer, (±)-linalool, displayed antimalarial activity (IC_{50} value: 76.81 ± 10.15 µM; Figure 3.2) as well as larvicidal activity (LC_{50} value: 0.025 ± 0.003 %; Figure 3.5). Toxicity was observed in the MTT cellular viability assay where the (-)-linalool enantiomer yielded an IC_{50} value of 0.19 ± 0.11 µM (Figure 3.11).

Only the (±)-isomer linalool possessed larvicidal activity, with a LC_{50} value of 0.025%. Pandey *et al.* (2013) reported a similar LC_{50} value for linalool as 0.024% on the *Ae. aegypti* larvae. It was also reported that linalool was a more active monoterpene than geraniol, due to linalool possessing a tertiary alcohol whereas geraniol only has a primary alcohol (Figures 2.1.7, 2.1.8 2.1.11, and 2.1.12) (Pandey *et al.*, 2013). Linalool was shown to be an effective, reversible inhibitor of AChE in insects. The specific toxic effect of linalool on animals is therefore likely to be caused by its neurotoxic specifically its neuro-pharmacological mode of action (López and Pascual-Villalobos, 2010).

4.2 *Artemisia afra*

Artemisia afra belonging to genus *Artemisia* is widely used in many parts of the world either alone or in combination with other plants as a herbal remedy for a variety of ailments including the prevention and management of malarial symptoms (Roberts, 1990; Mander, 1998). There are more than 100,000 traditional healers practicing in South Africa and *A. afra* has been used as an infusion to treat malaria in the southern parts of Africa (Mander, 1998). However, a review found that up to the year 2000, *A. afra* did not attract researchers but only later, especially from 2005 onwards was there an increase in research. The scientific studies from 1993 onwards were based on investigating the antimalarial, antibacterial, antifungal,

anticancer, anti-tuberculotic, anti-trypanosomal, anti-ulcerative, anti-oxidant and protective myocardial activities of *A. afra* plant extracts and EOs (Patil *et al.*, 2011; Maroyi, 2017). Traditionally, an infusion of *A. afra* has been widely used along with *L. javanica* in the treatment of malaria (Fowler, 2006; Watt and Breyer-Brandwijk, 1962). It is also mentioned that *A. afra* has been used to treat fever and symptoms of an inflamed throat in children (Bally, 1937). *Artemisia afra* is rich in terpenes and is therefore likely to have valuable biological activities (Liu *et al.*, 2009).

In this study, the EO of *A. afra* was found to have antimalarial activity and possessed a good toxicity profile by only displaying toxicity towards the human kidney epithelial (HEK-293) cells, hence the integrity of the RBC membrane was maintained and no notable *Artemia* nauplii lethality was observed (Figure 4.9). However, no notable larvicidal activity or lipid peroxidation inhibition was noted for *A. afra*. Figure 4.9 displays the antimalarial and cytotoxic properties of *A. afra* and its major EOCs, as well the other variable activities of these major EOCs. *Artemisia afra* was composed of the main EOCs of eucalyptol, *artemisia* ketone, α-pinene, as well as the (-)-α-thujone and β-thujone isomers (Table A.2). The latter EOCs are discussed below; but it was noted that eucalyptol was also a major EOC in *O. basilicum* (Section 4.3) and α-thujone was such in *L. javanica* (Section 4.1).

In this study, the antimalarial activity observed by *A. afra* on the *P. falciparum* NF54 chloroquine-sensitive strain yielded an IC_{50} value of 4.1 $x10^{-4}$% (Figure 3.3). Research on the EO of *A. afra* was limited; however, there have been investigations on the extracts of *A. afra*. Clarkson *et al.* (2004) studied 134 species of plants native to South Africa representing 54 families for *in vitro* anti-plasmodial activity against the chloroquine-sensitive *P. falciparum* strain (D10) using the pLDH assay. Of the species assayed, 49% showed promising anti-plasmodial activity (IC_{50} ≤1.0 $x10^{-3}$ %) while 17% were found to be highly active (IC_{50} ≤5.0 $x10^{-4}$%). The IC_{50} value for *A. afra* leaf extract in (i) (DCM), (ii) DCM/MeOH (1:1), (iii) MeOH and (iv) water were respectively, 5.0 $x10^{-4}$, 7.3 $x10^{-4}$, 8.0 $x10^{-4}$ and >1.0 $x10^{-2}$%. This indicated that the non-polar solvent DCM extract had the highest activity against the *P. falciparum* parasite. The MeOH and water solvent extracts displayed antimalarial effects on the chloroquine-sensitive (D6) and chloroquine-resistant (W2) *P. falciparum* strains. Antimalarial activities of the D6 strain of MeOH and water extracts were presented with IC_{50} values of 9.00 $x10^{-4}$ and 1.12 $x10^{-3}$%, respectively. The IC_{50} values of the W2 strain of MeOH and water extracts were 3.98 $x10^{-4}$ and 4.65 $x10^{-4}$%, respectively (Clarkson *et al.*, 2004). Eucalyptol was the only EOC of *A. afra* that displayed antimalarial activity (IC_{50} value: 34.64 µM; 6.19 $x10^{-6}$%; Figure 3.2). Artemisinin is known to display antipyretic properties in the treatment of malaria. However, artemisinin is not present in the plant *A. afra* but in that of *A.*

annua. *Artemisia annua* is an annual herb containing sesquiterpene lactones, flavonoids and EOs. Similarly to *A. afra*, *A. annua* contained the EOs of (-)-*trans*-caryophyllene, eucalyptol and *artemisia* ketone and is used for the treatment of fever, malaria, skin diseases, jaundice and haemorrhoids (Klayman *et al*., 1984).

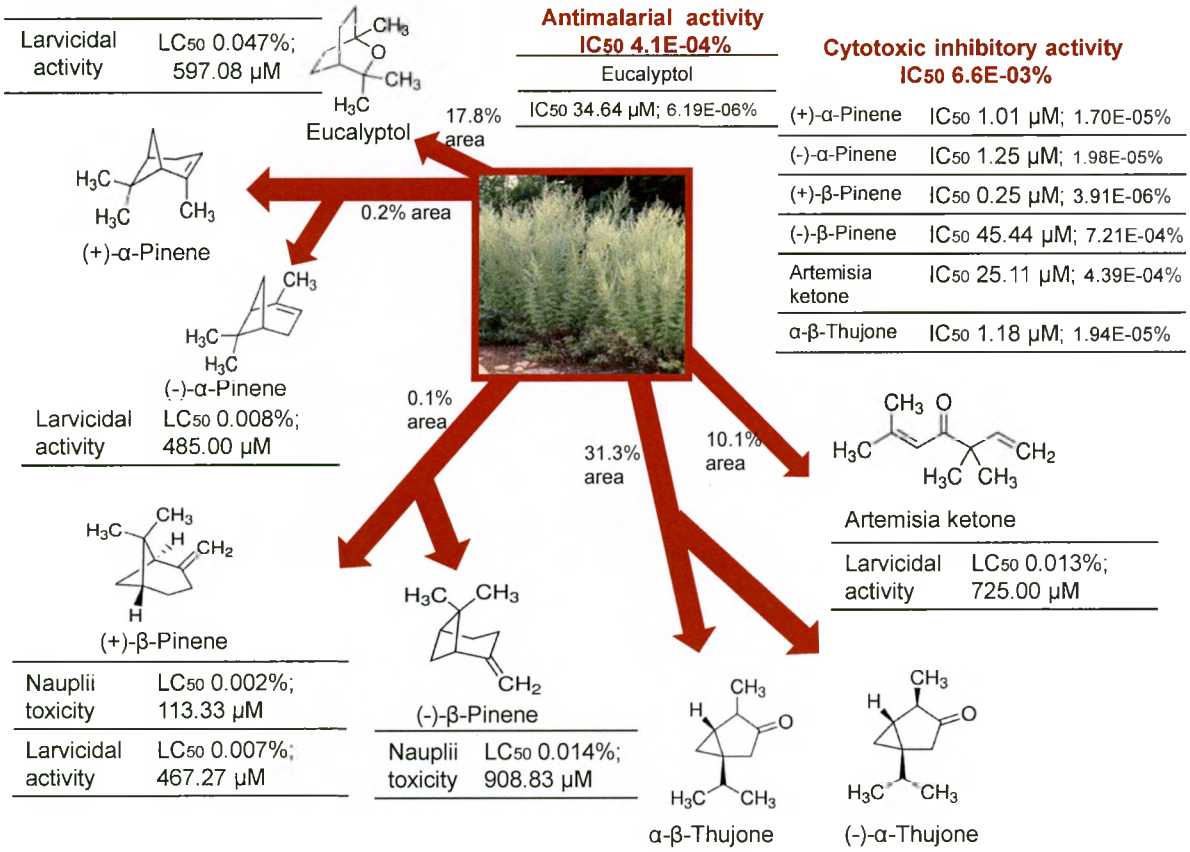

Figure 4.9: The properties displayed by the EO, *A. afra*, and its major EOCs.

