Larvicidal and Toxicological Properties of Insect-Repellent Essential Oils and their Main Constituents

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

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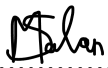
Declaration

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



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Suné Malan

Signed at…Medical School, WITS, 7 York Road, Parktown…… on the…22nd… day of……October……2025

Dedication

I would like to thank my parents for their unwavering support and love. Words cannot describe how thankful I am. Thank you for always believing in me. I also want to thank my husband for standing by my side through this degree. Thank you for all of the words of encouragement and support. Thank you to my cousin, who accepted me into their home as part of their family for the duration of my studies and always encouraged me.

Congress Presentations

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Abstract

The female *Anopheles* mosquito, the principal vector of malaria, is increasingly developing resistance to conventional insecticides. As malaria cases continue to rise globally, there is a critical need to explore safer and more sustainable alternatives. In this context, natural products have emerged as a valuable source of bioactive molecules with potential insecticidal activity. Essential oils derived from plants possess diverse biological properties, including insecticidal activity, which is attributed to their chemical constituents. The aim of this study was to determine the larvicidal properties of insect-repellent essential oils and their key constituents, whilst determining their preliminary toxicity profile (haemolysis, *Artemia franciscana* nauplii lethality, free radical scavenging activity), possible mechanism of action (metal chelating properties) and *in silico* pharmacokinetic and toxicity studies. The essential oils selected for this study include black pepper (*Piper nigrum*), clove-leaf (*Syzygium aromaticum*), sweet basil (*Ocimum basilicum*), thyme (*Thymus vulgaris*) and oregano (*Origanum vulgare*). The main constituents of these essential oils were identified using Gas Chromatography-Mass Spectrometry (GC-MS). Additionally, eugenol, a common essential oil constituent, along with its derivatives, was investigated. Out of 17 tested compounds - including essential oils, essential oil constituents, eugenol and its derivatives, 16 exhibited potential larvicidal activity with LC_{50} values ranging from 8.66 µg/mL to 66.11 µg/mL at 72 hours of exposure, compared to standard insecticide, propoxur (LC_{50} value: 0.04 ± 0.01 µg/mL). Eugenol and its derivatives displayed low to moderate larvicidal activity, with isoeugenol exhibiting potential time-dependent mortality. Among the essential oils oregano (LC_{50} value: 10.42 ± 1.56 µg/mL) and thyme (LC_{50} value: 17.00 ± 4.58 µg/mL) were the most effective against the *An. arabiensis* larvae at 72 hours of exposure; however, they exhibited greater toxicity towards the *Artemia franciscana* with LC_{50} value of 4.21 ± 0.55 µg/mL and 2.42 ± 0.00µg/mL, respectively, compared to propoxur. The essential oils constituents, γ-terpinene (LC_{50} value: 8.66 ± 2.35 µg/mL at 72 hours) and *p*-cymene (LC_{50} value: 12.68 ± 1.28 µg/mL at 72 hours) emerged as the most potent larvicidal compounds while demonstrating lower toxicity to *A. franciscana* (% mortality range: 22.27% to 41.66%; from 24 to 72 hours) and minimal haemolytic (<22%) effects and low antioxidant activity (<0.1% to 0.20%) at high concentrations. When combined with propoxur at various concentration ratios, they demonstrated enhanced larvicidal activity. However, their mode of action against *Anopheles* vectors remains unclear, as they showed no significant metal-chelating activity (iron chelation: <0.1% to 0.60%; copper(II) chelation: <0.1 ± 0.01%). *In silico* studies found that the essential oil constituents are low class I toxic hazards with ECOSAR toxicity predicting higher toxicity than *Artemia* nauplii lethality results. Further research is necessary to optimise the efficacy of these essential oil constituents and elucidate their mechanism of action.

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Table of contents

Nomenclature/ List of abbreviations or symbols

Abbreviation	Description
A	Absorbance
ADME	Absorption, Distribution, Metabolism and Excretion
ACD-B	Acid citrate dextrose solution B
ANOVA	Analysis of variance
An.	*Anopheles*
A.	*Aspergillus*
BBB	Blood brain barrier
Cu/Zn-SOD	Copper/zinc superoxide dismutase
COX	Cyclooxygenase
Cyp	Cytochrome
°C	Degrees celsius
DOH	Department of Health
DDT	Dichlorodiphenyl-trichloroethane
DMSO	Dimethyl sulphoxide
$DPPH^{\bullet}$	2,2-diphenyl-2-picrylhydrazyl
ECOSAR	Ecological structure-activity relationship model
EDTA	Ethylenediaminetetraacetic Acid tetrasodium disodium salt
EOC	Essential oil constituent
EO	Essential oil
Fe^{3+}	Ferric iron
$FeCl_2$	Ferrous chloride
Fe^{2+}	Ferrous iron
GC-MS	Gas chromatography-mass spectrometry
GIT	Gastrointestinal tract
g	Gravitational force
IC_{50}	Half maximal inhibitory concentration
HEPES	4-(2-hydroxyethyl)-1-piperazine
IRS	Indoor residual spraying
IV	Intravenous
ITN	Insecticide treated bed nets
IARC	International Agency for Research on Cancer
Kdr	Knockdown resistance
LCD_{50}	Lethal dose concentration 50% mortality
µg/mL	Microgram per millilitre
µL	Microlitre
µm	Micrometre
mg/L	Milligram per litre
Mg/mL	Milligram per millilitre
mL	Millilitre
mM	Millimolar
MCOs	Multicopper oxidases
nm	Nanometre
n.d.	Not determined
#	Number
n	Number of repeats
%	Percentage

% (v/v)	Percentage volume weight per volume
% (w/v)	Percentage weight per volume
PBS	Phosphate buffered saline
PBO	Piperonyl butoxide
PPARs	Peroxisome proliferator-activated receptors
P.	*Plasmodium*
$K_2Cr_2O_7$	Potassium dichromate
ppm	Parts per million
RRI's	Relative retention indices
RPMI-1640	Roswell Park Memorial Institute medium
SI	Selectivity Index
SA	South Africa
sp.	Species
s.d.	Standard deviation
TRPA1	Transient receptor potential cation channel subfamily A member 1
WHO	World Health Organisation
WRIM	Wits Research Institute for Malaria

List of figures

List of tables

List of Equations

Chapter 1 – Introduction

1.0 Malaria

Malaria is a global problem caused by the protozoan, *Plasmodium* transmitted by the bite of a female *Anopheles* mosquito (Burke *et al.*, 2017; Gwarinda *et al.*, 2021). The vectors *An. funestus* and *An. gambiae* are the main vectors to transmit malaria in South Africa (Gwarinda *et al.*, 2021). The malaria vectors responsible for residual malaria are *An. arabiensis* and *An. merus*, of the *An. gambiae* complex, and *An. vaneedeni*, of the *An. funestus* complex (Christian *et al.*, 2017). *Plasmodium* species that cause malaria include *P. falciparum, P. vivax, P. knowlesi, P. ovale* and *P. malariae* (Sato, 2021). In South Africa, *P. falciparum* is the species that accounts for approximately 90% of malaria cases and is the most responsible for mortality (Gwarinda *et al.*, 2021; Munzhedzi *et al.*, 2021). Uncomplicated malaria symptoms include fever, headache, chills, fatigue, nausea, myalgia and can cause mild anaemia (Mace, 2021).

1.1 Epidemiology

There was an increase in global malaria cases from 252 million cases in 2022 to 263 million cases in 2023. This increase is mostly from the WHO African Region accounting for 94% of the cases in 2023 with approximately 246 million cases. *Plasmodium vivax* related cases have increased from 2.5% to 3.5% from the year 2022 to 2023. Estimated global deaths declined from 600 000 in 2022 to 597 000 in 2023. Estimated malaria deaths in the WHO African Region in 2023 were 569 000 an increase from 2022. In South Africa, there was an increase of 44% of indigenous cases from 2022 to 2023 (WHO, 2024).

In South Africa the three main malaria endemic provinces are KwaZulu-Natal, Limpopo and Mpumalanga where there is active malaria transmission (SA DOH prevention guidelines; Gwarinda *et al.*, 2021). In these endemic areas, heterogeneous transmission of malaria occurs despite vector transmission control and intervention efforts (Gwarinda *et al.*, 2021). In the Gauteng and Northwest provinces, malaria cases have also been reported (Munzhedzi *et al.*, 2021). Increased malaria cases and fatality in the Gauteng province was mostly due to patients traveling to and from Mozambique where there is a high incidence of malaria (Fox, Karstaedt and Menezes, 2021). Cross-border migration from Mozambique and Zimbabwe has accounted for several malaria cases in Limpopo and KwaZulu-Natal, accounting for 26% of Limpopo's malaria cases between 1998 and 2017 (Abiodun *et al.*, 2020).

Peak malaria season in South Africa is from November to April, during the summer, with highest transmission between September and May (Van Zyl, 2016; Christian *et al.*, 2017; Dahan-Moss *et al.*, 2019; Gwarinda *et al.*, 2021). This is due to the hot weather and the rain

season (Abiodun *et al.*, 2020; Gwarinda *et al.*, 2021). Hot weather and rain are favourable conditions for the parasite and vector, and an increase in malaria cases are expected when temperatures rise. It enhances feeding behaviour of the vector and is favourable for reproduction of the parasite and vector (Wright *et al.*, 2021).

1.1.1 Life Cycle and Therapeutic Management of the Malaria Parasite

The malaria parasite's life cycle consists of two stages, the infective stage and diagnostic stage, and three cycles, the sporogonic cycle, exo-erythrocytic cycle and intra-erythrocytic cycle. The transmission of malaria requires two hosts, namely the human host and *Anopheles* vector (Figure 1.1) (Centers for Disease Control and Prevention, 2024a). The formation of male and female gametocytes has been identified as promising targets to interfere with the transmission of malaria (Chawla, Oberstaller and Adams, 2021).

Prevention of a malaria infection is key to interrupt the transmission of malaria. The use of bed nets and, insect repellents are primary non-pharmacological interventions which have shown success. Pharmacological interventions recommended for use in South Africa include doxycycline, mefloquine and atovaquone-proguanil (SA DOH prevention guidelines). Whilst if an infection is verified microscopically or via a rapid antigen detection diagnostic test, recommended treatment for uncomplicated malaria is artemether-lumefantrine or quinine-clindamycin. Whilst intravenous artesunate is recommended for severe malaria infections until the patient can tolerate oral medication at which point they are administered artemether-lumefantrine (van Zyl, 2016).

In an effort to reduce the high mortality rates among African children, the first malaria vaccine was approved for use by the WHO, and has recommended the vaccine as prevention of *P. falciparum* infections in children living in regions with moderate to high malaria transmission (WHO, 2022). The RTS,S/AS01 stands for a recombinant malaria vaccine, with "RTS" referring to the repeat T epitopes derived from the circumsporozoite protein, "S" for the S antigen derived from hepatitis B surface antigen, and "AS01" being a proprietary adjuvant (Egbewande, 2022).

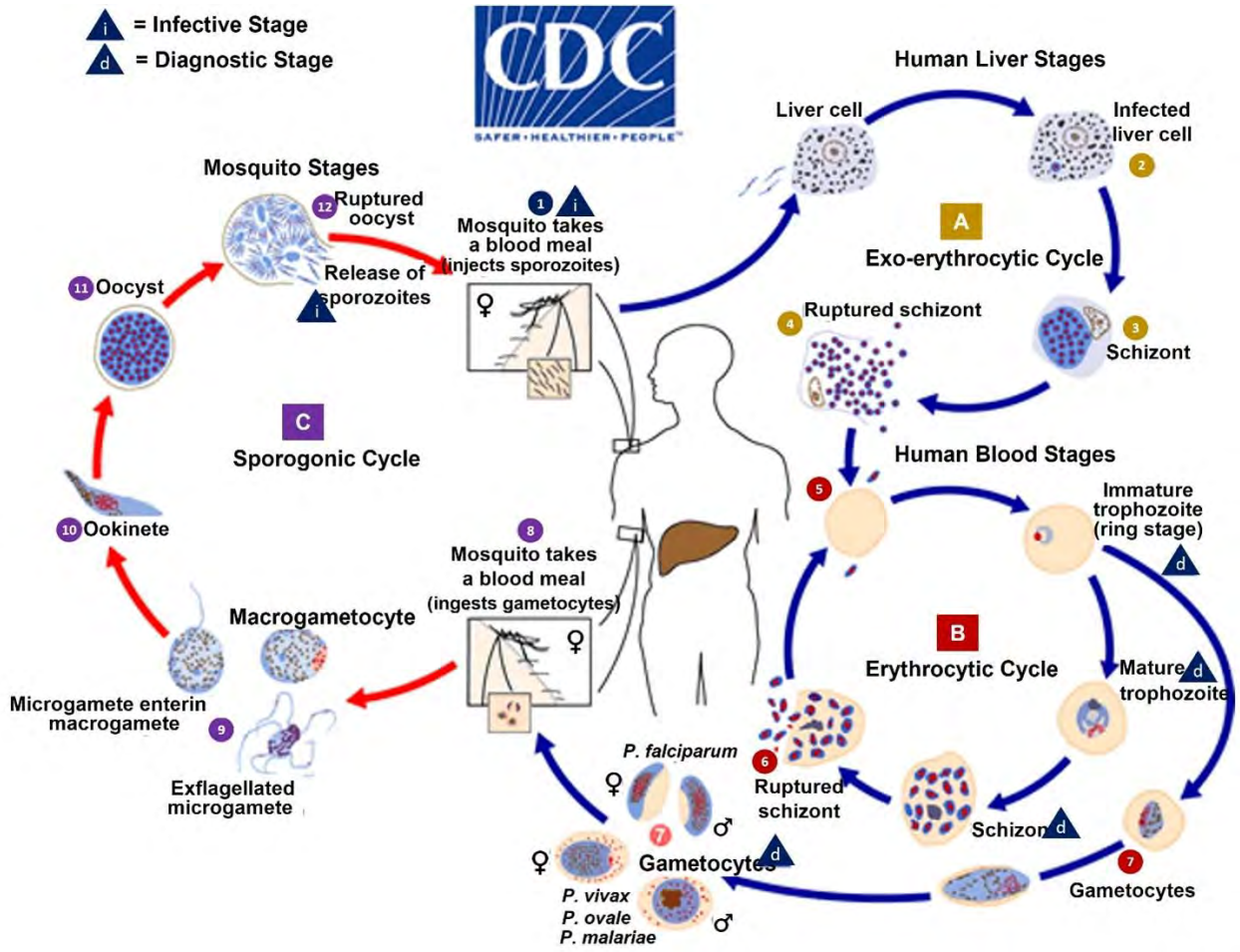


Figure 1.1. Sporozoites is injected into the host when the mosquito feeds on blood. **A.** This stage is the exo-erythrocytic cycle in the liver cells **2.** The liver cells are infected with the sporozoites. **3.** The sporozoites mature and develops into schizonts. **4.** The schizonts rupture and release merozoites into the bloodstream. **B.** The next stage is the erythrocytic cycle in the red blood cells. **5.** The merozoites infects the red blood cells. The merozoites develops into ring stage trophozoites that mature into schizonts. **6.** The schizont ruptures and release merozoites. **7.** Gametocytes, microgametocytes and macrogametocytes, are formed by some parasites. **8.** When the mosquito feeds on blood, the gametocytes are ingested by the mosquito. **C.** The sporogonic cycle takes place in the mosquito where the parasite multiply. **9.** The microgametocytes penetrate the macrogametocytes forming zygotes in the stomach of the mosquito. **10.** The zygotes develop into ookinetes. **11.** The ookinetes develop into oocysts when they mosquito's midgut wall. **12.** The oocysts will grow and rupture, releasing sporozoites, that will migrate to the salivary glands of the mosquito, which can be inoculated into a new host. (Centers for Disease Control and Prevention, 2024a).

1.2 Vector Biology

The two main *Anopheles* groups are the *Anopheles gambiae* complex and *An. funestus group. An. arabiensis, An. merus* and *An. quadriannulatus* are the three *Anopheles* species found in the *An. gambiae* complex, and the three *Anopheles* species in the *An. funestus group* are *An. leesoni, An. parensis, An. rivulorum, An. rivulorum-like* and *An. vaneedeni.* (Christian *et al.*, 2017; Dahan-Moss *et al.*, 2019). According to the Malaria Vector Surveillance report of Dahan-Moss *et al.* (2019), *An. arabiensis, An. merus* and *An. vaneedeni,* were present in all three of the endemic provinces of South Africa. Also present in all endemic provinces were *An. rivulorum*, a potential malaria vector. During this surveillance, 18 species of *Anopheles* were seen in the three endemic provinces of South Africa, some of which are malaria vectors in South Africa and other vectors in Africa locations. In the KwaZulu-Natal province during late summer, *An. arabiensis* were more present, whereas in Mpumalanga, *An. merus* were more present in spring through early summer (Dahan-Moss *et al.*, 2019). It has been concluded that the ongoing residual malaria transmission is due to the *An. vaneedeni, An. merus* and *An. arabiensis* malaria vectors (Christian *et al.*, 2017). *An. funestus*, especially groups *An. leesoni*, *An. parensis*, *An. rivulorum* and *An. vaneedeni*, has a possible contribution to malaria transmission in Mpumalanga and KwaZulu-Natal (*Burke et al.*, 2017). *Anopheles arabiensis* is one of the main malaria vectors responsible for residual malaria transmission and have variable feeding (on human and animals) and resting behaviours (indoor or outdoor) (Brooke, 2013; Kaiser, 2021).