Cytotoxicity tests are an essential part of developing a potential pharmaceutical product into a clinically acceptable drug. These tests provide a screening method to determine whether the test compounds are not more harmful to the normal biological processes than the effects they are being tested for (Mativandlele, 2008). With regard to the toxicity of *A. afra*, a cytotoxic activity was observed on the HEK-293, yielding an IC_{50} value of 6.6 $x10^{-3}$% compared to the anticancer drug, camptothecin, IC_{50} value of 9.6 $x10^{-5}$% (Figure 3.12). *Artemisia afra* was also found to affect other cell lines with IC_{50} values greater than 0.03% and thus were considered to be non-toxic to confluent Chang liver cells and human hepatoma carcinoma (Hep-G2) cells (Pruissen, 2013). Hubsch (2014) also observed no inhibitory effects on *Artemia* nauplii viability within 24 hours of exposure, however, 0.01% aqueous and organic extracts of *A. afra* demonstrated a potential toxicity with an IC_{50} value of 68.28 ± 4.64% against the human kidney epithelial (HEK-293) cells. The pinene isomers,

artemisia ketone, *p*-cymene and the α-β-thujone isomer displayed toxic properties against the human kidney epithelial (HEK-293) cells (Figure 3.10). The most commonly known toxic effects of *A. afra* are central nervous system effects which are due to the thujone content of the plant (Watt and Breyer-Brandwijk, 1962; Van Wyk *et al.*, 2002). In this study, thujone comprises of 31.3% of *A. afra* (Table A.2), however only the α-β-thujone isomer displayed cytotoxicity against the human kidney epithelial cells (Figure 3.11). As such, the safety profile of *A. afra* remains controversial due to its high thujone content (Patil *et al.*, 2011).

Although *A. afra* did not display significant larvicidal activity, it can be noted that its major EOCs namely; (-)-α-pinene, (+)-β-pinene, eucalyptol, *p*-cymene and *artemisia* ketone, individually had lethality effects against the larvae (Figures 3.5 and 4.9). There is a lack of information based on clinical trial data on the safety and efficacy of *A. afra* (Dube, 2006). This could be one of the reasons why this plant has as yet not attracted too much attention by the industry and validated scientifically through clinical trials (Patil *et al.*, 2011). Therefore, there is a need for further *in vivo* studies and better reporting from clinical trials and from doctors/healers (Hubsch, 2014).

4.2.1 Eucalyptol

Eucalyptol, an oxygenated monoterpene cyclic ether (Figure 2.1.19), has been identified as a promising major EOC as it possesses antimalarial and larvicidal activity (Figures 3.2 and 3.5). Eucalyptol has been used to treat bronchitis, sinusitis and chronic rhinitis and also for the treatment of asthma (Juergens *et al.,* 1998). It constituted a larger proportion of *A. afra* (17.8 %) than *O. basilicum* (5.3 %) (Tables A.2 and A.3). Eucalyptol was the only EOC of *A. afra* that displayed antimalarial activity (IC_{50} value: 34.64 µM; 6.19 $x10^{-6}$ %, Figure 3.2), along with its larvicidal activity (LC_{50} value: 597.08 µM; 4.70 $x10^{-2}$ %; Figure 3.5). This was compared to the antimalarial activity as determined by Seatlholo (2008), whereby an IC_{50} value of 70.20 ± 4.00 µM of eucalyptol on the chloroquine resistant *P. falciparum* FCR-3 strain was displayed. With regard to larvicidal activity, Oliveira *et al.* (2013) determined the activity of the EO, *Piper aduncum* L. with eucalyptol as the main constituent (40.5%) to yield a LC_{50} value of 0.065% on the *An. aegypti* larvae and had been reported due to its AChE activity. No notable toxicity was reported in this study (Tables 3.4. and 3.5) and a review mentioned that no negative effects from animal experiments have been reported so far, but data are rather insufficient. No reproductive, mutagenicity, developmental toxicity or carcinogenicity has been reported until now and sub-acute hepatotoxic and nephrotoxic effects in animal experiments appeared only after the application of high doses (Bhowal and Gopal, 2016).

4.2.2 Thujone

Although thujone, a monoterpene ketone, constituted 31.3% of the *A. afra* EO (Table A.2), with only the α-β-thujone isomer (Figure 2.1.10) displaying cytotoxic properties (IC_{50} value 1.18 µM; 1.94 $x10^{-5}$ %; Table 3.10) compared to (-)-α-thujone (Figure 2.1.9). Seatlholo (2008) determined that the (±)-α-β-thujone isomer display cytotoxic properties on human kidney epithelial (HEK-293) cells with an IC_{50} value of 81 µM, as well as antimalarial activity with an IC_{50} value of 528.60 µM on *P. falciparum* FCR-3 strain. Whereas no antimalarial activity was displayed in this study with parasite viabilities of 84.42 and 99.33% for α-β-thujone (-)-α-thujone, respectively compared to the 5.98% of quinine (Table 3.1). Thujone EOCs are known to be toxic and have been associated with convulsions, mental confusions, epileptic seizures and abortive effects (Cheremisinoff, 2003; Zhou *et al.*, 2004). Toxicity may have been due to reactive metabolites which bound to cellular proteins and DNA, which led to toxicity through cytotoxicity, oncogene activation and hypersensitivity (Zhou *et al.*, 2004).

4.2.3 *Artemisia* ketone

Artemisia ketone is the major EOC of many *Artemisia* plants, often up to 60%. Lutgen (2013) mentioned that scientific literature on *artemisia* ketone was absent. The EOC, *artemisia* ketone constituted 10.1% of *A. afra* (Figure 2.1.20; Table A.2) which could have also contributed to the larvicidal (LC_{50} value: 0.013%; 725 µM; Figure 3.5) and cytotoxic activities (25.11 µM; 4.39 $x10^{-4}$%; Figure 3.10). Published larvicidal studies on *artemisia* ketone are lacking. However, Ali *et al.* (2014) analysed the EO of *A. aborenscens* from Yemen which contained 51.05% *artemisia* ketone and observed cytotoxic properties. An IC_{50} value of 1.69 $x10^{-3}$% was obtained on human colonic adenocarcinoma HT29 tumour cells (Ali *et al.*, 2014).

4.2.4 Pinene

Pinene derivatives have been detected in oils of many plant species. The variations in the EOCs composition of the same plant species may be attributed to a variety of factors such as harvesting season, geographic location, climatic condition, altitude, chemotype or subspecies, reproductive stage, choice of plant part and extraction method (Kamatou *et al.*, 2008). Pinene is a bicyclic monoterpene consisting of α- and β-pinene constitutional isomers, with both structural isomers having enantioners known as (+)-α-pinene, (-)-α-pinene, (+)-β-pinene and (-)-β-pinene (Figures 2.1.1 - 2.1.4; Silva *et al.*, 2012). According to the GC-MS analysis, the α- and β-pinene isomers were present in the tested EO of *A. afra* and only β-pinene was present in *L. javanica* (Tables A.1 and A.2). The α- (0.2%) and β-pinene (0.1%) isomers constituted more in *A. afra* than *L. javanica,* whereas, in *L. javanica*, only the α-pinene (0.2 %) was a major EOC. Varying properties were also observed amongst the isomers. The (+)-β- and (-)-α-pinene isomers displayed larvicidal activity (Figure 3.5), with all

four pinene isomers possessing cytotoxicity and only the β-pinene isomers killing the *Artemia* nauplii (Figure 3.8).

Similar larvicidal activity was observed between (+)-β-pinene and (+)-α-pinene with LC_{50} values of 0.0073 ± 0.01% and 0.0077 ± 0.002%, respectively, whereas the (-)-β- and (+)-α-pinene were inactive (Figure 3.5). Govindarajan *et al.* (2016) determined the effects of α- and β-pinene against *An. subpictus, Ae. albopictus* and *Cx. tritaeniorhychus* larvae, with LC_{50} values for α-pinene reported as 0.0032, 0.0034, and 0.0037%, respectively, and for the β-isomer, as 0.0042, 0.0045 and 0.0048%, respectively. The EOCs were effective at low doses which could be an advantageous alternative to develop newer and safer mosquito control tools (Govindarajan *et al.,* 2016), as the LC_{50} values were comparable to the activity determined in this study (Figure 3.9). In conjunction with the inhibition of AChE being a potential larvicidal mechanism of action, Miyazawa and Yamafuji (2005) determined the AChE inhibition activity of bicyclic monoterpenes which included the pinene isomers. Both (+)-α-pinene and (-)-α-pinene isomers showed strong inhibition with IC_{50} values of 0.40 and 0.44 mM, respectively. Thus it is mentioned that inhibition of AChE by bicyclic monoterpenes is reported to have insecticidal activity (Miyawaza and Yamafuji, 2005).

Toxicity was noted against the *Artemia* nauplii for the (+)-β- and (-)-β-pinene with LC_{50} values of 113.30 and 908.93 µM (0.0012 and 0.014 %) but not for the α-pinene isomers (Figure 3.8). Greater toxicity was observed for the α-pinene isomers against the human kidney epithelial (HEK-293) cells yielding IC_{50} values of 45.44, 1.07 and 1.25 µM for (-)-β-pinene, (+)-α- and (-)-α- pinene, respectively (Figure 3.10). Low safety indices were present for all pinene isomers against the larvae and *P. falciparum* parasites ranging from 0.02 to 1.44 (Table 3.8).

Silva *et al.* (2012) evaluated the cytotoxic effect of (+)-α-pinene and (+)-β-pinene standards against murine macrophages (Swiss mouse peritoneal) at concentrations ranging from 6.25 $x10^{-3}$ to 0.10% using the MTT assay. A comparison of both enantiomers revealed that (+)-α-pinene was more cytotoxic, reducing cell viability by 33.5% with a concentration of 0.01% and by 100% with 5% pinene. Despite being cytotoxic, (+)-β-pinene reduced macrophage viability by 57% at concentrations of 0.03, 0.05 and 0.1%, and was not toxic at 0.01%. Seatlholo (2008) determined the IC_{50} value of 172.20 and 164.10 µM on (+)-α- and (+)-β-pinene on human kidney epithelial (HEK-293) cells, respectively, compared to 71.20 µM quinine.

4.3 *Ocimum basilicum*

Ocimum basilicum is rich in EOs that has been the subject of numerous chemical studies and has been grown as a medicinal plant, culinary herb and as an antimicrobial agent (Grayer *et al.,* 1996). Traditionally uses as a medicinal plant are for the treatment of coughs, headaches, constipation, diarrhoea, kidney malfunction, warts and worms (Simon *et al.*, 1999). It has also been reported for its excellent larvicidal properties (Govindarajan *et al.*, 2013). In this study it was observed that *O. basilicum* displayed antimalarial activity while maintaining the integrity of the RBC membrane. No toxicity was observed on the *Artemia* nauplii; however; *O. basilicum* was highly toxic to the human kidney epithelial (HEK-293) cells and displayed potent lethality effects on the larvae vector. The antimalarial, larvicidal, lipid peroxidation and toxicity properties of eugenol of *O. basilicum* are discussed below.

Ocimum basilicum was the only EO that displayed lipid peroxidation inhibitory properties compared to Trolox (IC_{50} value: 1.2 $x10^{-3}$%; Figure 3.14). The major EOCs of *O. basilicum* included eugenol, eucalyptol and linalool (Figure 4.10; Table A.3). Eugenol was present in the EO of *C. nardus* as well, eucalyptol in *A. afra* (Section 4.2) and linalool in *L. javanica* (Section 4.1). Lipid peroxidation is due to the oxidative stress caused by reactive oxygen species which include free radicals (e.g. nitric oxide, nitrogen dioxide, peroxly, hydroxyl superoxide) and non free radicals (e.g. hydrogen peroxide and oxygen) (Hamzah *et al.*, 2013.) The process alters membrane permeability and causes tissue damage; however the presence of anti-oxidant enzymes (e.g. superoxide dismutase, glucose-6-phosphate dehydrogenase (G6PD) and tissue glutathione) protected the tissue from free radical attack (Rahman, 2007).The ability of the EO/EOCs to inhibit oxidative stress is a protective effect in the host and a potentially beneficial therapeutic effect in pathophysiological disorders causing ischemia, traumatic injury and leukocyte-mediated inflammation (Halliwell *et al.*, 1994; Minotti and Aust, 1989). It was observed in this study that the EO of *O. basilicum* and its major EOC, eugenol, an allylbenzene, (Figure 2.1.17) inhibited lipid peroxidation with IC_{50} values of 0.0018% and 0.0003%, (18.90 µM), respectively (Figure 3.13 and 3.14).

Kumaravelu *et al.* (1996) reported that eugenol possessed hepatoprotective properties against carbon tetrachloride intoxication. It was suggested that with eugenol incorporated into the cell membrane, it was able to protect the membrane from free radical attack (i.e. inhibit lipid peroxidation products in RBCs), and thereby facilitating the normal activities of the anti-oxidant enzymes and induced glutathione-S-transferase activity to remove the oxidative stress imposed by the carbon tetrachloride.

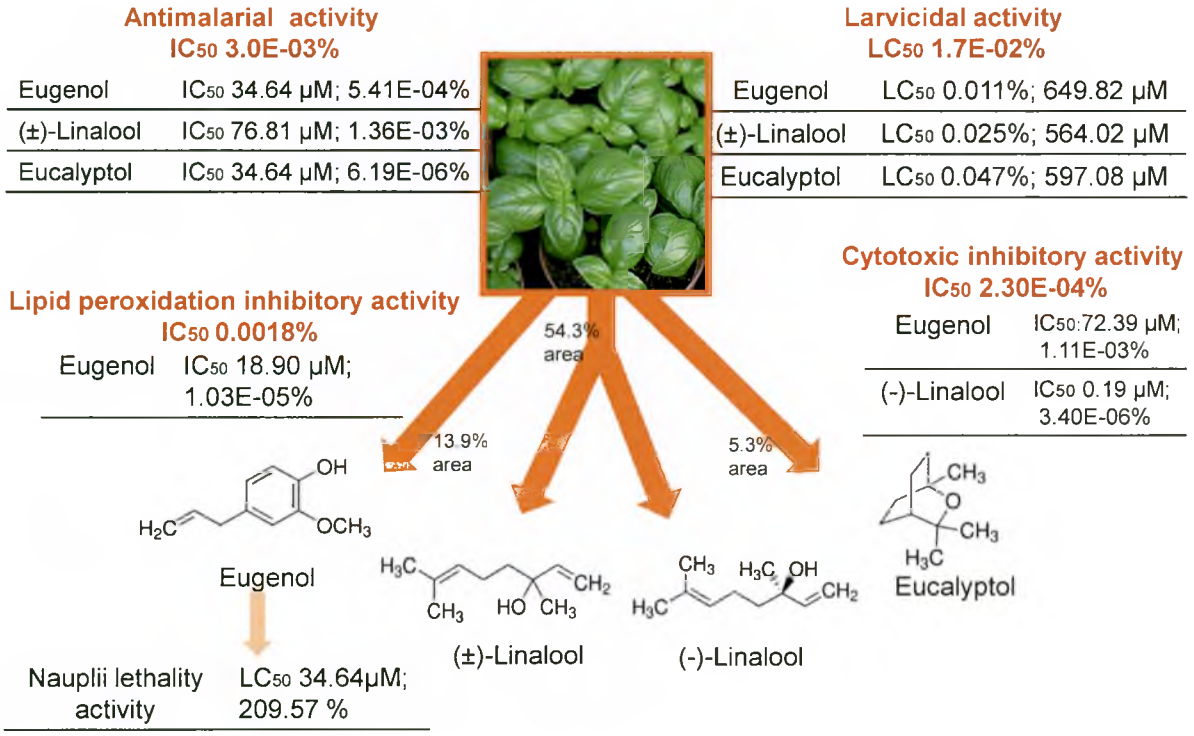

Figure 4.10: The properties displayed by the EO, *O. basilicum*, and its major EOCs.