1.2.1 Life Cycle of the *Anopheles* Mosquito

The *Anopheles* mosquito requires bodies of water, possibly small and seasonal, for their aquatic larvae and pupae. Suitable habitats range from ponds to water tanks, swamps, open containers, tyres and ditches. The adults can however live in dry regions where both male and female require plant nectar to survive and the female requires blood to develop her eggs (Figure 1.2) (Van Zyl, 2016).

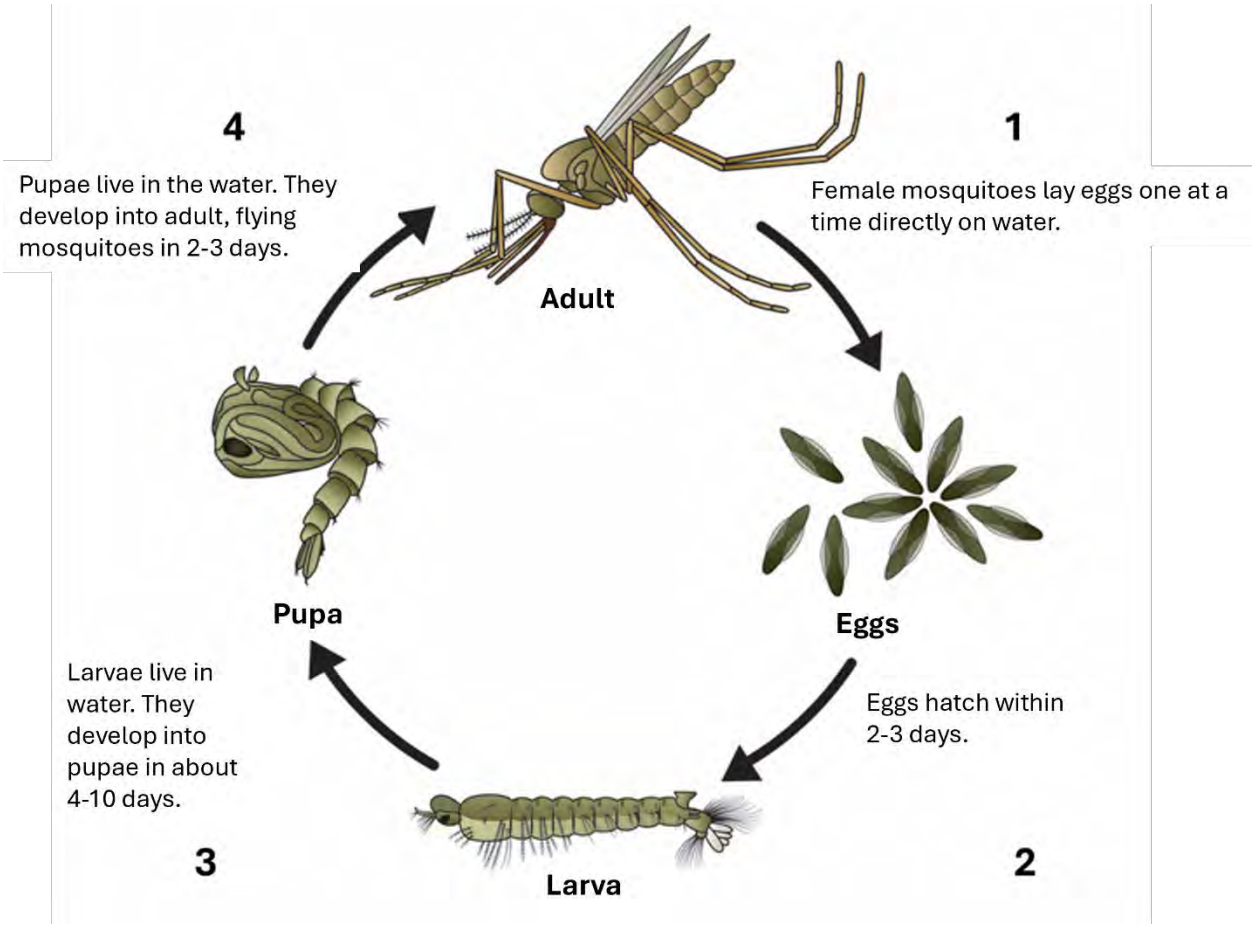


Figure 1.2. The life cycle of the *Anopheles* mosquito. 1. Adult female *Anopheles* mosquitoes lay their eggs in the water.**2.** The eggs then hatch into larvae 2 to 3 days later. **3.** Larvae develop into pupae in the water after 4 to 10 days. **4.** Adult mosquitoes develop form pupae within 2 to 3 days (Centres for Disease Control and Prevention, 2024b).

1.2.2 Metal Homeostasis in *Anopheles* Mosquitoes

Metal homeostasis in *Anopheles* mosquitoes is essential for their survival, reproduction, and ability to support *Plasmodium* infection. Key metals such as iron, copper, and zinc play crucial roles in physiological processes, including oxidative stress regulation, metabolism, and immune responses (Jeanrenaud, Brooke and Oliver, 2020; Cardoso-Jaime, Broderick and Maya-Maldonado, 2022). Maya-Maldonado *et al*. (2021) provides key insights into the interplay between *Plasmodium berghei* infection, metal homeostasis, and mosquito physiology. It was observed that after a blood meal, metal levels in *Anopheles albimanus* increased post-digestion and excretion, suggesting active regulation of metal availability in response to nutrient intake.

1.2.2.1 Iron and Copper Homeostasis

Iron is vital for mosquito reproduction, metabolism, and immunity. After a blood meal, iron levels rise significantly due to haemoglobin digestion. Excess iron can be toxic, so mosquitoes regulate its levels through storage proteins like ferritin and excretion mechanisms (Zhou *et al.*, 2007). In *Anopheles*, iron is also involved in ovarian follicle development, and its chelation has been shown to impair reproduction and reduce *Plasmodium* infection (Maya-Maldonado *et al.*, 2021; Rani *et al.*, 2021).

Copper is required for oxidative stress management, as it is a cofactor for enzymes like copper/zinc superoxide dismutase (Cu/Zn-SOD). It is also important for cuticle formation and immune responses. Unlike iron, copper chelation can reduce *Plasmodium* infection, indicating its role in parasite development within the vector (Rasoloson *et al.*, 2004; Maya-Maldonado *et al.*, 2021; Cardoso-Jaime, Broderick and Maya-Maldonado, 2022).

Multicopper oxidases (MCOs) are a family of enzymes that play a crucial role in metal homeostasis by binding and oxidizing substrates, including copper and iron. These enzymes are essential for iron metabolism, detoxification, and immune function. Multicopper oxidase is an enzyme that binds to certain substrates including copper and iron. *Anopheles gambiae* have orthologs of two multicopper oxidase's (Gorman *et al.*, 2008).

MCO1 (ortholog of ceruloplasmin/hephaestin) is involved in iron transport and homeostasis, where it assists the oxidisation of Fe^{2+} (ferrous iron) to Fe^{3+} (ferric iron), which facilitates iron binding to transferrin for safe transport in the haemolymph (Lang *et al.*, 2012; Knutson, 2017). MCO1 may play a role in iron detoxification after a blood meal, preventing oxidative stress (Gorman *et al.*, 2008).

Whilst multicopper oxidase 2 (MCO2), (ortholog of laccase-type enzymes) is known to be a factor of cuticle sclerotization and immune defence. It also plays a role in melanisation, a key immune response in mosquitoes against pathogens, including *Plasmodium.* As well as being involved oxidative stress regulation (Lambrechts *et al.*, 2007; Lang, Kanost and Gorman, 2012; Asano, 2024). Four amino-terminal von Willebrand factor domains is present in *An. gambiae* which may be involved in protein-protein interactions (Gorman *et al.*, 2008).

Both MCOs are likely crucial for *An. gambiae* survival, metal balance, and interactions with *Plasmodium* parasites. Disrupting MCO function could affect iron and copper homeostasis, potentially influencing mosquito fitness and vector competence, making them an interesting target for malaria control strategies.

1.3 Malaria Vector Control

1.3.1 Control and Elimination

In South Africa, the endemic areas have control operations in place (Public Health Bulletin, 2024). This includes routine vector control consisting of indoor residual spraying (IRS), a widespread control mechanism, that reduce malaria transmission, by reducing adult vector density and longevity (Christian *et al.*, 2017; Dahan-Moss *et al.*, 2019; WHO, 2022). Larval source management is also used as control mechanism (Christian *et al.*, 2017; Dahan-Moss *et al.*, 2019).

Although there are control operations in place, residual malaria transmission continues (Christian *et al.*, 2017; Dahan-Moss *et al.*, 2019). Indoor residual spraying would not be efficient for the prevention of vectors feeding and resting outdoors, like *An. arabiensis* vectors, and can be the reason for residual malaria transmission (Brooke *et al.*, 2013; Christian *et al.*, 2017; Dahan-Moss *et al.*, 2019). In 2021, 128 million insecticide treated bed nets (ITN's) were distributed. The primary insecticide class used in ITN's are pyrethroids. The main malaria vector control tool is long-lasting insecticidal nets as they are effective. Compared to ITN'S, IRS is much more expensive and challenging (WHO, 2022).

1.4 Development of Resistance in the *Anopheles* Vector

In 57 of the 66 malaria endemic countries globally, at least one vector collected at a collection site has showed insecticide resistance to at least one insecticide (WHO, 2024). Resistance to carbamates, neonicotinoids, organophosphates and pyrethroids has been detected in the WHO African region. In the WHO African Region, *An. stephensi* is present and poses a threat as it is resistant to many insecticides (WHO, 2024).

In north Kwazulu-Natal mostly, *An. funestus* and *An. arabiensis* have become resistant to insecticides, with *An. funestus* also have become resistant to pyrethroid-carbamate insecticides (Christian *et al.*, 2017; Dahan-Moss *et al.*, 2019). As the primary insecticide used in ITN's, the widespread resistance to pyrethroids is problematic. High intensity of the resistance to pyrethroids was reported in 293 sites of 27 countries. Susceptibility has been seen to be fully restored by piperonyl butoxide (PBO) (WHO, 2022). Pre-exposure of mosquitoes to PBO restores susceptibility to pyrethroids by inhibition of the Cytochrome P450 monooxygenases, responsible for defence against pyrethroids (Kabula *et al.*, 2024). *An. arabiensis* vectors have developed resistance to permethrin, with the first incident reported in South Africa 2009. These vectors had an increase in monooxygenase enzymes, an enzyme correlated to vector resistance to permethrin (Mouatcho, 2009).

Another problem is that some vectors are undergoing behavioural changes like biting, feeding and resting behaviour in attempt to reduce insecticide contact (WHO, 2022). Resistance of the malaria vector in many populations is also a result of exposure to ongoing agricultural pesticides as well as insecticidal use for malaria vector control. This leads to gene selection for resistance (Brooke 2013). Therefore, there is a need for new novel agents that can be used for either insecticidal control or against the malaria parasites.

Anopheles malaria vectors have developed molecular mechanisms and enzymatic processes that confer resistance to various insecticidal agents. Key resistance mechanisms include increased enzymatic detoxification and decreased target-site sensitivity, particularly to pyrethroids. Cytochrome P450 oxidases, glutathione-S-transferases and esterases are known to detoxify insecticides, while target site sensitivity is decreased when the voltage-gated sodium channel is mutated, known as knockdown resistance (*kdr).* The malaria vectors, *An. funestes, An. gambiae* and *An. arabiensis* have shown the ability to enzymatically detoxify pyrethroids (Nardini *et al.*, 2013). Resistance to DDT has been reported in KwaZulu-Natal to *An. arabiensis* through the L1014F *kdr* mutation as well as enzymatic detoxifying mechanisms (Nardini *et al.*, 2013).

As such novel insect repellents, adulticidal or larvicidal agents are needed. Pyrethrum, a naturally occurring plant oil that originates from two kinds of pyrethrum daisies, namely *Chrysanthemum coccineum* and *Chrysanthemum cinerariifolium*, is used for its pyrethrin extracts (Shimira *et al.*, 2021). Citronella and lemon grass are also commonly used as mosquito repellents (Halim, Lesmana and Sitepu, 2021). Similarly in South Africa several aromatic plants are used to repel and kill *Anopheles* larvae (Ganesan *et al.*, 2023). This long-standing use of plant-based products highlights the potential for further research into novel larvicidal agents or synergistic combinations that could benefit malaria-affected communities.

1.5 Essential Oils

Essential oils (EO) are aromatic volatile liquid oils distilled from various plant parts by various different methods (Ebadollahi, 2013; Tongnuanchan and Benjakul, 2014). Essential oils are already in use by the pharmaceutical industry, and many have insecticidal and insect repellent properties (Ebadollahi, 2013). Essential oils have a variety of actions on insects from plants ranging from attracting, repelling and killing and insects (Karpouhtsis *et al.*, 1998). Adulticidal, larvicidal and ovicidal properties of various essential oils on *Anopheles* have been reported by Rants'o *et al.* (2022a). Based on existing literature, essential oils previously reported to exhibit insecticidal activity against various *Anopheles* species were selected for further evaluation against *An. arabiensis* (KWAG strain) and determine their safety profile on the ecosystem

(Amer and Mehlhorn, 2006; Pandey, Upadhyay and Tripathi, 2009; Murugan *et al.*, 2015; Govindarajan *et al.*, 2016; Osanloo *et al.*, 2018). The focus of this study is to investigate the essential oils black pepper (*Piper nigrum*), clove (*Syzygium aromaticum*), sweet basil (*Ocimum basilicum*), oregano (*Origanum vulgare*) and thyme (*Thymus vulgaris*) and their main essential oil constituents (EOCs).

1.5.1 Black pepper oil (*Piper nigrum*)

Black pepper (*Piper nigrum)* is derived from the dried peppercorns of the plant and is widely used as a spice due to its pungent aroma and flavour-enhancing properties. The main constituents of black pepper essential oils are reported to be β-caryophyllene, γ-terpinene-4-ol, pinene and limonene (Asadi, 2021; Saleem *et al.*, 2022; Pop, Marc and Mureșan, 2024). Black pepper essential oil has demonstrated antibacterial activity, against *Escherichia coli* isolated from meat, with its mechanism involving disruption of the bacterial cell wall and leakage of intracellular contents, leading to reduced cell viability (Zhang *et al*., 2017). Black pepper contains polyphenols which possess anti-oxidant effects (Wang *et al.*, 2021). In addition, black pepper essential oils have reported inhibitory effects on the HeLa cell viability and possibly has anticancer therapeutic properties (Wang *et al.*, 2021).

The active compound in black pepper is piperine. The therapeutic properties of black pepper such as anti-oxidant, antimicrobial and anti-inflammatory properties, is attributed to piperine. It can inhibit enzymes responsible for metabolization that leads to an increase oral bioavailability (Saleem *et al.*, 2022; Pop, Marc and Mureșan, 2024). Both black pepper and piperine have demonstrated toxicity against mosquito larvae (Samuel *et al.*, 2016). Piperine derivatives are predicted to be acetylcholinesterase inhibitors by molecular docking (Tantawy *et al.*, 2020).

1.5.2 Clove oil (*Syzygium aromaticum*)

Clove oil, derived from the clove plant (*Syzygium aromaticum*), is a versatile essential oil with a wide range of uses in medicine and cosmetics. Clove oil is extracted from the plants buds stems and leaves with the principal constituent eugenol and a smaller percentage of acetyleugenol. It is used in dental practices because of its antiseptic and analgesic properties (Jirovetz *et al.*, 2006; Thosar *et al.*, 2013). Clove oils poses antibacterial properties against *Staphylococcus aureus, Escherichia coli* and *fungi, Candida albicans, Aspergillus niger* and *A. flavus* (Viuda-Martos *et al.*, 2006; Thosar *et al.*, 2013). It has anti-oxidant activity mainly attributed to eugenol, and lipid peroxidation inhibitory and ferric (Fe^{3+}) chelating properties (Jirovetz *et al.*, 2006). Clove oil has been reported to possess insect-repellent properties and

often used in natural repellents for mosquitoes, ants, and other insects (Sanga *et al.*, 2023). However, there is limited direct evidence specifically linking clove oil to a reduction in *Anopheles* mosquitoes.

1.5.3 Sweet basil (*Ocimum basilicum*)

Sweet basil essential oil, derived from the leaves of the basil plant, is known for its fresh, herbaceous aroma and a variety of therapeutic properties. Some of its uses include: insecticidal, digestive problems, anti-inflammatory, antibacterial, antiviral, antifungal and anxiolytic (Ch *et al.*, 2015; Hyldgaard *et al.*, 2015; Azizah *et al.*, 2023). In addition, sweet basil oil has a dose-dependent inhibitory effect on alpha-amylase, alpha-glucosidase, angiotensin-converting enzyme and lipid peroxidation (Ademiluyi, Oyeleye and Oboh, 2016). The anti-oxidant activity of the oil in scavenging hydroxide (˙OH) free radicals is attributed to some of the main constituents, namely eugenol and methyleugenol (Li *et al.*, 2017; Miele *et al*., 2001).

Although there is not a vast body of research specifically linking sweet basil essential oil to repelling *Anopheles* mosquitoes, basil oil in general has insect-repellent properties, and it is known to deter diverse types of insects, including mosquitoes. Studies have also indicated that basil oil have larvicidal properties (Nour *et al.*, 2022; Đukić *et al.*, 2023). The other major constituents in sweet basil oil are reported to be linalool (>30%), estragole, eugenol, bicyclosesquiphellandrene, and methyl cinnamate (Ahmed *et al.*, 2019).

1.5.4 Oregano (*Origanum vulgare*)

Oregano essential oil is made by different drying methods of aerial parts of the plant, that affects the quality of the oil (Caputo *et al.*, 2022). It is a highly valued for its powerful antimicrobial properties including respiratory and digestive issues, antifungal activity against *Aspergillus niger* and *A. flavus*, anti-oxidant, and anti-inflammatory properties (Viuda-Martos *et al.*, 2006; Assiri *et al.*, 2016; Leyva-López *et al.*, 2017). Oregano essential oil have shown insecticidal activity against the *Drosophila* species, and have insect-repellent and antimicrobial properties (karpouhtsis *et al.*, 1998; Patel *et al.*, 2023).The active compounds in oregano essential oil are responsible for its medicinal and insect-repellent properties. Of the main constituents of oregano essential oil including thymol, terpinene, *p*-cymene, β-caryophyllene, linalool and carvacrol; the latter is the primary active compound in oregano oil, accounting for a significant portion of its antimicrobial, anti-inflammatory, and insect-repelling effects (Patel *et al.*, 2023).

1.5.5 Thyme (*Thymus vulgaris*)

The genus *Thymus* includes over 300 species, and the essential oil is extracted from leaves and flower tops from plants. Thyme is useful in a variety of conditions ranging from gastric and respiratory conditions, reduce inflammation and alleviate pain; as well as infections caused by bacteria, *Staphylococcus aureus, Escherichia coli,* and fungi, *Candida albicans, Aspergillus niger* and *A. flavus* (Viuda-Martos *et al.*, 2006; Thosar *et al.*, 2013). In addition, thyme essential oil has been reported to possess strong antibacterial activity against strains of *Staphylococcus* sp. *Escherichia* sp. and *Pseudomonas* sp. resistant to many antibiotics (Vassiliou *et al.*, 2023). The main reported constituents of thyme essential oil are carvacrol, linalool, cymene, γ-terpinene, β-myrcene, terpinene-4-ol and thymol with the latter attributed to the insect repellent properties and therapeutic properties of thyme (Youssefi *et al.*, 2019; Kim, Sowndhararajan and Kim, 2022; Vassiliou *et al.*, 2023).

1.6 Eugenol

1.6.1 Chemistry

Eugenol also known as 4-allyl-2-methoxyphenol or 2-methoxy-4-(2-propenyl)phenol (Pramod, Ansari and Ali, 2010; Marchese *et al.*, 2017) is a chemical compound with a short hydrogen tail and is classified as a methoxyphenol (Ito, Murakami and Yoshino, 2005). This compound is a guaiacol with an allyl chain substitute. Eugenol is weakly acidic and slightly water soluble (Kamatou, Vermaak and Viljoen, 2012). *Lamisceae*, *Myrtaceae*, *Myristicaceae* and *Lauraceae* are a few plant families in which eugenol can be found, such as clove, cinnamon, turmeric, thyme, clover pepper, betel pepper and nutmeg plants (Khalil *et al.*, 2017; Ulanowska and Olas, 2021). Clove oil, basil oil, cinnamon oil, pimento oil and bay oil contains eugenol as a major constituent (Schmidt *et al.*, 2007; Marchese *et al.*, 2017).

1.6.1.1 Uses and Pharmacological Properties of Eugenol

Eugenol is found in bay leaves, clove oil and allspice and used in flavouring, food and cosmetic agents. The food and drug administration also rendered eugenol as safe to use (Fujisawa *et al.*, 2002; Ito, Murakami and Yoshino, 2005). Eugenol has a detergent-like effect and thus is used as antiseptic in dentistry as well as, in the form of zinc oxide eugenol cement (Fujisawa *et al.*, 2002; Ito, Murakami and Yoshino, 2005).

Eugenol have been reported to have numerous pharmacological properties including anti-mutagenic and insecticidal properties, as well as anti-carcinogenic (Sukumaran, Unnikrishnan and Kuttan, 1994) and anti-allergy properties (Thosar, 2013). A review by Kamatou, Vermaak

and Viljoen (2012) reported that eugenol possess various pharmacological properties including antibacterial, antiplasmodial, antiviral, antihemintic, analgesic, anti-oxidant, anticancer, antimutagenic, antigenotoxic and anti-inflammatory activity.

This phenolic compound has been widely described as an anti-oxidant to scavenge free radicals, superoxide and nitric oxide radicals along with inhibiting iron-mediated lipid peroxidation (Fujisawa *et al.*, 2002; Ito, Murakami and Yoshino, 2005). Eugenol is also reported to be an analgesic by interacting with vanilloid receptors and neurotransmitters; as well as possessing agonistic and antagonistic effects on γ-aminobutyric acid and *N*-methyl-D-aspartate glutamate receptors, respectively (Guénette *et al.*, 2007a). Kim *et al.* (1997) reported that the anaphylactic properties of eugenol were due to a dose-dependent inhibition of histamine release from mast cells. Eugenol reportedly has potential anticonvulsant effects (Fajemiroye *et al.*, 2014).

Eugenol also possess insecticidal properties against *Rhyzopertha dominica*, *Oryzaephilus surinamensis* and *Callosobruchus chinensis* as well as repellent properties. The phenol also exhibited potent insecticidal properties as well as repellence towards *Sitophilus granaries*, *Sitophilus zeamaris*, *Tribolium castaneum* and *Prostephanus truncalus* (beetles). Eugenol also has potential as a fumigant against mould and yeast (Kamatou, Vermaak and Viljoen, 2012). Studies by Rants'o *et al.* (2022a) and Rants'o, Koekemoer and Van Zyl (2023) on eugenol exhibited insecticidal properties on *Anopheles* larvae.

1.6.2 Eugenol Derivatives

1.6.2.1 Isoeugenol

Isoeugenol can be found in several plants, amongst others nutmeg (*Myristica fragrans*), basil (*Ocimum basilicum*), allspice (*Pimenta dioica*), cinnamon (*Cinnamomum verum*) and clove (*Syzygium aromaticum*) (Janssens *et al.*, 1990; Koeduka *et al.*, 2006; Hyldgaard *et al.*, 2015). It is often used as a fragrant food additive (Ito, Murakami and Yoshino, 2005). This phenolic compound has anti-oxidant properties (Ito, Murakami and Yoshino, 2005); as well as mild analgesic (pain-relieving) and anti-inflammatory properties (Kaur and Sultana, 2012). It has been reported to possess antibacterial and antifungal activity (Galvão *et al.*, 2022; Fajdek-Bieda *et al.*, 2025).

1.6.2.2 MethylIsoeugenol, Eugenyl Acetate and *O*-eugenol

(*E*)-Methylisoeugenol is used as a food flavouring additive and can be found in the essential oils of *Pimenta pseudocaryphyllus* (Tan and Nishida, 2012). (*E*)-Methylisoeugenol has been

reported to possess anti-inflammatory, *in vivo* anxiolytic and antidepressant effects (Fajemiroye *et al.*, 2014).

Eugenyl acetate is an ester derived from eugenol that is found in clove buds, *Syzygium aromaticum* (Safrudin, Maimulyanti and Prihadi, 2015; Dos Santos *et al.*, 2016). Eugenyl acetate is found in clove and cinnamon essential oils (Lopez *et al.*, 2025). It also has potential antimicrobial effects against Gram-positive and -negative microbes, anti-inflammatory and analgesic properties (Vanin *et al.*, 2014)**.** Eugenyl acetate has also been reported to have larvicidal properties (Machado *et al.*, 2017).

σ-Eugenol is an isomer of eugenol, where the hydroxyl group is between the allyl and methoxy group (Moraes *et al.*, 2020; Macêdo *et al.*, 2022). Macêdo *et al.* (2022) revealed σ-eugenol to have potential antibacterial activity against *S. aureus* by efflux pump inhibition. As for eugenol, σ-eugenol has been reported to possess anti-oxidant, anti-inflammatory and antifungal activity and to be moderately toxic against *Drosophila melanogaster* (Macêdo *et al.*, 2022).

1.6.2.3 Methyleugenol

The phenylpropanoid, methyleugenol is a methylated derivative of eugenol. Eugenol and its methyl derivative, methyleugenol, are found in almost all spices. This compound is a product from the shikimate pathway by phenylalanine. Methyleugenol can be found in *Citronella* grass and over 450 plant species. It can be found in essential oils in different amounts from the leaves, stems, roots, flowers or whole plants. Essential oils from plant species that contain ≥90% methyleugenol are amongst others *Croton malambo, Cinnamomum cordatum, Melaleuca bracteate, Pimenta racemose*, *Ocimum basilicum (*basil), *Cymbopogon citratus* (lemongrass), *Zingiber officinale (*ginger) and *Myristica fragrans (*nutmeg) (Tan and Nishida, 2012). Methyleugenol has anti-oxidant activity by scavenging free radicals, analgesic and anti-inflammatory properties (Choi *et al.*, 2010). It also has anticonvulsant properties (Fajemiroye *et al.*, 2014). Methyleugenol has been shown to have some insect-repellent properties against pests (Xu *et al.*, 2015). This makes it a potential component of natural insect repellents.

1.7. Main Constituents of Essential Oils Under Investigation

1.7.1 Monoterpenes

γ-Terpinene**,** *ρ*-cymene, linalool, carvacrol and thymol are all classified as monoterpenes and have some pesticidal properties, including mosquito killing properties as well as insect repellent properties. Thymol has antibacterial properties and monoterpenes have been

considered antifungal. Linalool acts by disruption of insect nervous system as do most monoterpenes by various mechanism that can be considered targets for larvicides (Qasim *et al.*, 2024).

γ-Terpinene is found in essential oils from *Origanum vulgare* L., *Thymus capitatus* (L.) and *Satureja montana* L. (Ibraliu *et al.*, 2011). Carvacrol can be found in essential oils from *Oreganum, Thymus* and *Satureja* plants. It has vast biological properties including antimicrobial, antifungal, antiprotozoal, anti-oxidant and anti-inflammatory and is larvicidal against *Aedes aegypti* as well as *T. castaneum.* Anticholinesterase activity has also been detected (Eltalawy *et al.*, 2025). Thymol can be found in *Thymus* plants and has demonstrated larvicidal properties against *Aedes aegypti* and have several properties including antimicrobial and anti-oxidant properties (Kowalczyk *et al.*, 2020; Eltalawy *et al.*, 2025). Interestingly, all four of these essential oil constituents are the main constituents of thyme essential oil. These constituents are formed by biosynthesis when γ-terpinene undergoes aromatisation into *p*-cymene. Hydroxylation of *p*-cymene results in carvacrol and thymol, where hydroxyl groups are on different carbons of the benzene ring (Kowalczyk *et al.*, 2020).

1.7.2 Sesquiterpenoid

β-Caryophyllene is a sesquiterpenoid and the mayor constituent in many essential oils including black pepper, thyme, basil, oregano, cinnamon and rosemary. It is an agonist to the cannabinoid (CB2-R) receptor, found in the central nervous system, which engages in neurotransmitter regulation and immunity. It also has anti-inflammatory and antimicrobial properties with toxicity in animal studies only at extremely high dosages (Francomano *et al.*, 2019).

Given the increasing resistance of *Anopheles* mosquitoes to currently used synthetic insecticides, there is a pressing need for new bioinsecticides with potent insecticidal properties and safer toxicity profiles. Essential oils and their constituents have the potential to serve as novel insecticides or provide valuable strategies to combat *Anopheles* vector proliferation.

1.8 Aim, Objectives and Dissertation outline

1.8.1 Aim

The aim of the study was to determine larvicidal activity of insect-repellent essential oils black pepper, clove, sweet basil, thyme and oregano and their major constituents, whilst determining a preliminary toxicity profile and possible mechanism of action.

1.8.2 Objectives

1.8.2.1 To evaluate the insecticidal properties and morphological effects of the test phenol compounds and essential oils on the developmental stages of *Anopheles*.

1.8.2.2 To determine whether the test phenol compounds augments or reduce the insecticidal properties of propoxur.

1.8.2.3 To assess the preliminary toxicity profile of all test phenol compounds and essential oils to induce haemolysis and *Artemia* mortality or possess protective anti-oxidative properties.

1.8.2.4 To determine the *in silico* pharmacokinetic parameters, possible targets and toxicology profile

1.8.2.5 To investigate the potential interference of test phenol compounds on metal homeostasis as a potential mechanism of action.

1.8.3 Dissertation Outline

1.8.3.1 Chapter 1: Introduction

In the introduction, an overview of malaria as well as epidemiology is given. Also included is the malaria parasite and vector biology and currently used treatments and insecticides, and the development of resistance. The importance of vector metal homeostasis is discussed. An overview of the essential oils and essential oil constituents properties used in this assay is given.

1.8.3.2 Chapter 2: Methods

In the methods section the materials used in the study is outlined. The experimental protocols, data analysis, equations and statistical analysis is discussed in detail.

1.8.3.3 Chapter 3: Results

The results section contains data reported at the highest screening concentration as well as IC_{50}/ LC_{50} values where applicable. The essential oils and essential oil constituents are

compared to the positive controls in the applicable experiment. Data is reported in tables, bar graphs or log sigmoidal dose-response curve.

1.8.3.4 Chapter 4: Discussion

The discussion consists of the interpretation of the data and the implications of the findings, compared to previous studies. The larvicidal activity, toxicological profile and properties of the essential oils and essential oil constituents are put together to get an overview of the results.

1.8.3.5 Chapter 5: Conclusion

This chapter contains concluding remarks on the study as well as recommendations for future studies.

Chapter 2 – Methodology

2.0 Overview

In this chapter the materials and methods used in this study are discussed. The properties of the control compounds as well as the EO and EOCs are reported with details on how they were prepared in each respective experiment. The principle of the assays as well as the execution of experiments, with details of the data and statistical analysis are discussed. The execution of the *in silico* work is also detailed.

2.1 Materials

2.1.1 Compound Properties

The best quality of the standard controls and EOCs used in the various assays were purchased commercially from Fluka, (Switzerland), Merck® (Germany), Sigma-Aldrich, (United States). The essential oils were purchased from SOIL (South Africa), Phyto-force (South Africa), Escentia (South Africa) and Burgess and Finch (South Africa).

Table 2.1 The properties of control compounds used in the respective assays. 1: Sigma-Aldrich (United States); 2: Supelco® (PESTANAL™; United States/ Germany); 3: LabChem (United States); 4: Rochelle Chemicals (South Africa); 5: Riedel-de Haën (Switzerland); 6: Fluka (Switzerland).