In support of the anti-oxidant properties of eugenol, Tsujimoto *et al.* (1988) determined that in the presence of eugenol (200 µM), up to 50% lipid peroxidation catalyzed by benzoyl peroxide /Cu^{2+} or hydrogen peroxide /Cu^{2+} was inhibited in human erythrocyte membranes. In contrast, Suzuki *et al.* (1985) reported that short time exposure to eugenol (2 mM) caused superoxide generation and cytotoxicity in neutrophils.

Notable toxicity of *O. basilicum* was only observed on the human kidney epithelial (HEK-293) cells with an IC_{50} value of 2.3 x10^{-4}% (Figure 3.11). Kathirvel and Ravi (2012) conducted studies to identify the chemical composition and *in vitro* activity of the EO of *O. basilicum* against the human cervical cancer (HeLa) and human laryngeal epithelial carcinoma (HEp-2) cell lines and it was found that the EO was cytotoxic to both cell lines with IC_{50} values of 0.009 and 0.010%, respectively. The major EOCs of *O. basilicum* that were found to be cytotoxic included (-)-linalool and eugenol (Figure 3.10). Eugenol was regarded as cytotoxic with an IC_{50} value of 72.4 µM (0.001%) against the human kidney epithelial cells; whilst also displaying toxicity towards the *Artemia* nauplii (LC_{50} value: 0.003%) (Figures 3.10 and 3.8). Comparatively, Wafai *et al.* (2017) also determined eugenol to possess a dose-dependent decrease on the viability of human breast cancer (oestrogen positive) MCF-7 cells (EC_{50} 0.9

mM). The effect was then compared to another breast cancer cell line (oestrogen negative), MDA-MB- 231, and an estimated EC_{50} of 1.6 mM was obtained (Wafai *et al.* 2017). Hume (1984) also reported that 0.2 mM eugenol inhibited cellular viability of mouse fibroblasts by 50% when compared to the controls following a 24 hour exposure. Whilst also inhibiting cellular respiration at concentrations >0.1 mM.

Antiplasmodial activity by *O. basilicum* was observed with an IC_{50} value of 3.0 $x10^{-3}$%, however, being higher than that of the control quinine, 1.7 $x10^{-5}$% (Figure 3.3). The major EOCs of *O. basilicum* that displayed antimalarial activity included (±)-linalool, eugenol and eucalyptol (Figure 3.2). It has been recognized that studies on antiplasmodial activities with *Ocimum* species are too limited. Ntonga *et al.* (2014) determined the antiplasmodial IC_{50} value of 0.002% on a chloroquine-resistant FcB1/Colombia *P. falciparum* strain using the radioisotopic method.

Ocimum basilicum displayed larvicidal properties on the *An. arabiensis* larvae yielding a LC_{50} value of 0.017% compared to the control DDT (LC_{50} value: 2.47 $x10^{-10}$%) (Figure 3.5). The major EOCs, eucalyptol, (±)-linalool and eugenol, displayed larvicidal properties as well with LC_{50} values of 0.047, 0.025 and 0.011%, respectively (Figure 3.9). Similarly, Govindarajan *et al.* (2013) reported larvicidal activity by *O. basilicum* with LC_{50} values of 0.98 $x10^{-3}$, 1.20 $x10^{-3}$ and 1.40 $x10^{-3}$% on *An. subpictus, Ae. albopictus* and *Cx. tritaeniorhynchus* larvae, respectively.

4.4 *Cymbopogon citratus*

Cymbopogon citratus has been extensively utilised globally for its medicinal, cosmetic, and nutritional benefits. Studies on its phyto-constituent composition have documented the presence of tannins, saponins, flavonoids, phenols, anthraquinones, alkaloids, deoxysugars, and various EOCs in the plant. *Cymbopogon citratus* is an economically important aromatic perennial plant of the *Poaceae* family that has been used to extract its EOs for a wide range of applications based on its antibacterial, antifungal, antiprotozoal, anticarcinogenic, anti-inflammatory, anti-oxidant, cardioprotective, antitussive, antiseptic, and anti-rheumatic activities. It has also been used to inhibit platelet aggregation, treat diabetes, dyslipidemia, gastrointestinal disturbances, anxiety, malaria, flu, fever, and pneumonia, as well as in aromatherapy (Ekpenyong *et al.*, 2014). The EO of *C. citratus* has been applied in the control of pathogens and insects and used in traditional medicine as a herbal infusion to treat fever (Masamba *et al.*, 2003). The major EOC of *C. citratus* namely citral, as discussed in this section, constituted 78.5% of the EO. The other major EOCs of *C. citratus* were (-)-*trans*-

caryophyllene as seen in Section 4.1 (*L. javanica*), geraniol and geranyl acetate (Section 4.5.1).

The EO of *C. citratus* presented with antimalarial, larvicidal and variable toxicity properties (Figure 4.11). *Cymbopogon citratus* is commonly used as an insect repellent and has shown promising lethality effects against the mosquito larvae (Figure 3.5). The treatment of *C. citratus* on the *An. arabiensis* larvae yielded a LC_{50} value of 0.019% (Figure 3.5). Ntonga *et al.* (2014) determined the effect of *C. citratus* on *An. funestus* s.s. larvae to have LC_{50} values of 0.0036 and 0.0035% for 4th and 3rd instar larvae, respectively. However, several synergistically interacting compounds could have also been the source of the larvicidal effectiveness of the EO and not necessarily linked to the nature of the dominant compound. This is similar to the finding by Freitas *et al.* (2010) who attributed the larvicidal activity against *Ae. aegypti* to the EOC citral. Citral, a simple a cyclic monoterpene, is constituted of two geometrical isomers, geranial and neral (Figure 2.1.16) (Kpoviessi *et al.*, 2014). However, no notable larvicidal effects by citral against the *An. arabiensis* larvae at a 0.01% (v/v) concentration were observed in this study (Figure 3.4). Although, the other major EOCs, *trans*-geraniol and geranyl acetate of *C. citratus* displayed larvicidal activity (Figure 4.11).

The most potent EO antimalarial activity was exhibited by *C. citratus* against the *P. falciparum* NF54 strain yielding an IC_{50} value of 5.10 x10^{-4}% (Figure 3.3). *Cymbopogon citratus* displayed antiplasmodial activity when evaluated by Ntonga *et al.* (2014) on the *P. falciparum* chloroquine-resistant FcB1/Colombia strain with an IC_{50} of 4.20 x10^{-4} ± 5.0 x10^{-5}%. Whilst a tenfold difference was observed with an IC_{50} value of 4.80 x10^{-3} ± 1.31 x10^{-3}% when the pLDH assay was performed on the *P. falciparum* chloroquine-sensitive-3D strain (Kpoviessi *et al.*, 2014). The major EOCs of *C. citratus* that possessed antiplasmodial activity were (-)-*trans*-caryophyllene and citral (Figure 3.2).

As observed, *C. citratus* possessed larvicidal and antimalarial properties; however there is a concern about the toxic properties of the EO as it was the most cytotoxic against the human kidney epithelial (HEK-293) cells with potent *Artemia* nauplii lethality properties (Figure 3.12). Where *C. citratus* had a LC_{50} of 7.0 x10^{-2}% against the *Artemia* nauplii and an IC_{50} value of 1.2 x10^{-3}% against the human kidney epithelial (HEK-293) cells (Figures 3.8 and 3.12). A similar cytotoxic profile was reported for *C. citratus* on the macrophage-like CHO cell line and human non-cancer fibroblast WI38 cell line with IC_{50} values of 1.06 x10^{-3} and 3.98 x10^{-3}%, respectively (Kpoviessi *et al.*, 2014).