Control Compound	Molecular weight (g/mol)	Empirical formula	SMILES	Source
Dichlorodiphenyl-trichloroethane (4,4'-DDT)	354.49	$C_{14}H_9Cl_5$	[13CH]1=[13CH][13C](=[13CH][13CH]=[13C]1C([13C]2=[13CH][13CH]=[13C]([13CH]=[13CH]2)Cl)C(Cl)(Cl)Cl)Cl	2
Propoxur	209.24	$C_{11}H_{15}NO_3$	CC(C)OC1=CC=CC=C1OC(=O)NC	2
Potassium Dichromate	294.19	$K_2Cr_2O_7$	[O-][Cr](=O)(=O)O[Cr](=O)(=O)[O-].[K+].[K+]	3
Triton X-100	564.8	$C_{16}H_{26}O_2$	CC(C)(C)CC(C)(C)C1=CC=C(C=C1)OCCO	1
Ethylenediaminetetra acetic Acid tetrasodium (EDTA) disodium salt	372.24	$C_{10}H_{14}O_6N_2.2H_2O$	C(CN(CC(=O)O)CC(=O)[O-])N(CC(=O)O)CC(=O)[O-].[Na+].[Na+]	4
Trolox	250.29	$C_{14}H_{18}O_4$	CC1=C(C2=C(CCC(O2)(C)C(=O)O)C(=C1O)C)C	1
Ammonium tetrathiomolybdate	260.28	$H_8MoN_2S_4$	N.N.[SH-].[SH-].S=[Mo+2]=S	1
L(+) Ascorbic acid	176.12	$C_6H_8O_6$	C([C@@H]([C@@H]1C(=C(C(=O)O1)O)O)O)O	5
2,2'-Bipiridyl	156.19	$C_{10}H_8N_2$	C1=CC=NC(=C1)C2=CC=CC=N2	6
Butylhydroxyansiole	180.24	$C_{22}H_{32}O_4$	CC(C)(C)C1=C(C=CC(=C1)OC)O	6
n-Propyl gallate	212.2	$C_{10}H_{12}O_5$	CCCOC(=O)C1=CC(=C(C(=C1)O)O)O	1
Bathophenanthroline	590.55	$C_{24}H_{16}N_2Na_2O_9S_2$	C1=CC(=CC=C1C2=C3C=CC4=C(C=CN=C4C3=NC=C2)C5=CC=C(C=C5)S(=O)(=O)[O-])S(=O)(=O)[O-].O.O.O.[Na+].[Na+]	1

Properties in Table 2.1 were determined using PubChem (2025): Dichlorodiphenyl-trichloroethane (4,4'-DDT) (PubChem, 2025a), Propoxur (PubChem, 2025b), Potassium Dichromate (PubChem, 2025c), Triton X-100 (PubChem, 2025d), Ethylenediaminetetraacetic Acid tetrasodium (EDTA) disodium salt (PubChem, 2025e), Trolox (PubChem, 2025f), Ammonium tetrathiomolybdate (PubChem, 2025g), L(+) Ascorbic acid (PubChem, 2025h), 2,2'-Bipiridyl (PubChem, 2025i), Butylhydroxyansiole (PubChem, 2025j), n-Propyl gallate (PubChem, 2025k), Bathophenanthroline (PubChem, 2025l).

Table 2.2 The properties of the essential oil constituents. 1: Sigma-Aldrich (United States); 2: Fluka (Switzerland); 3: Merck® (Germany)

Essential oil constituent	Molecular weight (g/mol)	Density (g/mL)	Empirical formula	SMILES	Source
β-Caryophyllene	204.35	0.910	$C_{15}H_{24}$	C=C1CC\C=C(\C)CCC2C1CC2(C)C	1
Carvacrol	150.22	0.976	$C_{10}H_{14}O$	Cc1ccc(cc1O)C(C)C	1
ρ-Cymene	134.22	0.861	$C_{10}H_{14}$	Cc1ccc(cc1)C(C)C	1
Eugenol	164.21	1.570	$C_{10}H_{12}O_2$	COc1cc(ccc1O)CC=C	2
Eugenol acetate	206.24	1.668	$C_{12}H_{14}O_3$	COc1cc(ccc1OC(C)=O)CC=C	1
Isoeugenol	164.21	1.085	$C_{10}H_{12}O_2$	COc1cc(ccc1O)\C=C\C	3
Linalool	154.25	0.87	$C_{10}H_{18}O$	CC(O)(CC\C=C(\C)C)C=C	1
Methyleugenol	178.23	1.036	$C_{11}H_{14}O_2$	COc1cc(ccc1OC)CC=C	1
Methylisoeugenol	178.23	1.060	$C_{11}H_{14}O_2$	COc1cc(ccc1OC)\C=C/C	3
σ-Eugenol	164.20	1.068	$C_{10}H_{12}O_2$	COc1cccc(CC=C)c1O	1
γ-Terpinene	136.23	0.850	C10H16	CC=1CC=C(CC=1)C(C)C	2
Thymol	150.22	0.965	$C_{10}H_{14}O$	Cc1cc(O)c(cc1)C(C)C	1

Table 2.3 Properties of the essential oils sourced from various South African companies.

Essential oil	Species	Density (g/mL)	Company
Clove-leaf oil	*Syzygium aromaticum*	1.438	Phyto-Force® (South Africa)
Sweet basil oil	*Ocimum basilicum*	1.272	SOIL® (South Africa)
Black pepper oil	*Piper nigrum*	1.270	SOIL® (South Africa)
Thyme oil	*Thymus Vulgaris*	1.195	Burgess & Finch® (South Africa)
Oregano oil	*Origanum vulgare*	1.530	Escentia® (South Africa)

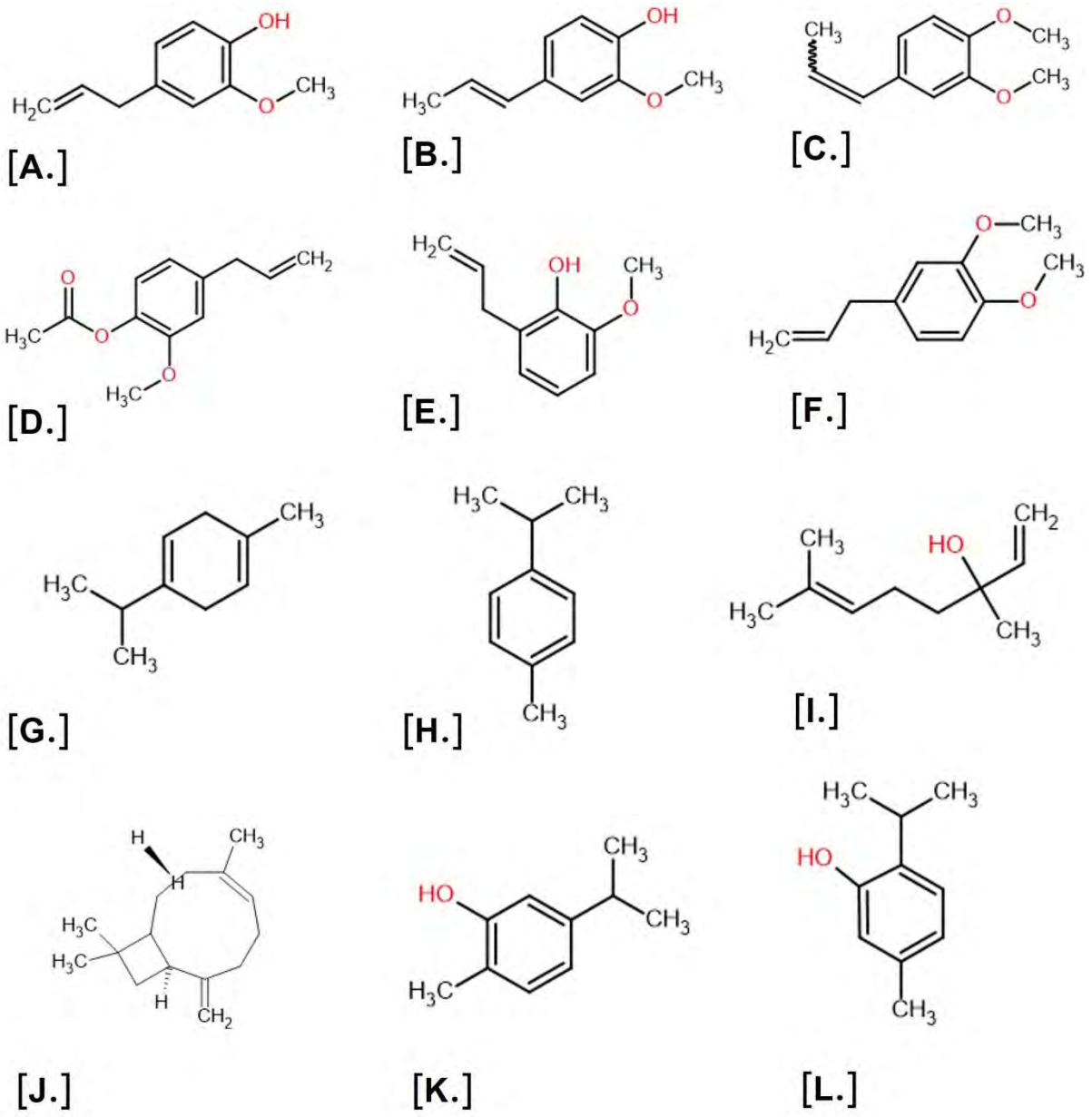


Figure 2.1: Chemical structures of essential oil constituents under investigation. **A**. Eugenol (PubChem, 2025A), **B**. Isoeugenol (PubChem, 2025B), **C.** Methylisoeugenol (PubChem, 2025C), **D.** Eugenyl acetate (PubChem, 2025D), **E.** σ-Eugenol (PubChem, 2025E), **F.** Methyleugenol (PubChem, 2025F), **G.** γ-Terpinene (PubChem, 2025G), **H.** *p*-Cymene (PubChem, 2025H), **I.** Linalool (PubChem, 2025I), **J.** β-Caryophyllene (PubChem, 2025J), **K.** Carvacrol (PubChem, 2025K), **L**. Thymol (PubChem, 2025L).

2.1.2 Preparation of Essential Oils and Essential Oil Constituents

EO and EOCs were weighed out before each experiment and dimethyl sulfoxide (DMSO) added. Different stock concentrations of the EO/EOCs were prepared as appropriate for each assay. Taking

the dilution factor into account, the EO and EOCs were diluted in DMSO to screening concentrations of 0.1, 1, 10 and 100 µg/ml. The EO/EOCs that exhibited more than 60% inhibitory activity were further diluted to seven serial concentrations to obtain a log sigmoidal dose response curve to determine the 50% inhibitory concentration (IC_{50} value) using GraphPad Prism version 5 or 50% lethal concentration (LC_{50} values) using International Business Machines Corporation Statistical Package for the Social Sciences (IBM SPSS) version 29.0.2.0 (20).

2.1.3 Preparations of Positive Control Standards

All positive control standards were prepared at a standard concentration of 10 mM, and converted to µg/mL, using each positive control's respective molecular weight (Table 2.1). Triton X-100, in liquid form was converted to µg/mL, using density (1.07 g/mL). Stock concentrations from this point forward were represented as µg/mL, to match EO/EOC stock concentrations to compare in the same SI units.

2.2 Gas Chromatography Mass Spectrometry Analysis

The gas chromatography-mass spectrometry (GC-MS) of the five essential oils were conducted by Prof Viljoen's research team at the Tshwane University of Technology, South Africa to determine the EOCs of the EO. Briefly, the EO were diluted to 20% (v/v) with hexane and analysed by GC-MS. Major components were tentatively identified by comparing the mass spectra to that of the NIST05 and Wiley libraries and comparison of retention indices. Relative retention indices (RRI's) obtained from Massfinder (Maree *et al.*, 2014).

2.3 Malaria Vector Activity

The EOs and EOCs were tested for insecticidal activity against the 3rd instar larval life stage of the *Anopheles arabiensis* vector (KWAG strain, originally resistant to pyrethroids) obtained from KwaZulu Natal (Mouatcho *et al.*, 2009). This assay was done to determine whether the EO/EOCs could be considered as a possible insecticide against *Anopheles* larvae. The assay was conducted according to the WHO 2005 determination of biological activity protocol (WHO, 2005). Animal ethics clearance (Reference number Waiver 18-02-2025-O; Appendix A) was obtained from the Animal Ethics Research Committee of the University of the Witwatersrand, permitting the use of *Anopheles* mosquito larvae.

2.3.1 *Anopheles* Vector Maintenance

Anopheles arabiensis (KWAG-strain) larvae were housed in the Maureen Coetzee insectary, Wits Research Institute for Malaria (WRIM), University of the Witwatersrand, Johannesburg. Larvae were reared on a 3:1 ratio of mixed powdered dog biscuits (Beano™) and Brewer's yeast (Hunt *et al.*, 2005). Larvae in the Maureen Coetzee insectary were reared at 25°C at a 80% relative humidity with a 12 hour day/night cycle with 1 hour dusk to dawn cycle (WHO, 2005).

2.3.2 Preparation of EO/EOCs and Control Compounds

EO and EOCs were weighed out before each experiment with a stock concentration of 2000 µg/mL were prepared in DMSO. Taking into account the dilution factor of 100, the EO and EOCs were diluted in DMSO to a screen concentration of 0.1, 1, 10 and 100 µg/mL. The positive controls were Dichlorodiphenyltrichloroethane (4,4'-DDT; Supelco® PESTANAL™) and propoxur (Supelco® PESTANAL™) with a stock concentration of 3544.9 µg/mL and 2092.4 µg/mL, respectively, prepared in DMSO, with a starting concentration of 1 µg/mL (100% mortality).

2.3.3 Larvicidal Assay

As per the WHO guidelines (2005), each cup contained 20 larvae, 99 ml of distilled water, and 1 ml of the test compound, making up a total volume of 100 ml. Each cup was closed with a net and elastic to ensure if adult *Anopheles* developed they were trapped in the cup. The room temperature of 27 ºC was maintained to ensure optimal larvae development. Larval food (3:1 ratio of mixed powdered dog biscuits (Beano™) and Brewer's yeast) was added daily (Hunt *et al.*, 2005). Mortality was recorded at 24, 48 and 72 hours, with morphological alterations recorded.

The positive controls were DDT and propoxur, with the negative control consisting of 100 ml distilled water with 20 larvae. DMSO, the solvent used to prepare the EO/EOCs was tested to rule out DMSO-induced toxicity. The final % DMSO was maintained at 1% to ensure no DMSO lethality. Each EO/EOC/control was tested in 5 replicates to account for inter-variability between larvae generations (WHO, 2005; Rants'o *et al.*, 2022b).

2.3.4 Data Analysis

From the 5 replicates, the percentage mortality was calculated taking the dead and moribund larvae into account as per Equation 2.1. The EO/EOCs that exhibited more than 60% activity at 100 µg/mL were diluted further to obtain LC_{50} values, with Probit analysis (IBM SPSS Statistical version 28.0.2.0 (20) Software package, IBM Corp). Morphological changes were recorded using an Olympus Model

SZ2-ILST microscope, with a 12x magnification and 20 000 µm scale, with pictures recorded using the Dino Capture version 2.0.

$$\%\ Larvae\ mortality = \frac{Number\ of\ dead\ and\ moribund\ larvae}{Total\ number\ of\ larvae} x\ 100$$

Equation 2.1: Larvae % mortality as an indicator of EO and EOC larvicidal activity (WHO, 2005)**.**

2.3.5 Larvicidal Combination Assay

After analysis of the larvicidal activity of the individual EO/EOCs/controls, the EO/EOC with the highest activity (with the lowest LC_{50} value), were further tested in combination with positive control, propoxur at specific concentrations. This was done to determine whether the activity of the EO/EOC could be enhanced and to determine whether it had a synergistic or antagonistic effect on larvae mortality. Taking the dilution factor of 200x into account, serial dilutions were prepared before the EO/EOC was combined with propoxur in a ratio of 1:1. Experimental setup was done as described in Section 2.3.4. The EO/EOC and propoxur were evaluated in each experiment for direct comparison to the various combinations. An untreated water control was set up as the negative control. Data analysis and morphological changes were recorded as per Section 2.3.4. Data was reported as % mortality (Equation 2.1).

To determine whether the combinations had an additive, antagonistic or synergistic effect on % larvae mortality, the combination data (% larvae mortality) was imported into a bar graph. This was done to visualise the difference in mortality across the different combinations. This data was then compared to the % mortality rates of the individual most active EO/EOC and propoxur data to determine if there was an increase in % mortality.

2.4 Toxicity Activity

2.4.1 Red Blood Cell Toxicity

The haemolysis assay was undertaken to determine the red blood cell lytic (haemolytic) properties of the EO/EOCs according to Hayat *et al.* (2011) and Ansari *et al.* (2017). When EO/EOCs interfere with the integrity of the red blood cells and change their morphology, haemoglobin would leak out resulting in a pink to red hue of the supernatant to dark red, whereas intact red blood cells the supernatant remained clear (‘Red blood cells: Hemolysis’, 2025). A human ethics waiver was obtained from the Human Research Ethics committee of the University of the Witwatersrand (Protocol reference: M240945; Appendix A) for the requitement and blood collection from healthy volunteers.