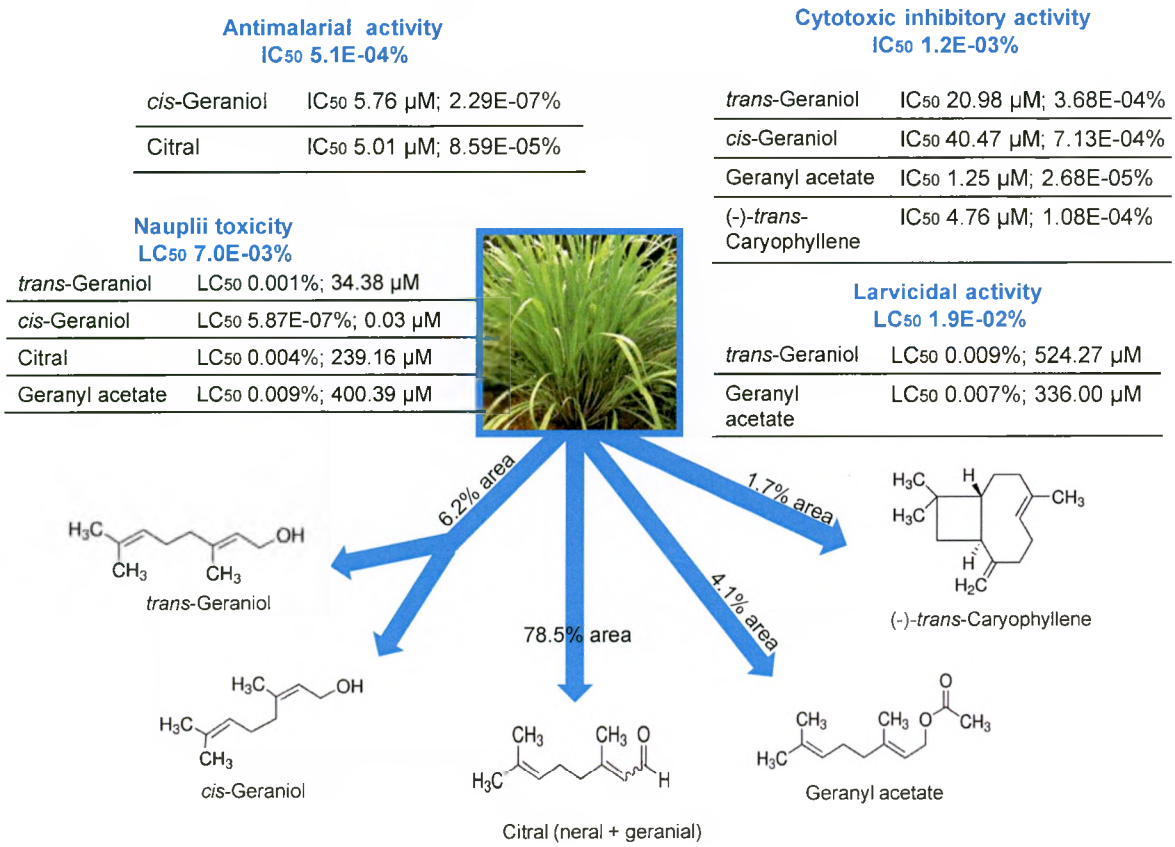

Figure 4.11: The properties displayed by the EO, *C. citratus,* and its major EOCs.

The major EOCs that affected cell viability included (-)-*trans*-caryophyllene, *trans*- and *cis*-geraniol (Figure 3.11). In contrast to the low toxic effect of citral against human kidney epithelial (HEK-293) cells with 73.83 ± 4.00% cell viability at 50 uM (Table 3.6), citral was reported to have an IC_{50} values of 2.06 $x10^{-3}$ and 3.95 $x10^{-3}$% on the macrophage like CHO and human non-cancerfibroblast WI38 cell lines, respectively (Table 3.6; Kpoviessi *et al.*, 2014).

The major EOCs of *C. citratus* that were active against the *Artemia* nauplii were *cis*-geraniol, *trans*-geraniol, geranyl acetate and citral (Figure 3.9). In contrast to the LC_{50} value of 7.0 $x10^{-2}$% determined in this study against the *A. franciscana* nauplii (Figure 3.8), Dosumu *et al.* (2010) obtained an LC_{50} value of 3.56 $x10^{-3}$% on *A. salina* nauplii. Whilst a LC_{50} value of 9.83 $x10^{-4}$% on the *A. salina* nauplii was also determined (Parra *et al.*, 2001). More empirical studies evaluating the effect of *C. citratus* are needed to substantiate its use therapeutically (Ekpenyong *et al.*, 2014).

4.5 *Cymbopogon nardus*

Cymbopogon nardus is used for cooking, perfumery, rheumatism and in the treatment of fevers, intestinal parasites, digestive and menstrual problems (Konwar and Gohain, 1999; Abena *et al.*, 2007). It is one of the most widely used natural repellents on the market, used at concentrations of 5-10% (Trongtokit *et al.*, 2005). *Cymbopogon nardus*, belonging to the same genus as *C. citratus,* possessed the same biological profile by displaying antimalarial, larvicidal, cytotoxic and toxicity properties (Figure 4.12). The EOCs; geraniol, citronellal and geranyl acetate were found in majority in *C. nardus* (Table A.5). Eugenol was also a major EOC with various properties discussed under *O. basilicum* (Section 4.3).

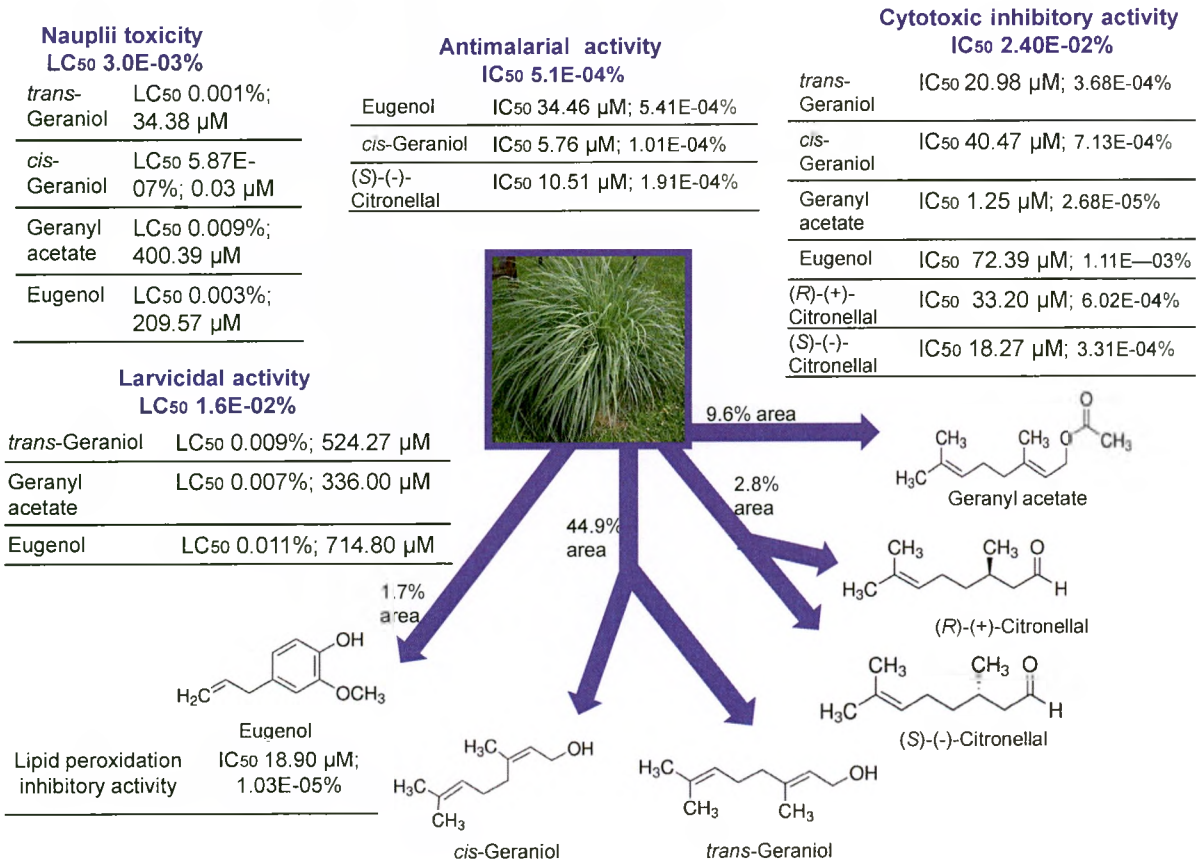

Figure 4.12: The properties displayed by the EO, *C. nardus*, and its major EOCs.

The EO of *C. nardus*, commonly known as citronella oil, has been widely used as an insect repellent (Trongtokit *et al.*, 2005). In this study, *C. nardus* displayed activity against the *An. arabiensis* larvae with a LC_{50} value of 0.0016% in comparison the control, DDT (LC_{50} value: 2.5 $x10^{-10}$%) (Figure 3.5); which is similar in activity towards *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti* larvae with LC_{50} values of 0.005, 0.009 and 0.005% respectively, also after a 24 hour exposure period (Manimaran *et al.*, 2012). The major

EOCs present in the EO, such as geranyl acetate, *trans*-geraniol and eugenol have also exhibited larvicidal activity (Figure 3.5).

As observed, *C. nardus* possessed less potent larvicidal (0.2 fold) and antiplasmodial (24 fold) activity than *C. citratus* (Figures 3.5 and 3.3). This has previously been noted where *C. nardus* yielded a higher IC_{50} value of 5.26 $x10^{-3}$ ± 4.97 $x10^{-4}$% on *P. falciparum* chloroquine-sensitive 3D7 strain compared *to C. citratus* (IC_{50} value: 4.80 $x10^{-3}$ ± 1.31 $x10^{-3}$%) (Kpoviessi *et al.*, 2014). The EOCs of *C. nardus* that displayed antimalarial activity included (S)-(-)-citronellal, *cis*-geraniol and eugenol (Figure 3.2).

The *Cymbopogon* species have potential for the development of antimalarial and larvicidal agents; however a more extended safety profile needs to be established. As it was noted in this study that there was toxicity against the *Artemia* nauplii by *C. nardus* with an LC_{50} value of 3.10 $x10^{-3}$% (Figure 3.8). *C. nardus* was less cytotoxic than *C. citratus* with an IC_{50} value of 2.40 $x10^{-2}$% (Figure 3.11). The major EOCs of *C. nardus* that were toxic against the *Artemia* nauplii included geranyl acetate, *cis*-geraniol, *trans*-geraniol and eugenol, and those toxic against the human kidney epithelial (HEK-293) cells included (R)-(-)-citronellal, (S)-(-)-citronellal, eugenol, *cis*-geraniol and *trans*-geraniol (Figure 3.8). Meyer *et al.* (1982) classified crude extracts/EOs into toxic (LC_{50} <0.1%) and non-toxic (LC_{50} >0.1%) according to the levels required to attain the LC_{50} value against nauplii. Based on this classification, *C. nardus* oil was found to be toxic (LC_{50} <0.1%). These results are similar to the findings whereby *C. nardus* oils were found to be toxic to *A. salina* nauplii (LD_{50} value: 0.012 x 10^{-2}%; Brasileiro *et al.*, 2006). In addition, cellular viability was affected by *C. nardus*, but to a less extent than *C. citratus*. *Cymbopogon nardus* was found to be toxic not only to human kidney epithelial (HEK-293) cells, but also to macrophage-like CHO and human non-cancer fibroblast WI38 cells, where the latter two had an IC_{50} value >5 $x10^{-3}$% (Kpoviessi *et al.*, 2014).

4.5.1 Geraniol

The major EOC, geraniol is an acyclic monoterpene alcohol and is present in the *trans*-geraniol (geraniol) and *cis*-geraniol (nerol) geometric isomeric forms (Figures 2.1.12 and 2.1.13). Geraniol has been reported to exhibit various biochemical and pharmacological properties including anti-oxidant, anti-inflammatory and insecticidal properties (Chen and Viljoen, 2010). Geraniol was also present in the EOs of *C. citratus* (Table A.4). Only the *trans*-geraniol isomer displayed larvicidal activity (LC_{50} value: 524.27 µM; 0.0092%) compared to DDT (LC_{50} value: 2.47 $x10^{-10}$%) (Figure 3.5). This was compared to the LD_{50} value of 0.005% as determined by Ali *et al.* (2013) on *Ae. aegypti.* Whilst Pandey *et al.* (2013) reported a tenfold difference to the Ali *et al.* (2013) study with LC_{50} value of 0.042% on

Ae. Aegypti. Liu *et al.* (2013) determined the larvicidal effect of geraniol on *Ae. albopictus* and determined a LC_{50} value of 0.003%.

The toxicity of *trans*-geraniol was also observed on the *Artemia* nauplii although a factor of nine folds lower (LC_{50} value: 34.38 µM; 0.001%). In contrast, although the *cis*-isomer did not possess any larvicidal properties, it did inhibit the most *Artemia* nauplii viability (LC_{50} value: 0.03 µM; $5.87x10^{-7}$%) in comparison to the control potassium dichromate (LC_{50} value: 0.01%; Figure 3.8). This similar toxicity profile was observed for both isomer when human kidney epithelial (HEK-293) cells were exposed to *trans*- and *cis*-geraniol with IC_{50} values of 20.98 and 40.47 µM, respectively ($3.86 x10^{-4}$ and $7.13 x10^{-4}$%; Figure 3.10). In comparison, it would appear as if the *cis*-isomer was slightly safer than *trans*-geraniol. With regard to previous cytotoxicity studies, Seatlholo (2008) determined that *trans*-geraniol, resulted in an IC_{50} value of 128.5 ± 4.70 µM, when exposed to human kidney epithelial (HEK-293) cells. However, with regard to antimalarial properties, only the *cis*-geraniol isomer depicted antimalarial properties against the chloroquine-sensitive *P. falciparum* strain with an IC_{50} value of 5.76 µM (Figure 3.2) compared to 0.18 µM of quinine. Similarly, Seatlholo (2008) obtained an IC_{50} value of 135.40 µM for *trans*-geraniol on the chloroquine-resistant *P. falciparum* (FCR-3) strain compared to the control quinine (IC_{50} value: 0.29 µM).

4.5.2 Geranyl acetate

Geranyl acetate, a monoterpene ether, is prepared by the acetylation of geraniol (Figure 2.1.15). Larvicidal activity was displayed with a LC_{50} value of 0.007%. No notable antimalarial activity was noted in comparison to geraniol (Figures 3.2 and 3.5) as the antimalarial control (quinine) had an IC_{50} value of 0.18 µM. However, an IC_{50} value of 114.2 µM on the chloroquine-resistant *P. falciparum* FCR-3 strain was determined by Seatlholo (2008) in comparison to quinine (0.29 µM). Geranyl acetate displayed toxicity against the *Artemia* nauplii (LC_{50} value: 0.009%; 400.39 µM) (Figure 3.8). Cytotoxicity on human kidney epithelial HEK (293) cells with an IC_{50} value of 1.25 µM ($2.68 x10^{-5}$ %; Figure 3.11), however an IC_{50} value of 796.40 µM on the same cell line was determined by Seatlholo (2008).

4.5.3 Citronellal

Citronellal is a monoterpene unsaturated aldehyde, with stereoisomers, (+)- and (-)-citronellal (Figures 2.1.5 and 2.1.6) have been used as an ingredient in insect repellents (Corona *et al.*, 1984; Toxnet, 2017). Antimalarial activity was displayed by (*S*)-(-)-citronellal (IC_{50} value: 10.51 µM, $1.91 x10^{-4}$%; Figure 2.3). Yoshida *et al.* (2005) noted that acyclic monoterpenes which included (*R*)-(+)-citronellal and (*S*)-(-)-citronellal were potent inhibitors of *p*-glycoprotein in the parasite, resulting in accumulation of the EO and eventual death.

However, in the study conducted on chloroquine-resistant *P. falciparum* FCR-3 strain by Seatlholo (2008), (-)-citronellal was relatively inactive with an IC_{50} value of 698.5 ± 36.5 µM (Quinine, IC_{50} value: 0.29 µM). Cytotoxicity was displayed in this study by (*S*)-(-)-citronellal as well as (*R*)-(+)-citronellal yielding IC_{50} values of 18.27 ± 4.62 µM (3.31 $x10^{-4}$%) and 33.2 ± 5.68 µM (6.02 $x10^{-4}$%), respectively in comparison to camptothecin (IC_{50} value: 0.10 µM) (Figure 2.12). In contrast, Usta *et al.* (2009) found no significant cell death (20%) induced by 500 µM citronellal on hepatocellular carcinoma cell line (Hep-G2) cells. (*R*)-(+)-citronellal has been used as a frangrance ingredient in formulae and thus mentioned with regard to application to the skin, a maximum daily exposure of 0.0005 mg/kg for high end users is used (Lapczynski *et al.*, 2008).