2.4.1.1 Preparation of Phosphate Buffered Saline and Complete Culture Medium

Autoclaved deionised MilliQ® water (200 ml) was added to a phosphate buffered saline (PBS) tablet (Sigma, United States) and then the pH adjusted to 7.4. Each tablet prepared as such yielded 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4, at 25 °C (Sigma, 2025). The PBS solution was autoclaved (pressure: 121 kpa, temperature: >100 °C) for 30 minutes and stored at 4 °C until required (van Zyl, 1997).

Incomplete culture medium liquid RPMI-1640 medium (Roswell Park Memorial Institute medium) 500 mL (product number: R7388) purchased from Sigma-Aldridge (United States) comprised of 20 mM 4-(2-hydroxyethyl)-1-piperazine (HEPES) and L-glutamine, without sodium bicarbonate, liquid, sterile-filtered. This product was stored at 4 °C. The complete culture medium was prepared by adding 82 ml RPMI-1640 (Sigma-Aldrich, United States), 3 ml 5% (w/v) bicarbonate and 15 ml ALBUMAX™, filtered through a 0.22 µm syringe filter (Acrodisc® PF Syringe filter).

2.4.1.2 Preparation of Uninfected Red Blood Cells

The human blood was obtained from healthy volunteers who matched the inclusion criteria of not having taken antimicrobials, anticoagulants, antivirals or antimalarial medication three weeks prior, and had not travelled to a malaria-endemic area in the month before donation. Prospective volunteers completed a self-administered questionnaire and consent form to confirm eligibility based on the inclusion and exclusion criteria (Appendix A). Human whole blood was collected in 6 ml blood tubes containing an anticoagulant, acid citrate dextrose solution-B (ACD-B; BD Vacutainer®). The blood was washed by centrifugation at 671 *g* for 5 minutes to remove plasma and buffy coat. The supernatant was discarded, and RBCs washed three times with phosphate-buffered saline (PBS, pH 7.4) by centrifugation. Thereafter incomplete culture media was added to maintain a 50% haematocrit and stored at 4 °C for no longer than 10 days.

Red blood cells, no more than three days old, were used in the haemolytic assay to maintain optimal membrane integrity. A 2% haematocrit suspension was then prepared from the pelleted red blood cells using complete culture medium (Section 2.4.1.1).

2.4.1.3 Preparation of EO/EOCs and Positive Controls

EO/EOCs were weighed out before each experiment to prepare a stock concentration of 3000 µg/mL in DMSO. Taking into account, the dilution factor of 10, the EO/EOCs were further diluted in DMSO to screening concentrations of 0.1, 1, 10 and 100 µg/ml. The positive control with 100% haemolysis consisted of a final concentration of 0.2% (v/v) (2.14 µg/ml) Triton™ X-100 (Sigma-Alrich, United

States) prepared in deionised water, that was sterilized with a 0.22 µm filter and stored at 4 °C until required.

2.4.1.4 Experimental Protocol

In a sterile 96-well plate, 20 µL of the EO/EOCs, 0.2% (v/v) Triton™ X-100 and DMSO (negative control) were added to the plate, whereafter 180 µL of the red blood cell suspension (Section 2.4.1.2) was added to each well. The plates were incubated for 48 hours at 37 °C in a candle jar, under conditions of 3% O_2, 5% CO_2 and 92% N_2. After 48 hours the plate was centrifuged (Thermo Scientific Heraus Megafuge 8 Centrifuge) at 931 *g* for 5 minutes to disrupt the pellet. Then the supernatant (50 µL) of each well, was added into a non-sterile 96-well microtitre plate along with 150 µL deionised water. The absorbance was read at 414 nm using the ThermoScientific Multiskan Go plate reader (SKANIT software 3.2).

2.4.1.5 Data Analysis

The EO/EOCs/controls were screened three times and the mean and standard deviation determined. Percentage haemolysis was calculated using Equation 2.2 taking the 4x dilution factor into account after replating the supernatant. The EO/EOCs that exhibited more than 60% haemolytic activity at 100 µg/mL were serially diluted further to obtain a log sigmoidal-dose response curve, to determine the IC_{50} value using GraphPad Prism version 5.

$$\%\, Haemolysis = \left(\frac{A_{Test\ compoundl} - A_{blank}}{A_{positive} - A_{blank}}\right) x100$$

Equation 2.2: Percentage haemolysis as a marker of EO/EOCs affecting red blood cell membrane integrity. Where A is absorbance read, blank is the negative control and positive is the difference between the 100% haemolysis control and the negative control (Mustapha, 2017).

2.4.2 *Artemia franciscana* Nauplii Lethality Assay

To assess the toxicity of the EO and EOCs on the ecosystem, the *Artemia franciscana* nauplii lethality assay was conducted. The assay was adapted from Ruebhart, Wickramasinghe and Cock, (2009). Animal ethics clearance was obtained from the Animal Ethics Research Committee of the University of the Witwatersrand (Reference number Waiver 18-02-2025-O; Appendix A), permitting the use of *Artemia franciscana* nauplii.

2.4.2.1 Preparation of Saltwater and Hatching of Eggs

To make up the saltwater for optimal hatching and development of the nauplii, 32 grams of sea salt (Instant Ocean) was dissolved in one litre of distilled water. *Artemia franciscana* eggs (Ocean Nutrition™) were hatched in aerated saltwater for 24 hours at 25 ± 2 °C with a 2000 lux artificial light. The hatched nauplii along with the saltwater was poured into a sorting tray and densified using a point light source (Ruebhart, Wickramasinghe and Cock, 2009).

2.4.2.2 Preparation of EO/EOCs and Positive Controls

EO/EOCs were weighed out before each experiment. A stock concentration of 2000 µg/mL were prepared in DMSO. Taking into account, the dilution factor of 100x, the EO and EOCs were diluted in DMSO to a screen concentration of 0.1, 1, 10 and 100 µg/ml. The EO/EOCs that exhibited more than 60% activity at 100 µg/mL were diluted further to obtain LC_{50} values. The positive controls were DDT (stock concentration: 3544.9 µg/mL) and propoxur prepared in DMSO and Potassium dichromate prepared in deionised water with a stock concentration of 2941.9 µg/mL.

2.4.2.3 Experimental Protocol

In a 48-well plate, 50 µL of densified nauplii, containing about 25-30 nauplii, was plated into each well. Saltwater (197.5 µL) was added to each well along with 2.5 µL EO/EOC. The negative untreated control was saltwater and separately 1% DMSO, to rule out toxicity due to DMSO, and the positive control, potassium dichromate. The plate was incubated at room temperature (25 ± 2 °C). Dead nauplii were counted on a 10X microscope at 0, 24, 48 and 72 hours and morphology photographically with an Olympus SC50 microscope (Entry cellSens software) at 24, 48 and 72 hours. After 72 hours once the counts were finished, three drops of 50% (v/v) acetic acid were added to each well to kill of remaining live nauplii. Total number of nauplii were counted and recorded per well.

2.4.2.4 Data Analysis

The EO/EOCs/controls were screened three times and the mean and standard deviation determined and reported as % mortality. Percentage mortality was determined using Equation 2.3. Compounds that resulted in more than 60% mortality after 24 hours were tested further to determine the LC_{50} values using Probit analysis (IBM SPSS Statistical version 29.0.2.0 Software Package, IBM Corp.).

$$\%\ Artemia\ nauplii\ mortality = \left(\frac{Total\ dead\ nauplii\ in\ three\ wells}{Total\ nauplii\ in\ three\ wells}\right) x\ 100$$

Equation 2.3: Percentage *Artemia franciscana* nauplii mortality as in indicator of EO and EOCs environment toxicity (Mustapha, 2017).

2.4.3 *Artemia* Combination Assay

The two most active larvicidal compounds identified in Section 2.3.4, that was used for combination studies in Section 2.3.5, were tested on *Artemia franciscana* nauplii as well. The combination was done in a 1:1 ratio with the same concentrations as described in Section 2.3.5, to determine preliminary toxicity of the EO/EOC in combination with propoxur. The *Artemia* combination assay was done according to Section 2.4.2.3 and data analysis according to Section 2.4.2.4. To determine whether the combinations increased % mortality on *Artemia*, the combination data (% nauplii mortality) was imported into a bar graph. This was done to visualise the difference in mortality compared to the % mortality rates of the individual most active EO/EOC and propoxur.

2.4.4 Selectivity Index

The selectivity index (SI) for the EO/EOCs were determined for *Anopheles* larvae and *Artemia* toxicity by using their LC_{50} values determined by the Larvicidal assay (Section 2.3.3) and *Artemia franciscana* nauplii lethality assay (Section 2.4.2). A selectivity index above 10 is an indication of non-toxicity to non-target organisms, indicating that the EO/EOC is more selective (toxic) towards the target organism (*Anopheles*) and less selective (less toxic) toward non-target organisms (*Artemia*) (Rants'o ***et al.***, 2022b). Equation 2.4 was used in calculating the selectivity indices for the EO/EOCs. In the event that EO/EOCs that did not warrant an LC_{50} determination in the *Artemia franciscana* nauplii lethality assay, the highest concentration in screening was used.

$$Selectivity\ Index = \frac{LC_{50}\ Artemia\ nauplii}{LC_{50}\ Anopheles\ larvae}$$

Equation 2.4. Equation used in the determination of the SI of EO/EOCs (Mustapha, 2017; Rants'o ***et al.***, 2022b).

2.5 Metal Homeostasis Activity

2.5.1 Iron Chelation Assay

The iron chelation assay was undertaken to determine the ferrous iron chelating ability of the EO/EOCs in comparison to known iron chelators. Using the ferrozine assay as described by Gülçin, Elmastaş and Aboul-Enein (2007), the colour change from clear to dark purple detected when

ferrozine chelates iron was used as the basis of this assay. As iron metabolism is important in the physiology of the *Anopheles* vector (Geiser ***et al.***, 2015), the iron chelating properties of the EO/EOCs were investigated as possible mode of larvicidal action.

2.5.1.1 Preparation of Iron Chelating Assay Reagents

Taking the dilution factor of 7.5x for this assay, the reagents were prepared as follows. A concentration of 1.2 mM ferrozine (Fluka, Switzerland) was prepared in deionised MilliQ® water and stored at 4 °C for a maximum period of 2 weeks. A 5% (w/v) ammonium acetate (Riedel-de Haën, Switzerland) solution was prepared in deionised MilliQ® water in which 0.48 mM $FeCl_2$ (Fluka, Switzerland) was dissolved. The positive control, EDTA (Rochelle Chemicals, South Africa) was made up in deionised MilliQ® water at a stock concentration of 3722.4 µg/mL.

2.5.1.2 Preparation of EO/EOCs and Positive Controls

The EO/EOCs were weighed out fresh before each experiment to prepare a stock concentration of 2000 µg/mL in DMSO. Taking a dilution factor of 7.5 into account, the EO/EOCs were diluted in DMSO to screening concentrations of 0.1, 1, 10 and 100 µg/mL. The stock solutions of the positive controls, EDTA and 2,2-bipiridyl were prepared in deionised water to a concentration of 3722.4 µg/mL and 1561.9 µg/mL, respectively.

2.5.1.3 Iron Chelation Assay

A volume of 20 µL of the EO/EOCs and control compounds were added in triplicate to the 96-well plate. $FeCl_2$ (30 µL) was added to all the wells and incubated for 5 minutes in the dark. Appropriate controls were prepared with DMSO/water with $FeCl_2$ and ferrozine used as the 100% chelation control and the blank untreated control contained 100 µL DMSO/water and 30 µL $FeCl_2$. Ferrozine (100 µL) was added to all but the blank control wells that contained only $FeCl_2$ and water, The plates were incubated for a further 30 minutes in the dark before the absorbance was read at 562 nm using a UV-spectrophotometer (Thermo Scientific MULTISCAN GO, SKANIT Software 3.2).

2.5.1.4 Data Analysis

The EO/EOCs/controls were screened three times and the mean and standard deviation determined and reported as reported as % iron chelation. Percentage iron chelation was calculated using Equation 2.5. The EO/EOCs that exhibited more than 60% activity at 100 µg/mL were diluted further to obtain a log sigmoidal-dose response curve to determine the IC_{50} value using GraphPad Prism version 5.

$$\boldsymbol{Iron\ chelation\ abillity}\ (\%) = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) x\ 100$$

Equation 2.5: Iron chelating ability (%) determined using the ferrozine assay. Where *A* is absorbance, *control* the average of 100% iron chelation control minus the blank and *sample* is the EO/EOC/positive control (Gülçin, Elmastaş and Aboul-Enein, 2007).

2.5.2 Copper (II) Chelation

Metal ions play distinct roles in living organisms, including mosquitoes as essential co-factors in enzymes (Dow, 2017). Compounds with copper chelating properties have the potential to influence larvae mortality. The copper chelation assay utilizes the copper (I) chelating properties of neocuproine to detect a colour change from clear to yellow for the copper chelating abilities of EO/EOCs (Říha *et al.*, 2013). Hydroxylamine was added to oxidize the copper (II) ions to copper (I) to optimise the assay (Syed and Syeda, 2007).

2.5.2.1 Preparation of Copper Chelation Reagents

To prepare the HEPES buffer, 1.25 mM (3.575 g/L) HEPES was dissolved in deionised MilliQ® water. The HEPES buffer was pH to 7.4 and stored at 4ºC for up to 6 months.

A 5 mM stock of copper (II) sulphate pentahydrate was prepared in deionised MilliQ® water and further diluted in deionised MilliQ® water to a concentration of 1 mM.

Hydroxylamine and neocuproine with a 5 mM concentration were prepared in deionised MilliQ® water. These reagents were prepared fresh for every experiment.

2.5.2.2 Preparation of EO/EOCs and Positive Controls

EO/EOCs were weighed out before each experiment to prepare a stock concentration of 3000 µg/mL in DMSO. Taking into account the 6 x dilution factor for the assay, the EO/EOC/controls were diluted in DMSO to screening concentrations of 0.1, 1, 10 and 100 µg/mL. A 2602.8 µg/mL stock solution of the positive control, tetrathiomolybdate (TMB) was prepared in DMSO.

2.5.2.3 Experimental Protocol

EO/EOCs/controls (50 µL) were added to the 96-well plate in triplicate, with 50 µL DMSO in the blank and negative untreated wells. Copper (II) sulphate (50 µL) and HEPES buffer (100 µL) were then added to all wells in the 96-well plate before being incubated for 15 minutes at room temperature in the dark. Hydroxylamine (50 µL) was added to the plate and incubated for a further 15 minutes at

room temperature in the dark. Neocuproine (50 µL) was then added to all wells in the plate and incubated for 15 minutes at room temperature in the dark.

2.5.2.4 Data Analysis

The plates were read by a wavelength of 450 nm using a UV-spectrophotometer (Thermo Scientific MULTISCAN GO, SKANIT Software 3.2). The EO/EOCs/controls were screened three times and the mean and standard deviation determined and reported as % copper chelation. The percentage copper (II) was calculated using Equation 2.6. The EO/EOCs that exhibited more than 60% activity at 100 µg/mL were diluted further to obtain a log sigmoidal dose response curve and determine the IC_{50} values using GraphPad Prism version 5.

$$Copper\ chelation\ (\%) = \left(\frac{A_{test\ compound} - A_{blank}}{A_{positive} - A_{blank}}\right) x100$$

Equation 2.6: Percentage copper chelation to determine the copper II chelating ability of the EO/EOCs compared to the standard controls. Where A is absorbance, positive is 100 % copper chelation and blank is the negative control (Říha *et al.*, 2013).

2.6 Anti-oxidant Activity

2.6.1 1,1-Diphenyl-2-picryl-hydrazyl Free Radical Scavenging Assay

The 1,1-diphenyl-2-picryl-hydrazyl free radical (DPPH$^{\bullet}$) scavenging assay was used to determine the possible anti-oxidant properties of the EO/EOCs. DPPH$^{\bullet}$ is a stable free radical, such that when an anti-oxidant reacts with DPPH$^{\bullet}$, a hydrogen atom is received, reducing the nitrogen atom of DPPH$^{\bullet}$ (Kedare and Singh, 2011). DPPH$^{\bullet}$ is dark purple in colour and upon reduction the solution became clear indicating free radical scavenging activity. This assay was conducted according to Gülçin, Elmastaş and Aboul-Enein, (2007).

2.6.1.1 Preparation of DPPH$^{\bullet}$

A solution of 100 µM DPPH$^{\bullet}$ (Aldridge Chemistry, United states) was prepared in methanol 12 hours before the assay. The DPPH$^{\bullet}$ was stored at 4 °C covered with foil so it stays in the dark.

2.6.1.2 Preparation of EO/EOCs and Positive Controls

EO/EOCs were weighed out before each experiment. A stock concentration of 3000 µg/mL were prepared in DMSO. Taking into account, the different dilution factor of 5x, the EO/EOCs were diluted in DMSO to a screen concentration of 0.1, 1, 10 and 100 µg/mL. The positive controls, ascorbic acid

(Riedel-de Haën, Switzerland) and Trolox™, were prepared in double distilled water and DMSO, respectively at a stock concentration of 1761.2 µg/mL and 2502.9 µg/mL, respectively.

2.6.1.3 DPPH Experimental Setup

In accordance to the protocol of Gülçin, Elmastaş and Aboul-Enein (2007), 50 µL EO/EOC and controls were added in triplicate to the 96 non-sterile well plate. DMSO (50 µL) was used as a 100% free radical scavenging control, and 200 µL methanol as a blank control. Following which, 200 µL DPPH• was added to all the wells, except for the blank control, and incubated for 30 minutes in the dark. Absorbance was read at 562 nm using a UV-spectrophotometer (Thermo Scientific MULTISCAN GO, SKANIT Software 3.2).

2.6.1.4 Data Analysis

The EO/EOCs/controls were screened three times and the mean and standard deviation determined and reported as % free radical scavenging. Percentage free radical scavenging was calculated using Equation 2.7. The EO/EOCs that exhibited more than 60% activity at 100 µg/mL were diluted further to generate a log sigmoidal dose response curve to determine the IC_{50} values using GraphPad Prism version 5.

$$\boldsymbol{DPPH\ Scavenging\ activity}\ (\%) = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) x\ 100$$

Equation 2.7: DPPH• scavenging activity (%) to determine the free radical scavenging activity of the EO/EOCs compared to standard control. Where A is absorbance, control is 100% free radical scavenging minus the negative untreated control and sample is the EO/EOC/positive controls (Gülçin, Elmastaş and Aboul-Enein, 2007).

2.7 *In Silico* Studies

2.7.1 Chemical Structures and Identification

ACD Chemsketch© version C45E41 was used to draw the chemical structures of the EOCs and main control compounds (Appendix B). Through this application the EOCs conical Simplified Molecular Input Line Entry System (SMILES) were determined which was used as identifier for *in silico* pharmacokinetics (Swiss ADME) (Section 2.7.2), Toxtree version 3.1.0-1851-1525442531402 (Section 2.7.2), Target prediction (Swiss Targets) studies (Section 2.7.3), and Ecological Structure-Activity Relationship Model (ECOSAR) (Section 2.7.3).

2.7.2 *In silico* Pharmacokinetics and Toxicity Predictions

Swiss ADME available free online (http://www.swissadme.ch/) was used to determine the compounds *in silico* pharmacokinetic parameters. Pharmacokinetics were determined by entering compounds conical SMILES identifier (Section 2.7.1). Other properties such as blood brain barrier permeability, bioavailability, Lipinski rule adherence and permeability were also obtained (Daina, Michielin and Zoete, 2014, 2017; Daina and Zoete, 2016).

Toxtree software version 3.1.0.1851 was used to determine the toxicity level and metabolic pathways of the compounds (Mostrag-Szlichtyng, Zaldívar Comenges and Worth, 2010). The compounds conical smiles identifier (Section 2.7.1) was entered to determine the toxicity properties. Two pathway trees were selected namely, Cytochrome P450 metabolism and revised Cramers' rules. Toxic hazard class was determined using the revised Cramers' rules decision tree (Cramer, Ford and Hall, 1976; Munro *et al.*, 1996; Patlewicz *et al.*, 2008). Cytochrome P450 metabolism pathway was used to determine first metabolism reaction (Rydberg *et al.*, 2010; Rydberg, Gloriam and Olsen, 2010).

ECOSAR (Ecological Structure-Activity Relationship Model) Version 2.2 was used to further determine aquatic toxicity of the EOCs using their conical smile identifier (Section 2.6.1). The toxicity of the EOCs neutral organics functional group was used for comparison of toxicity. LC_{50} values against fish and daphnids were reported as predicted by the programme. The program included blue gill sunfish, common carp, fathead minnow, guppy, rainbow trout, red killifish or zebra fish in the determination of the fish model (Wright *et al.*, 2022).

2.7.3 *In silico* Molecular Target Predictions

The Swiss target prediction program (http://www.swisstargetprediction.ch/) was used to determine *in silico* target predictions of the compounds using their conical smile identifier (Section 2.7.1) in humans (Homo Sapiens). This helped to identify targets that can be problematic towards humans if ingested. Further literature research was done on the main targets predicted by this program (Gfeller, Michielin and Zoete, 2013; Daina, Michielin and Zoete, 2014, 2019).

2.8 Statistical Analysis

The analysis of variance (ANOVA) was used as statistical tool to determine the statistical significance of EO/EOCs on GraphPad Prism version 5. This tested whether there was a significant difference in activity between EO/EOC and their respective positive control compounds. A significant value was reported as $p < 0.05$ (DATAtab Team, 2025). A p-value of > 0.05 indicated that there was no statistical significant difference between the EO/EOC and the positive control. P-values < 0.05

indicated that there is a significant statistical difference between the EO/EOC and the positive control. The lower the *p*-value, the higher statistical difference was determined. Statistics were reported as follow: Analysis of Variance (ANOVA): Compared to positive control. (ns) not significant, *p*-value > 0.05; (*) significant, *p*-value ≤ 0.05; (**) Significant, *p*-value ≤ 0.01;(***) very significant, *p*-value ≤ 0.001; (****) very significant, *p*-value ≤ 0.0001.

Chapter 3 – Results

3.0 Results Overview

This chapter presents the findings of the study, beginning with the GC-MS analysis identifying the primary constituents of each essential oil. The study compounds (eugenols derivatives, EOs and EOCs) were first screened at four concentrations (0.1, 1, 10, and 100 µg/mL) to identify those to be further investigated to determine the LC_{50}/IC_{50} values in the various biological and chemical assays. To explore potential mechanisms of action, the anti-oxidant properties of the study compounds were evaluated using DPPH free radical scavenging, copper(II) chelation, and iron(II) chelation assays. To evaluate potential environmental safety issues, the lethality properties of the test compounds were assessed *in silico* toxicity predictions against fish and daphnids and using *Artemia franciscana* nauplii noting morphological effects. Toxicity to human was investigated to complement its safety profile with pharmacokinetics and potential human targets considered.

3.1. Gas Chromatography-Mass Spectrometry Data

The chemical composition of the clove leaf (*Syzygium aromaticum*) essential oil consisted of a total of 4 constituents representing 99.5% of the total oil (Table 3.1.A). GC-MS identified 85.7% eugenol and 11.7% β-caryophyllene as the major constituents.

Table 3.1.A: Clove leaf (*Syzygium aromaticum*) essential oil's EOCs as determined by GC-MS presented as % area. Massfinder was used to obtain RRI's.

Clove leaf oil (*Syzygium aromaticum*)		
RRI	**Compound Identification**	**% Area**
1613	**β-Caryophyllene**	**11.7**
1687	α-Humulene	1.4
2020	Caryophyllene oxide	1.0
2200	**Eugenol**	**85.7**
	Total	**99.8**

The chemical composition of the black pepper (*Piper nigrum*) essential oil is presented in Table 3.1.B, in which the compounds are listed in order of their elution. A total of 24 constituents, representing 99.5% of the total oil, were identified by GC-MS. The constituent that had the highest yield was β-caryophyllene, followed by limonene and sabinene (% area >10).

Table 3.1.B: The essential constituents recorded for black pepper (*Piper nigrum*) essential oil using GC-MS analysis presented as % area. Massfinder was used to obtain RRI's.

Black pepper (*Piper nigrum*)		
RRI	**Compound Identification**	**% Area**
1023	α-Pinene	8.7
1026	α-Thujene	1.4
1066	Camphene	0.3
1113	β-Pinene	8.4
1127	**Sabinene**	**10.3**
1153	δ-3-Carene	7.0
1170	Myrcene	3.1
1205	**Limonene**	**14.5**
1214	β-Phellandrene	2.1
1251	γ-Terpinene	0.3
1258	*trans*-β-Ocimene	0.2
1278	*p*-Cymene	0.5
1288	Terpinolene	0.5
1464	α-Cubebene	0.2
1478	δ-Elemene	2.3
1501	α-Copaene	3.7
1549	β-Copaene	0.3
1558	Linalool	0.5
1605	β-Elemene	0.5
1616	**β-Caryophyllene**	**29.7**
1686	α-Humulene	1.3
1737	β-Bisabolene	1.5
1771	δ-Cadinene	1.1
2018	Caryophyllene oxide	1.1
	Total	**99.5**

In comparison to the essential oil of black pepper (*Piper nigrum*), only 15 constituents were identified in sweet basil (*Oscimum basilicum*) essential oil (Table 3.1.C.). This represented 99.5% of the total oil as identified by GC-MS, with estragole and linalool the highest proportion of the essential oil.

Table 3.1.C: The results of sweet basil (*Oscimum basilicum*) essential oil after GC-MS analysis presented as % area. Massfinder was used to obtain RRI's.

Sweet basil (*Oscimum basilicum*)		
RRI	**Compound Identification**	**% Area**
1257	*trans*-β-Ocimene	0.2
1351	6-Methyl-5-heptene-2-one	0.1
1560	**Linalool**	**17.4**
1595	*cis*-α-Bergamotene	0.4
1613	β-Caryophyllene	0.4
1656	*p*-Menthan-1-ol	0.4
1678	*cis*-β-Farnesene	0.2
1694	**Estragole**	**75.9**
1704	Neral	0.4
1755	Geranial	0.6
1784	Unknown (*m/z* 93. 119. 121. 80)	1.2
2029	Unknown (*m/z* 177. 192. 131. 178)	1.8
2313	Unknown (*m/z* 135. 150. 91. 107)	0.4
2353	Unknown (*m/z* 135. 150. 91. 107)	0.4
	Total	**99.8**

The oregano (*Origanum vulgare*) essential oil was predominantly found to contain carvacrol with minor percentages of three other EOCs (Table 3.1.D). In total the 4 constituents. represented 99.5% of the total oil.

Table 3.1.D: The EOCs of oregano (*Origanum vulgare*) essential oil after GC-MS analysis presented as % area. Massfinder was used to obtain RRI's.

Oregano (*Origanum vulgare*)		
RRI	**Compound Identification**	**% Area**
1252	γ-Terpinene	3.2
1278	*p*-Cymene	2.8
2200	Thymol	5.5
2235	**Carvacrol**	**88.1**
	Total	**99.6**

The chemical composition of the thyme (*Thymus vulgaris*) essential oil consisted of a total of 14 constituents, representing 99.5% of the total oil (Table 3.1.E). The constituent with the highest proportion in this essential oil was thymol followed by *p*-cymene.

Table 3.1.E: The GC-MS analysis of thyme (*Thymus vulgaris*) essential oil presented as % area. Massfinder was used to obtain RRI's.

Thyme (*Thymus vulgaris*)		
RRI	**Compound Identification**	**% Area**
1021	α-Pinene	0.5
1066	Camphene	0.4
1168	Myrcene	1.0
1184	α-terpinene	1.0
1203	Limonene	0.3
1214	Eucalyptol	0.4
1252	γ-Terpinene	5.1
1280	***p*-Cymene**	**16.8**
1558	Linalool	4.8
1613	β-Caryophyllene	1.3
1620	Terpinen-4-ol	1.3
1721	Borneol	1.6
2201	**Thymol**	**61.6**
2232	Carvacrol	3.5
	Total	**99.6**

3.2. *An. arabiensis* Larvicidal Properties

3.2.1. Eugenol and Derivatives

Among the six eugenol-like EOCs to be tested, eugenol, methylisoeugenol, eugenyl acetate, σ-eugenol and methyleugenol at 100 µg/mL exhibited high larvicidal activity at 24 hours (Table 3.2). The eugenol derivatives (eugenol, isoeugenol) appeared to display a time-dependent increase in larval mortality over the three days (Figure 3.1). In contrast, the control insecticide propoxur (1 µg/mL) achieved 100% mortality within 24 hours. Derivatives that induced more than 60% larval mortality after 72 hours were selected for LC_{50} value determination.

Table 3.2: The lethality of *An. arabiensis* of eugenol and derivatives at a 100 µg/mL, compared to positive control propoxur and DDT at 24-. 48- and 72- hours.

Compound	% Larvae mortality (Average ± s.d.; *n=5*)		
	24 hours	48 hours	72 hours
Propoxur (1 µg/mL)	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
DDT (1 µg/mL)	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
Eugenyl Acetate	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
σ-Eugenol	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
Methyleugenol	96.67 ± 6.06	100.00 ± 0.00	100.00 ± 0.00
Methylisoeugenol	91.67 ± 7.64	96.00 ± 4.18	99.00 ± 2.24
Eugenol	86.67 ± 11.55	95.00 ± 7.07	100.00 ± 0.00
Isoeugenol	32.00 ± 2.50	78.33 ± 2.89	86.25 ± 8.54

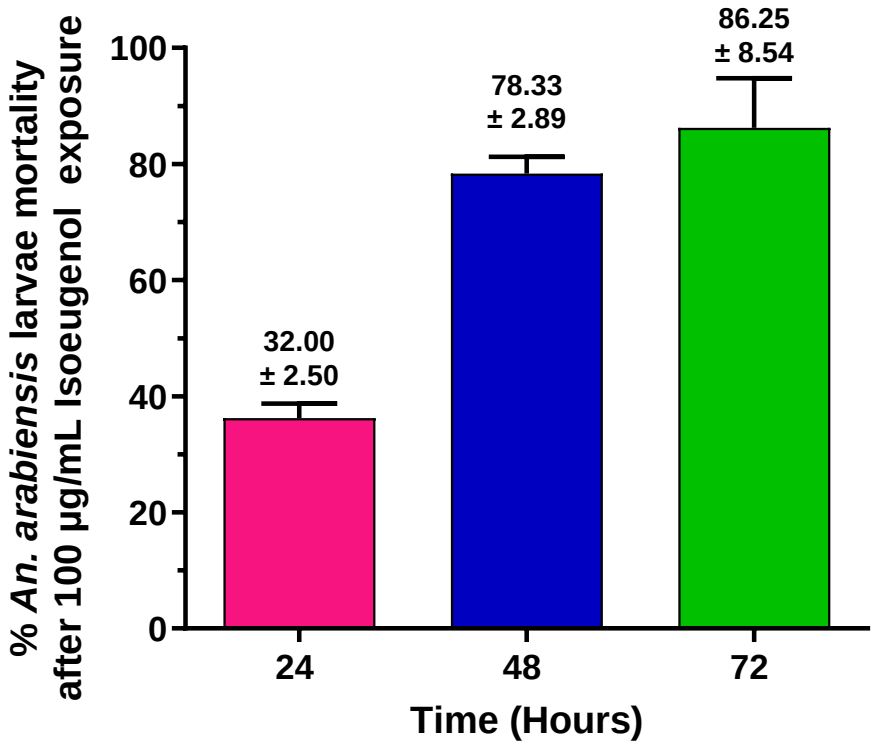


Figure 3.1. The time-dependant increase of larvae mortality over a 72-hour period from following exposure to 100 µg/mL isoeugenol.

The eugenol derivatives LC_{50} values were determined and compared to the standard controls, DDT and propoxur. The most active of the eugenol derivatives against *An. arabiensis* larvae was determined to be methylisoeugenol at all time intervals with comparable activity to σ-eugenol (Table 3.3). Of the six eugenol derivatives tested, eugenol and eugenol acetate displayed the greatest reduction in larvae viability over the 72 hour period; with eugenol acetate showing the most inhibition

after 72 hours of exposure. However, the inhibitory activity of these eugenol derivatives was not comparable to that of propoxur (LC_{50} value: 0.19 ± 0.06 µg/mL), exhibiting high significant difference (*p*-value ≤ 0.0001).