The various plant EOs and their major EOCs displayed notable antimalarial and larvicidal properties (Figures 4.1, 4.9 - 4.12). This does warrant further studies into the application of EOs and EOCs in the management of malaria, and the development of larvicidal agents. There is a lack of evidence with regard to certain plant's EOs and EOC. Due to the toxicity profiles obtained in the various assays; it is empirical that further toxicity studies be done. This would aid in the correct prescribing methods for traditional medicinal practitioners as well.

CHAPTER FIVE – CONCLUSION

Vector control and chemoprophylaxis are imperative aspects in the prevention of malaria. However, due to the emergence of resistance to antimalarial therapies and insecticides as a global issue, new compounds are required to ensure adequate therapy. Traditional phytomedicines have been globally used as effective malaria management and thus plant EOs and their EOCs are being researched to be used as new antimalarial and larvicidal agents. This study focused on five purchased or locally obtained EOs which GC-MS analysis were performed by Prof. S van Vuuren and Dr. GPP Kamatou, and twenty-two EOCs which were commercially purchased from Sigma-Aldrich®.

Based on the guidelines for laboratory and field testing of mosquito larvicides, the WHO (2005) protocol was utilized.

It was concluded that all five EOs displayed antimalarial activity; however, *O. basilicum* was the only EO that inhibited lipid peroxidation inhibition with notable larvicidal activities in contrast to having the least effect of the EOs on parasite growth. The antimalarial mechanism of action of these EOs and EOCs may have been due to the growth inhibition of the parasites through the non-mevalonate pathway of isoprenoid biosynthesis (Qidwai *et al.,* 2014). Haemolysis was not displayed by any EO or EOC, which eliminated the potential mechanism whereby the RBC would swell and in turn affect the membrane's integrity (Kirk, 2001); thereby killing the malaria parasite or inducing haemolysis of the uninfected RBCs.

In contrast, *C. citratus* possessed the most antimalarial activity with promising larvicidal properties, however was also toxic against the *Artemia* nauplii and human kidney epithelial (HEK-293) cells; indicating this oil would not be a potential candidate as an agent to safely target both the intra-erthyroctic RBC stages and vector. *Cymbopogon nardus* displayed the most promising larvicidal activity but was the most toxic to *Artemia* nauplii. There was notable antimalarial activity and the least toxicity against the kidney epithelial cells displayed, with a promising safety index. Amongst the EOs, all possessed antimalarial with larvicidal activity, except for *A. afra* lacking in larvicidal activity.

The most promising EO to be taken forward with regard to potent antimalarial and larvicidal activity with the least toxic properties was that of *L. javanica.* However, a more complete toxicity profile including more cell lines and toxicity or mutagenic assays should be considered. With regard to the EOCs, eucalyptol displayed the most potent antimalarial activity with some larvicidal activity and no toxicity. The most larvicidal activity was displayed by *cis*-nerolidol and *p*-cymene and thus their combined activity was investigated (Figure 3.5).

The molecular structures of *p*-cymene and *cis*-nerolidol differ, where *p*-cymene contains a benzene ring and isopropyl group, compared to the alcohol sesquiterpene of *cis*-nerolidol (Favre and Powel, 2013). However, it should be noted that the control, DDT, contained di-benzene structures (Sigma-Aldrich®). An additive interaction between the two EOCs was observed (ΣFIC of 0.94; Figure 3.4).The mainstay mechanism by which the EOs and EOCs displayed their activity may be due to their ability to inhibit AChE in the vector (Rattan, 2010). As observed, the additive effect was beneficial and may lead to further larvicidal studies. Seeing that phytochemical-combined formulations are considered advantageous in vector control management as the combination effect, improves activity and decreases the dose volume required (Intirach *et al.,* 2012). It was also observed that the reconstituted crude EO from the EO's major constituents did not display the same larvicidal activity as the crude EO (Table 3.3). It was mentioned that such a copy of the oil will not have the same therapeutic effect as the natural and pure EO; as the interaction of the numerous minor and major constituents is an essential interaction to be considered (Cuttle *et al*., 2009; Esoteric oils, 2017).

Eugenol was the only EOC which displayed lipid peroxidation inhibition activity, however; it possessed larvicidal and antimalarial activity and toxicity against the *Artemia* nauplii and human kidney epithelial (HEK-293) cells as well.

Taking this research forward, it would be a positive contribution to determine antimalarial activity on other *P. falciparum* parasite strains. The combination of EOCs on the lethality of larvae was beneficial with an additive interaction providing a constructive way forward with larvicides, as combinations would require a lower dose been delivered. The research reported opens the possibility for further investigation and isolation of EOs and EOCs with anti-parasitic properties in order to overcome the problem of insecticide resistance.

CHAPTER SIX – RECOMMENDATIONS

- This study focuses on five EOs and twenty-two EOCs, however, there are numerous plant EOs and EOCs that are used in the management of malaria which still needs to be evaluated in similar studies.

- The promising larvicidal results of the EOs and EOCs on the *An. arabiensis* species suggests that further larvicidal studies should be performed on different larvae species. The combination of the two most active EOCs warrants further combination studies being performed using different EOCs as well as combining the EOCs with the standard larvicidal agents (e.g. temephos).

- To determine a more complete morphological observation of the mosquito larvae, the morphological changes should be monitored at various intervals (e.g. six, twelve and twenty-four hourly).

- Furthermore, with regard to the antimalarial activity of the EOs and EOCs, only one parasite strain was used. Additional studies including more parasite strains and combination studies would be required.

- Other cell lines need to be considered (e.g. lung cells and liver cells) to elucidate a comprehensive toxicity profile

- Further *in vivo* studies with regard to safe routes of administration need to be addressed taking into consideration mechanism of action of these EOs and EOCs which still require thorough investigation to determine their safety profile.

- Certain plant EOs were obtained from surrounding areas (*A. afra* and *L. javanica*), it would be advantageous to explore the antimalarial and larvicidal activity and to elucidate a toxicological profile of more plant EOCs that are grown and commonly used in surrounding communities to bridge the gap between traditional healers and scientific practitioners to form a relationship to know the dosage and duration of treatment to prevent toxic side effects to humans.

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APPENDICES

Appendix A

Table A.1: Essential oil composition of *L. javanica* (Van Vuuren, 2007).

Major EOCs	Percentage area
α-Pinene	0.2
Camphene	0.3
Myrcene and α-phellandrene	2.6
Limonene	0.5
β-Phellandrene	0.5
(*Z*)-β-Ocimene	**13.0**
(*E*)-β-Ocimene	**6.2**
p-Cymene	0.9
Dihydrotagetone	0.2
cis-Alloocimene	0.2
Ipsenone (tentative, Wiley)	0.8
trans-Linalool oxide (*furanoid)*	0.2
1-Octen-3-ol	0.1
cis-Linalool oxide (*furanoid)*	0.2
α-Copaene	0.4
cis-Tagetone	0.2
trans-Tagetone	0.3
Camphor	0.2
Linalool	**65.2**
β-Caryophyllene	3.6
Alloaromadendrene	0.1
α-Humulene	0.2
γ–Muurolene	0.1
Borneol	0.4
Germacrene-D	1.5
α-Muurolene	0.1
Bicyclogermacrene	0.1
(*E*,*E*)-α-farnesene	0.2
δ-Cadinene	0.2
2,6-Dimethyl-3(*E*),5(*E*),7-octatriene-2-ol	0.1
Isocaryophyllene oxide	0.1
Caryophyllene oxide	0.4
(*E*)-Nerolidol	0.1
Humulene epoxide II	Trace < 0.1
Total	**99.4**

Table A.2: Essential oil composition of *A. afra* (Van Vuuren, 2007).