Table 3.3: The larvicidal properties of eugenol and derivatives on *An. arabiensis*, compared to the positive controls, propoxur and DDT at 24-, 48- and 72- hours. (LC_{50} values (average ± s.d.; *n*=3)

Compound	LC_{50} value (µg/mL) (Average ± s.d.; *n*=3)			Change in LC_{50} value activity from 24 to 72 hours
	24 hours	48 hours	72 hours	
Propoxur	0.19 ± 0.06	0.04 ± 0.01	0.04 ± 0.01	0.15
DDT	0.02 ± 0.00	0.02 ± 0.01	0.01 ± 0.00	0.01
Methylisoeugenol	**28.69 ± 5.91 (****)**	**20.19 ± 1.58 (***)**	**18.52 ± 5.60 (***)**	10.17
σ-Eugenol	**30.49 ± 9.19 (****)**	**22.71 ± 4.27 (***)**	**21.47 ± 2.13 (***)**	9.02
Methyleugenol	42.72 ± 2.54 (****)	37.12 ± 5.11 (****)	31.96 ± 2.10 (****)	10.76
Eugenyl Acetate	52.87 ± 4.05 (****)	37.62 ± 10.32 (****)	**18.09 ± 6.83 (**)**	34.78
Eugenol	72.02 ± 2.47 (****)	45.43 ± 5.28 (****)	36.21 ± 2.79 (****)	35.81
Isoeugenol	96.98 ± 5.57 (****)	70.86 ± 5.28 (****)	62.92 ± 9.82 (****)	34.06

Analysis of Variance (ANOVA): Compared to propoxur. (ns) not significant. *p*-value > 0.05; (*) significant. *p*-value ≤ 0.05; (**) Significant. *p*-value ≤ 0.01;(***) very significant. *p*-value ≤ 0.001; (***) very significant. *p*-value ≤ 0.0001.

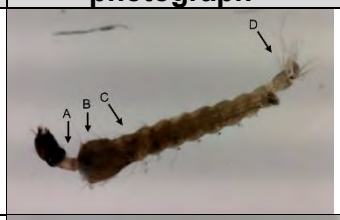
3.2.1.1. The Morphological Changes of *An. arabiensis* Observed After Treatment with Eugenol and Derivatives.

Compared to untreated *An. arabiensis* larvae (Table 3.4.A), eugenol and its derivatives generally caused head elongation (A), except for eugenyl acetate (Table 3.4.B). In contrast, propoxur treatment resulted in head enlargement (A). Alteration in thorax (B) was observed for most of the derivatives. whereas the larvae retained their posterior hair tufts (C), except when treated with eugenol and isoeugenol. Eugenol, isoeugenol, methylisoeugenol and σ-eugenol showed similarities in thorax alterations (B) to propoxur. Isoeugenol and methylisoeugenol showed a loss of normal tracheal gills (D), compared to propoxur that showed presence of normal tracheal gills (D) (Table 3.4.B).

Table 3.4.A: Morphology of *An. arabiensis* of untreated and positive controls propoxur and DDT at 1 µg/mL after 24 hours analysed microscopically using an Olympus Model SZ2-ILST microscope at a 12x magnification. (Scale bar: 20 000 µm).

Compound	Morphological photograph	Description
Untreated		A. Normal head size B. Normal thorax C. Presence of posterior hair tufts D. Presence of normal tracheal gills
Propoxur		A. Enlarged head B. Enlarged thorax C. Loss of some posterior hair tufts D. Presence of normal tracheal gills
DDT		A. Enlarged head B. Enlarged thorax C. Presence of posterior hair tufts D. Presence of normal tracheal gills

Table 3.4.B: Morphology of *An. arabiensis* of the eugenol and derivatives at 100 µg/mL after 24 hours analysed microscopically using an Olympus Model SZ2-ILST microscope at a 12x magnification. (Scale bar: 20 000 µm).

Compound	Morphological photograph	Description
Eugenol		A. Elongated head B. Elongated thorax C. Loss of posterior hair tufts D. Presence of normal tracheal gills
Isoeugenol		A. Elongated head B. Enlarged thorax C. Loss of posterior hair tufts D. Loss of normal tracheal gills
Methyl-isoeugenol		A. Normal head size B. Enlarged thorax C. Presence of some posterior hair tufts D. Loss of some normal tracheal gills
Eugenyl acetate		A. Normal head size B. Slightly enlarged thorax C. Presence of some posterior hair tufts D. Presence of normal tracheal gills
σ-Eugenol		A. Elongated head B. Small thorax C. Loss of posterior hair tufts D. Presence of normal tracheal gills
Methyl-eugenol		A. Slightly elongated head B. Normal thorax C. Presence of posterior hair tufts D. Presence of normal tracheal gills

3.2.2. Essential Oil Larvicidal Activity

All five of the whole EOs demonstrated significant *An. arabiensis* larval mortality within 24 hours at a concentration of 100 µg/mL with mortality rates ranging from 83.00% to 100.00%. Oregano and thyme essential oil were similar to that of propoxur and DDT at 1 µg/mL resulting in 100% mortality (Table 3.5). An increase of larvae mortality was seen after 48 and 72 hours, indicating a time dependent kill. Based on this promising inhibitory activity, all five EOs were further tested to determine their LC_{50} values.

Table 3.5: The percentage (Average ± s.d.; *n*=5) mortality of *An. arabiensis* of the EOs at a 100 µg/mL, compared to positive control propoxur and DDT, at 24-, 48- and 72- hours.

Compound	% Larvae mortality (Average ± s.d.; *n*=5)		
	24 hours	48 hours	72 hours
Propoxur (1 µg/mL)	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
DDT (1 µg/mL)	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
Oregano	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
Thyme	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
Sweet basil	99.00 ± 2.24	99.00 ± 2.24	99.00 ± 2.24
Clove	95.00 ± 8.66	95.00 ± 10.00	97.50 ± 5.00
Black pepper	83.00 ± 6.29	92.50 ± 8.66	98.00 ± 2.74

The LC_{50} values of the EOs were determined and compared to the standard controls, DDT and propoxur. Oregano essential oil (LC_{50} value: 15.16 ± 2.22 µg/mL) was the most active of the EOs after 24 hours followed by thyme essential oil (LC_{50} value: 23.40 ± 2.22 µg/mL) and black pepper essential oil (LC_{50} value: 42.40 ± 1.88 µg/mL) (Table 3.6). However, the inhibitory activity of the oregano essential oil was not comparable to that of propoxur with LC_{50} value of 0.19 ± 0.06 µg/mL after 24 hours, exhibiting 79.8-fold less activity (p-value < 0.0001).

3.2.2.2. The Morphological Changes of *An. arabiensis* Observed After Treatment with EOs

Clove, sweet basil, oregano and thyme essential oils showed elongation of the head and neck (A), when compared to untreated larvae and that of propoxur (Table 3.7). The larvae showed thorax (B) alterations, mainly enlargement, similar to that of propoxur. Sweet basil, clove and oregano essential

oils resulted in loss of some posterior hair tufts (C) compared to the untreated larvae (Table 3.4.A). Black pepper essential oil was the only essential oil to cause a loss of normal tracheal gills (D).

Table 3.6: The larvicidal LC_{50} values (Average ± s.d.; *n*=3) of the EOs on *An. arabiensis*, compared to the positive controls, propoxur and DDT, at 24-, 48- and 72-hours.

Compound	Larvicidal LC_{50} values (µg/mL) (Average ± s.d.; *n*=3)		
	24 hours	48 hours	72 hours
Propoxur	0.19 ± 0.06	0.04 ± 0.01	0.04 ± 0.01
DDT	0.02 ± 0.00	0.02 ± 0.01	0.01 ± 0.00
Oregano	**15.16 ± 2.22 (****)**	**13.47 ± 2.87 (*)**	**10.42 ± 1.56 (ns)**
Thyme	23.40 ± 2.22 (****)	21.05 ± 1.86 (****)	17.00 ± 4.58 (**)
Black pepper	42.40 ± 1.88 (****)	28.83 ± 7.56 (****)	22.73 ± 1.73 (***)
Sweet basil	45.22 ± 4.58 (****)	42.05 ± 7.84 (****)	35.21 ± 7.08 (****)
Clove	93.36 ± 5.14 (****)	79.29 ± 6.99 (****)	66.11 ± 10.38 (****)

Analysis of Variance (ANOVA): Compared to propoxur. (ns) not significant. *p*-value > 0.05; (*) significant. *p*-value ≤ 0.05; (**) Significant. *p*-value ≤ 0.01;(***) very significant. *p*-value ≤ 0.001; (****) very significant. *p*-value ≤ 0.0001.

Table 3.7: Morphology of *An. arabiensis* of the EOs at 100 µg/mL after 24 hours analysed microscopically using an Olympus Model SZ2-ILST microscope at a 12x magnification. (Scale bar: 20 000 µm).

Compound	Morphological photograph	Description
Black pepper		A. Normal head size B. Elongated thorax C. Presence of posterior hair tufts D. Decrease in tracheal gills
Sweet basil		A. Elongated head B. Enlarged thorax C. Loss of some posterior hair tufts D. Normal tracheal gills
Clove		A. Elongated head and neck B. Enlarged thorax C. Loss of some posterior hair tufts D. Normal tracheal gills
Oregano		A. Elongated head and neck B. Slightly elongated thorax C. Presence of some posterior hair tufts D. Presence of normal tracheal gills
Thyme		A. Elongated head and neck B. Enlarged thorax C. Normal posterior hair tufts D. Presence of normal tracheal gills

3.2.3. Main EOCs

Among the six main constituents tested, five exhibited a high mortality rate after 24 hours at a 100 µg/mL. With γ-terpinene, *p*-cymene, carvacrol and thymol showing 100% mortality comparable to the positive controls, propoxur and DDT at 1 µg/mL (Table 3.8). With β-caryophyllene inducing a mortality rate of 83.33 ± 7.64%, and while linalool was inactive.

Table 3.8: The percentage (Average ± s.d.; *n*=5) mortality of *An. arabiensis* of the main constituents at a 100 µg/mL, compared to positive control propoxur and DDT, at 24-, 48- and 72- hours.

Compound	% Larvae mortality (Average ± s.d.; *n*=5)		
	24 hours	48 hours	72 hours
Propoxur (1 µg/mL)	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
DDT (1 µg/mL)	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
γ-Terpinene	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
***p*-Cymene**	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
Carvacrol	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
Thymol	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
β-Caryophyllene	83.33 ± 7.64	95.00 ± 8.66	97.50 ± 5.00
Linalool	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

The five main constituents were further evaluated to determine their LC_{50} values. γ-Terpinene was the most inhibitory constituent with the LC_{50} values of 9.80 ± 1.60 µg/mL after 24 hours, but not significantly different from propoxur (*p*-value > 0.05). With thymol the second most active EOC, followed by *p*-cymene and carvacrol after 24 hours. With β-caryophyllene being the least active after 24 hours (Table 3.9). However, the EOCs larvicidal activity was not comparable to that of propoxur (*p*-value < 0.0001). However, following the time-dependent effect the order of potency altered from highest to lowest: γ-Terpinene > *p*-Cymene > Carvacrol > Thymol > β-Caryophyllene after 72 hours (Table 3.9).

Table 3.9: The larvicidal LC_{50} values (Average ± s.d.; *n*=3) of the main constituents on *An. arabiensis*, compared to positive control propoxur and DDT, at 24-, 48- and 72- hours.

Compound	Larvicidal LC_{50} values (µg/mL) (Average ± s.d.; *n*=3)		
	24 hours	**48 hours**	**72 hours**
Propoxur	0.19 ± 0.06	0.04 ± 0.01	0.04 ± 0.01
DDT	0.02 ± 0.00	0.02 ± 0.01	0.01 ± 0.00
γ-Terpinene	**9.80 ± 1.60 (ns)**	**9.10 ± 2.21 (*)**	**8.66 ± 2.35 (***)**
***p*-Cymene**	21.54 ± 1.06 (**)	19.30 ± 3.55 (****)	**12.68 ± 1.28 (****)**
Carvacrol	25.10 ± 6.05 (***)	18.02 ± 4.50 (****)	13.88 ± 3.08 (****)
Thymol	19.21 ± 4.02 (*)	17.10 ± 2.02 (****)	16.36 ± 3.26 (****)
β-Caryophyllene	118.92 ± 15.95 (***)	55.14 ± 7.28 (****)	25.44 ± 0.86 (****)

Analysis of Variance (ANOVA): Compared to propoxur. (ns) not significant. *p*-value > 0.05; (*) significant. *p*-value ≤ 0.05; (**) Significant. *p*-value ≤ 0.01;(***) very significant. *p*-value ≤ 0.001; (***) very significant. *p*-value ≤ 0.0001.

3.2.3.1. The Morphological Changes of *An. arabiensis* Observed After Treatment with the Main Constituents.

The main constituents resulted in elongation of the head (A), and *p*-cymene resulted in reduced head size (Table 3.10) compared to the untreated larvae (Table 3.4.A). Similar to propoxur, γ-terpinene and thymol resulted in enlargement of the thorax (B). Compared to the untreated control, *p* -cymene and carvacrol resulted in a decrease in thorax (B). γ-Terpinene and *p*-cymene resulted in loss of posterior hair tufts (C), whilst carvacrol still had some present. The γ-terpinene and *p*-cymene resulted in alterations of the tracheal gills (D).

Table 3.10: Morphology of *An. arabiensis* of the main constituents at 100 µg/mL after 24 hours analysed microscopically using an Olympus Model SZ2-ILST microscope at a 12x magnification. (Scale bar: 20 000 µm).

Compound	Morphological photograph	Description
γ-Terpinene		A. Elongated head and neck B. Enlarged thorax C. Loss of posterior hair tufts D. Loss of some tracheal gills
***p*-Cymene**		A. Decrease in head size B. Small thorax C. Loss of most posterior hair tufts D. Loss of some normal tracheal gills
β-Caryophyllene		A. Normal head B. Normal thorax C. Presence of posterior hair tufts D. Normal tracheal gills
Carvacrol		A. Decrease in head size B. Decreased thorax C. Presence of some posterior hair tufts D. Normal tracheal gills
Thymol		A. Elongated head and neck B. Enlarged thorax C. Normal posterior hair tufts D. Presence of normal tracheal gills

3.2.4. Larvicidal Combinations

Following the identification that *p*-cymene and γ-Terpinene were the most active against the 3rd instar larvae, they were combined to ascertain the interaction between them in order to augment the overall inhibitory activity of the two EOCs.

3.2.4.1. *p*-Cymene : Propoxur Combination

The combination of *p*-cymene with propoxur in a 1:1 ratio demonstrated strong larvicidal activity against *An. arabiensis* larvae, achieving 100% mortality within 24 hours at the following concentrations: *p*-cymene 100 µg/mL with propoxur 0.001 µg/mL, *p*-cymene 50 µg/mL with propoxur 0.01 µg/mL. and *p*-cymene 0.1 µg/mL with propoxur 1 µg/mL. Additionally, the combination of *p*-cymene (20 µg/mL) with propoxur (0.05 µg/mL) was highly effective, resulting in 95.0 ± 7.07% mortality at 24 hours. However, the combination of *p*-cymene 1 µg/mL with propoxur 0.25 µg/mL showed no larvicidal effect at 24 hours (0.00 ± 0.00%) (Figure 3.2).

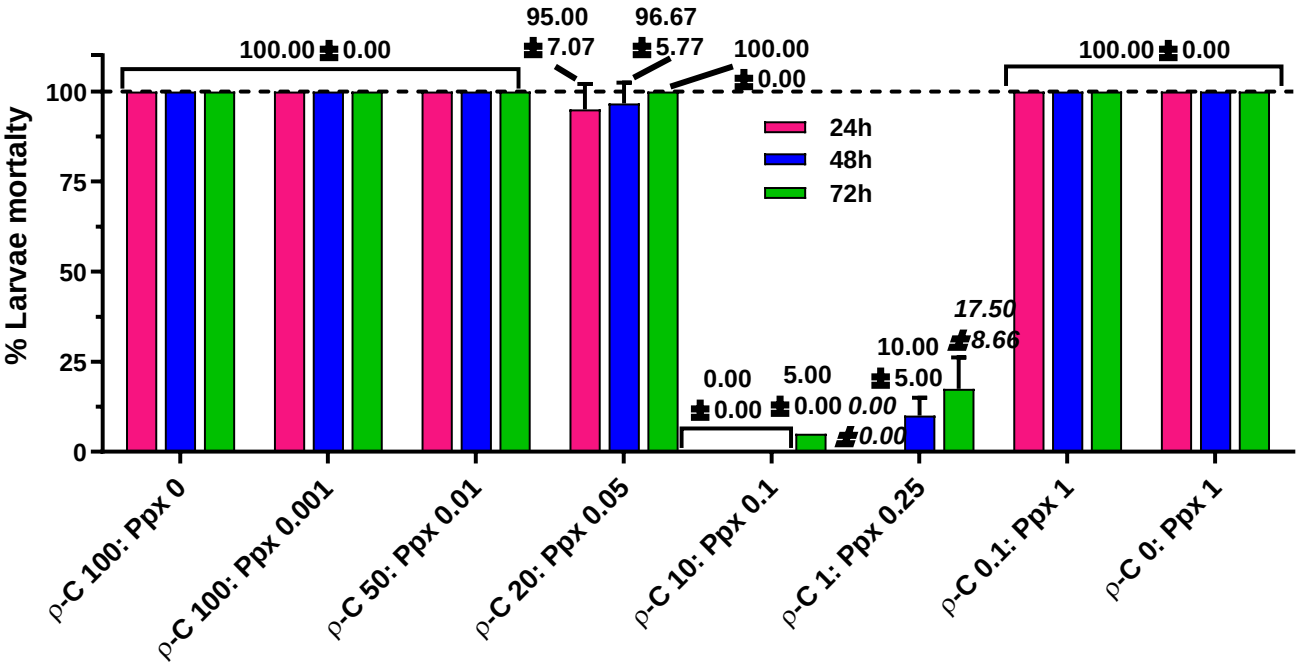


Figure 3.2: The percentage mortality of *An. arabiensis* larvae following exposure to different concentrations of *p*-cymene (*p*-C) and propoxur (Ppx) in a 1:1 ratio (µg/mL) over 24- 48- and 72-hours (*n* = 3).