Major EOCs	Percentage area	Major EOCs	Percentage area
α-Pinene	0.2	Terpinen-4-ol	2.1
Santolinatriene	0.1	*cis-p*-Menth-2-en-1-ol	0.7
Camphene	1	Dehydrosabina ketone	0.1
β-Pinene	0.1	Sabinaketone	Trace < 0.1
Sabinene	1	Sabinyl acetate	0.1
Myrcene	0.1	*trans*-Pinocarveol	0.1
Dehydro-1,8-cineole	Trace <0.1	β-Terpineol	0.1
1,8-cineole	**17.8**	*trans*-Piperitol	0.5
p-Cymene	2.2	α-Terpineol	0.2
***Artemisia* ketone**	**10.1**	Borneol	2.7
Yomogi alcohol	4.9	β-Selinene	0.2
Santolina alcohol	2	Piperitone	0.7
Santolinyl acetate	0.2	*cis*-Piperitol	0.8
α-Thujone	**18.8**	*Ar*-curcumene	0.1
Artemisyl acetate	1.8	Cumin aldehyde	0.1
β-Thujone	**12.5**	*p*-Cymen-8-ol	0.1
trans-Sabinene hydrate	0.3	Ascaridol	0.3
α-Copaene	0.1	*Epi*-cubebol	0.1
Artemisia alcohol	**5.5**	Caryophyllene oxide	0.3
Camphor	**8.2**	Cumin alcohol	0.1
Chrysanthenone	0.4	4-Hydroxy-4-methylcyclohex-2-enone	0.1
cis-Sabinene hydrate	0.3	Spathulenol	0.5
trans-p-Menth-2-en-1-ol	1	T-muurolol	0.5
Pinocarvone	0.2	Intermedeol	0.2
Bornyl acetate	0.2	**Total**	**99.7**

Table A.3: Essential oil composition of *O. basilicum* (Kamatou, 2014).

Major EOCs	Percentage area
1,8-Cineole	**5.3**
Linolool	**54.3**
α-Bergomotene	4.2
α-Guaine	1.8
β-Cubebene	2.7
δ-Guaine	1.2
δ-Cadinene	2.1
Eugenol	**13.9**
T-cadinol	3.2
Total	**88.7**

Table A.4: Essential oil composition of *C. citratus* (Kamatou, 2014).

Major EOCs	Percentage area
6-Merthyl-5-heptene-2-one	1.6
β-Caryophyllene	1.7
Neral	**30.7**
Geranial	**47.8**
Geraniol	**6.2**
Geranyl acetate	4.1
Total	**92.1**

Table A.5: Essential oil composition of *C. nardus* (Kamatou, 2014).

Major EOCs	Percentage area
Citronellal	2.8
β-Elemene	**6.2**
Citronellyl acetate	**5.6**
β-Cubebene	2.8
α-Muurolene	1.2
Geranyl acetate	**9.6**
δ-Cadinene	**17.7**
Geraniol	**44.9**
Eugenol	1.7
Total	**92.5**

Appendix B: Biosafety ethics clearance

Research Office

INSTITUTIONAL BIOSAFETY COMMITTEE
(R 14/16)

CLEARANCE CERTIFICATE - RENEWAL **PROTOCOL NUMBER: 20090503**

BRIEF DESCRIPTION OF APPLICATION:

Chemotherapeutic properties of novel synthetic and natural compounds

APPLICANT: Dr R van Zyl

SCHOOL/DEPARTMENT : Pharmacy/Pharmacology

DATE CONSIDERED: 20090528 and 20140327

DECISION OF COMMITTEE:

Approved unconditionally

1. This clearance certificate expires on 20190402 and may be renewed on application
2. An annual report must be provided on the anniversary date of this certificate, for as long as the project continues
3. Notification of any proposed modifications must be submitted on the attached form

DATE: 20140403 **CHAIRPERSON**: _____
(Professor A Capovilla)

DECLARATION BY APPLICANT:

To be completed in duplicate and **one copy** returned to the Secretary, Room 10004, 10th floor, Senate House, University.

1. I have read, understood and accepted the approval conditions above
2. I agree to submit a yearly progress report to the Committee and to submit an interim report on the form provided, in the event of any significant unforeseen event, *e.g.* suspension of a drug trial, temporary closure or relocation of my laboratory, etc
3. I note that the University Safety Officer, or his/her representative, may at any reasonable time inspect my laboratory or trial site to ensure compliance with current Health and Safety legislation. I undertake to offer my full co-operation in any such inspection
4. I have read, understood and will comply with the *recommended standard operating procedures for the handling of biohazardous materials* posted at http://web.wits.ac.za/Academic/Research/Biosafety.htm
5. I declare (delete as appropriate) that:
    a. I have all the approvals required by statute or regulation and by the funding agencies supporting this work, or
    b. that I will not begin work until such approvals are obtained

Signed: _____ **Date:** _____

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PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix C: Human Ethics clearance to use and purchase human plasma and blood

Human Research Ethics Committee (Medical)

Research Office Secretariat: Senate House Room SH 10005, 10th floor. Tel +27 (0)11-717-1252
Medical School Secretariat: Medical School Room 10M07, 10th Floor. Tel +27 (0)11-717-2700
Private Bag 3, Wits 2050, www.wits.ac.za. Fax +27 (0)11-717-1265

Ref: W-CJ-131030-1 30/10/2013

TO WHOM IT MAY CONCERN:

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

Investigator: Prof R van Zyl.

Project title: The chemotherapeutic properties of novel synthetics and natural compounds.

Reason: This is a laboratory study in which human blood and plasma for the in vitro maintenance of *Plasmodium falciparum* for experimental purposes such as drug sensitivity and toxicity studies. There are no human participants.

UNIVERSITY OF THE WITWATERSRAND
PROF PE CLEATON-JONES
HREC (MEDICAL)
2013 -10- 30
JOHANNESBURG

Professor Peter Cleaton-Jones

Chair: Human Research Ethics Committee (Medical)

Copy - HREC(Medical) Secretariat: Anisa Keshav, Zanele Ndlovu.

Appendix D: Human ethics clearance to obtain drug- free human whole from healthy volunteers

R14/49 Dr Robyn L van Zyl

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M140669

NAME: (Principal Investigator)	Dr Robyn L van Zyl
DEPARTMENT:	Pharmacy & Pharmacology Medical School
PROJECT TITLE:	The Chemotherapeutic Properties of Novel Synthetic and Natural Compounds (Renewal previously M090532)
DATE CONSIDERED:	29/05/2009 (Initial Approval) 26/06/2014 (Renewal)
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	
APPROVED BY:	Professor PE Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	26/06/2014

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**.

Principal Investigator Signature — Date 21-07-2014

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix E: Human ethics clearance certificate for use of human cell lines

Human Research Ethics Committee (Medical)

Golden Jubilee: October 1966 – October 2016

Research Office Secretariat: Faculty of Health Sciences, Phillip Tobias Building, 3rd Floor, Office 301,
29 Princess of Wales Terrace, Parktown, 2193 Tel +27 (0)11-717-1252 /1234/2656/2700
Private Bag 3, Wits 2050, email: zanele.ndlovu@wits.ac.za
Office email: hrec-medical.researchoffice@wits.ac.za
Website: www.wits.ac.za/research/about-our-research/ethics-and-research-integrity/

Ref: W-CJ-161129-2 29/11/2016

TO WHOM IT MAY CONCERN:

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

Investigator: **Ms Obaidiyah Mustapha (Student No 450187), Prof R van Zyl.**

Project title: **The effect of terpenes on the life-cycle of the malarial parasite.**

Reason: This is a laboratory study using commercial cell lines including HEK-293. There are no human participants.

UNIVERSITY OF THE WITWATERSRAND
PROF PE CLEATON-JONES
HREC (MEDICAL)
2016 -11- 29
JOHANNESBURG

Professor Peter Cleaton-Jones

Chair: Human Research Ethics Committee (Medical)

Copy – HREC (Medical) Secretariat: Zanele Ndlovu, Rhulani Mkansi.

Appendix F: Ethics clearance for the use of mosquitoes and mosquito parasites

Human Research Ethics Committee (Medical)

Research Office Secretariat: Senate House Room SH10005, 10th floor. Tel +27 (0)11-717-1252
Medical School Secretariat: Tobias Health Sciences Building, 2nd floor Tel +27 (0)11-717-2700
Private Bag 3, Wits 2050, www.wits.ac.za. Fax +27 (0)11-717-1265

Ref: W-CJ-150911-1 11/09/2015

TO WHOM IT MAY CONCERN:

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

Investigator: **Prof Maureen Coetzee.**

Project title: **Research on mosquitos.**

Reason: This waiver covers all research on mosquitos and mosquito parasites by Professor Coetzee, her staff and students as long as no humans or human tissues are involved. The waiver lasts 5 years and may be renewed.

UNIVERSITY OF THE WITWATERSRAND
PROF PE CLEATON-JONES
HREC (MED/CAM)
2015 -09- 11
JOHANNESBURG

Professor Peter Cleaton-Jones

Chair: Human Research Ethics Committee (Medical)

Copy – HREC (Medical) Secretariat : Zanele Ndlovu.