3.2.4.1.1. The Morphological Changes to *An. arabiensis* After Treatment at Combination Concentrations

Compared to the untreated larvae (Table 3.4.A), all *p*-cymene : propoxur combination ratios showed a general decrease in head size (A). The larvae had an increase in thorax size (B) and loss of some of the posterior hair tufts (C), except with the *p*-cymene 0.1 : Propoxur 1 combination where all hair tufts were lost. All except *p*-cymene 0 : Propoxur 1 and *p*-cymene 20: Propoxur 0.05 had loss of normal tracheal gills (D). Decomposition of larvae was observed (Table 3.11).

Table 3.11: Morphology of *An. arabiensis* of the *p*-cymene : propoxur combinations in a 1:1 ratio after 24 hours recorded microscopically using the Olympus Model SZ2-ILST microscope at a magnification at 12x (Sale bar: 20 000 µm). Where µm is: micrometre.

Combination	Morphological photograph	Description
***p*-Cymene 100: Propoxur 0**		A. Elongated head and neck B. Elongated thorax C. Some posterior hair tufts D. Decreased tracheal gills
***p*-Cymene 100: Propoxur 0.001**		A. Decreased head size B. Elongated thorax C. Some posterior hair tufts D. Decreased tracheal gills
***p*-Cymene 50: Propoxur 0.01**		A. Decreased head size B. Enlarged thorax C. Loss of some posterior hair tufts D. Decreased tracheal gills
***p*-Cymene 20: Propoxur 0.05**		A. Decreased head size B. Enlarged thorax C. Loss of some posterior hair tufts D. normal tracheal gills
***p*-Cymene 0.1: Propoxur 1**		A. Decreased head size and elongated neck B. Enlarged thorax C. Loss of posterior hair tufts D. Decreased tracheal gills
***p*-Cymene 0 /: Propoxur 1**		A. Decreased head size B. Normal thorax C. Loss of some posterior hair tufts D. Normal tracheal gills

3.2.4.2. γ-Terpinene : Propoxur Combination

The combination of γ-terpinene and propoxur in a 1:1 ratio demonstrated strong larvicidal activity against *An. arabiensis* larvae. At γ-terpinene 100 µg/mL with propoxur 0.001 µg/mL and γ-terpinene 0.1 µg/mL with propoxur 1 µg/mL 100% mortality was observed within 24 hours. Additionally, the combinations of γ-terpinene 25 µg/mL: Propoxur 0.01 µg/mL and γ-terpinene 17.25 µg/mL: Propoxur 0.05 µg/mL were also highly effective, resulting in mortality rates ranging from 91.67% to 99.00% at 24 hours. However, γ-terpinene 10 µg/mL: Propoxur 0.1 µg/mL (18.75 ± 8.54) showed lower efficacy; while γ-terpinene 1 µg/mL: Propoxur 0.25 µg/mL had no larvicidal effect (Figure 3.3).

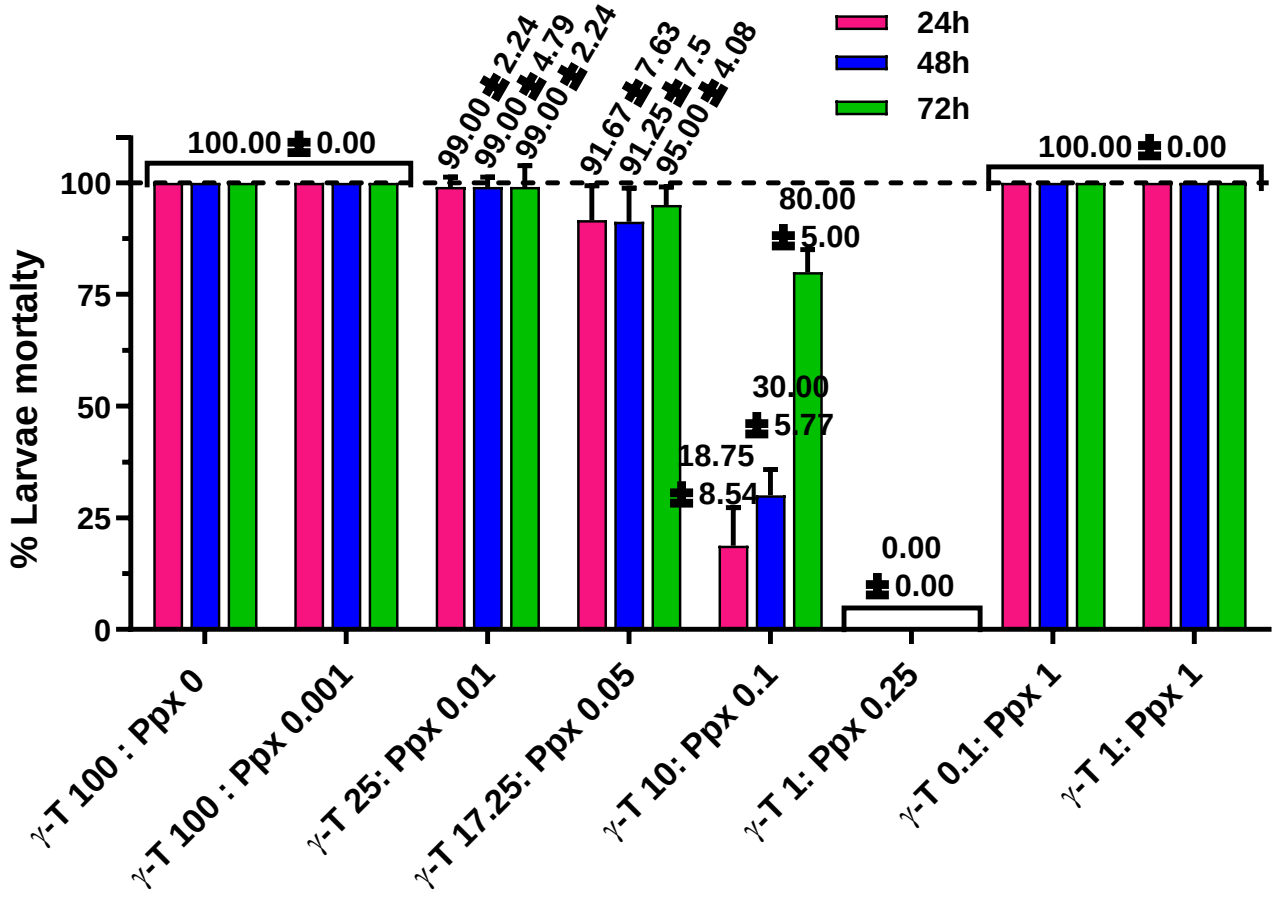


Figure 3.3: The percentage mortality of *An. arabiensis* larvae following exposure to different concentrations of γ-terpinene (γ-T) and propoxur (Ppx) in a 1:1 ratio over 24- 48- and 72- hours (*n*=3).

3.2.4.2.1. The Morphological Changes to *An. arabiensis* After Treatment at Combination Concentrations

All the combinations produced an increase in head size (A) except at the γ-terpinene 0.1: Propoxur 1 combination (Table 3.12), when compared to the untreated larvae (Table 3.4.A). The combinations

Table 3.12.: Morphology of *An. arabiensis* of the γ-terpinene: Propoxur combinations with a 1:1 ratio after 24 hours analysed microscopically using an Olympus Model SZ2-ILST microscope at a 12x magnification. (Scale bar: 20 000 µm).

Compound	Morphological photograph	Description
γ-terpinene 100: Propoxur0		A. Increased head size and elongated neck B. Enlarged thorax C. Normal posterior hair tufts D. Normal tracheal gills * Some decomposition
γ-terpinene 100: Propoxur 0.001		A. Enlarged head and elongated neck B. Normal thorax C. Normal posterior hair tufts D. Normal tracheal gills * Decomposition
γ-terpinene 25: Propoxur 0.01		A. Enlarged head B. Enlarged thorax C. Loss of some posterior hair tufts D. Normal tracheal gills
γ-terpinene 17.25: Propoxur 0.05		A. Enlarged head B. Enlarged thorax C. Normal posterior hair tufts D. Loss of tracheal gills
γ-terpinene 10: Propoxur 0.1		A. Enlarged head B. Decreased thorax C. Normal posterior hair tufts D. Normal tracheal gills
γ-terpinene 0.1: Propoxur 1		A. Normal head size B. Decreased thorax C. Loss of some posterior hair tufts D. Normal tracheal gills * Decomposition
Y-terpinene 0: Propoxur 1		A. Decreased head size B. Normal thorax C. Loss of some posterior hair tufts D. Normal tracheal gills

with higher γ-terpinene concentrations resulted in an enlarged thorax (B) and those with higher propoxur concentrations produced a decrease in thorax size. All concentrations except for the γ-terpinene 0.1: Propoxur 1 combinations, showed the larvae retaining their posterior hair tufts (C). The γ-terpinene 17.25: Propoxur 0.05 combination showed a loss of normal tracheal gills (Table 3.12).

3.3. Toxicology Profile

3.3.1. *Artemia franciscana* Nauplii Lethality

3.3.1.1. Eugenol and Derivatives

Among the six eugenol derivatives tested, three resulted in more than 60% *Artemia* lethality at 100 µg/mL after 24 hours, namely eugenol, methylisoeugenol, and σ-eugenol (Range: 100.00 ± 0.00%). Only eugenyl acetate and methyleugenol showed a favourable preliminary safety profile against the *A. franciscana* nauplii with percentage mortality of 14.99 ± 5.36% and 29.29 ± 1.01%, respectively after 24 hours. Propoxur showed the least toxicity against the *A. franciscana* nauplii (Table 3.13).

Table 3.13: The percentage (Average ± s.d.; *n*=3) mortality of *Artemia franciscana* nauplii of eugenol and derivatives at a 100 µg/mL, compared to positive control propoxur and DDT, at 24-, 48- and 72-hours.

Compound	% *Artemia* mortality (Average ± s.d.; *n*=3)		
	24 hours	48 hours	72 hours
Propoxur (10 µg/mL)	0.00 ± 0.00	0.00 ± 0.00	22.27 ± 10.10
Potassium dichromate (10 µg/mL)	5.63 ± 3.27	82.74 ± 3.32	95.53 ± 7.78
Eugenyl acetate	**14.99 ± 5.36**	**40.46 ± 14.53**	97.97 ± 1.96
Methyleugenol	**29.29 ± 1.01**	**43.17 ± 0.44**	**64.43 ± 8.32**
Isoeugenol	53.90 ± 7.33	94.21 ± 7.92	96.30 ± 6.42
Eugenol	96.01 ± 6.90	97.46 ± 4.39	99.13 ± 1.94
Methylisoeugenol	98.29 ± 2.96	100.00 ± 0.00	100.00 ± 0.00
σ-Eugenol	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00

The lethal eugenol derivatives (isoeugenol, eugenol, methylisoeugenol and σ-eugenol) LC_{50} values were determined and compared to the standard control, potassium dichromate. After 24 hours, the order of efficacy was methyleugenol > σ-eugenol > eugenol > methylisoeugenol with

methylisoeugenol 1.8-fold more toxic than potassium dichromate (*p*-value ≤ 0.05) (Figure 3.4). With eugenol, σ-eugenol and methyleugenol comparable to potassium dichromate with *p*-values of > 0.05. All eugenol derivatives were more toxic over time although methyleugenol and σ-eugenol were significantly less toxic than potassium dichromate (*p*-values: <0.001), eugenol and methylisoeugenol over 72 hours (Figure 3.4).

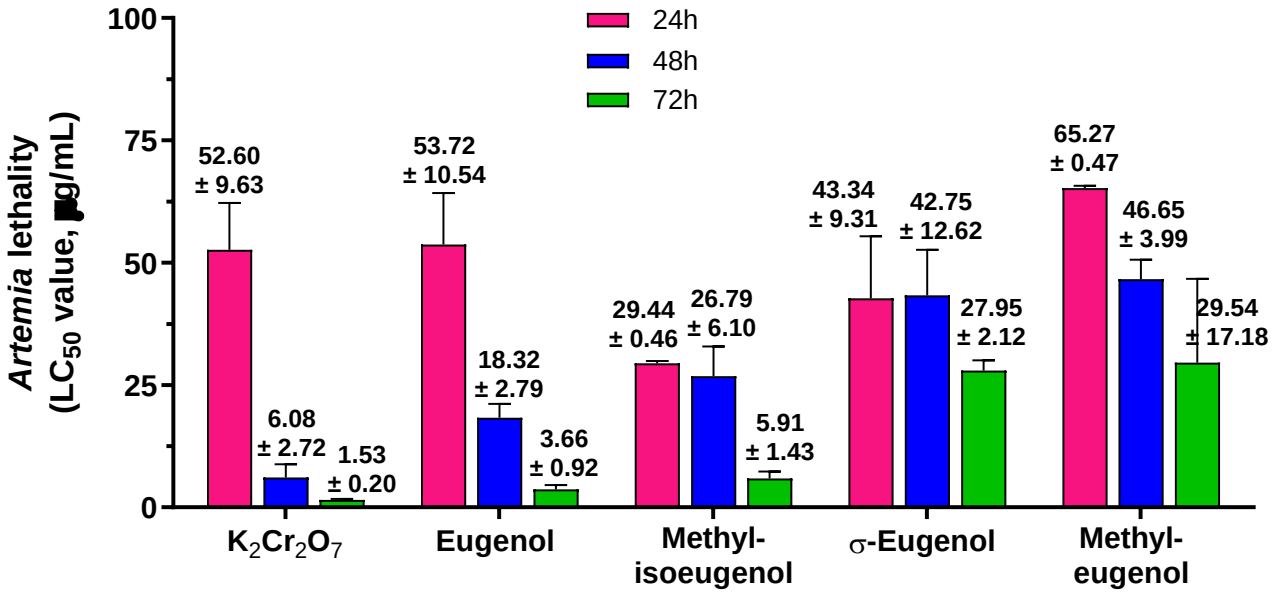
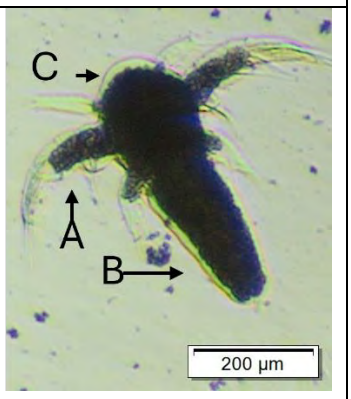
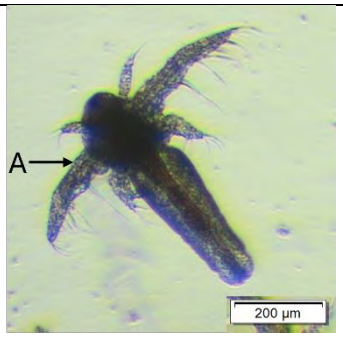
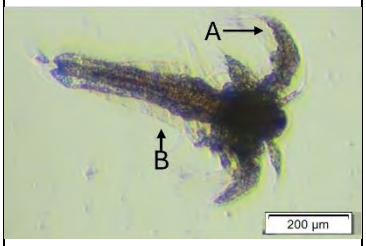


Figure 3.4. Lethal effects of eugenol and derivatives (methylisoeugenol, σ-eugenol and methyleugenol) compared to the positive control, potassium dichromate (*n*=3), represented as the LC_{50} values at over 72 hours on *A. franciscana* nauplii.

3.3.1.1.2. Effect of Eugenol and Derivatives *on Artemia franciscana* Nauplii Morphology

In comparison to the untreated control after 24 hours of exposure, eugenol, methylisoeugenol, σ-eugenol and methyleugenol all damaged the *Artemia* antennae (A) and a reduction of size (Table 3.14). Eugenol and methylisoeugenol showed damage to the abdomen (B), whilst methyleugenol resulted in a reduced size in the abdomen along with gut damage. Similarly to the observed morphology induced by the positive control, potassium dichromate. Eugenol and methylisoeugenol resulted in darkening of the nauplli and eyes (C) were not visible (Table 3.14).

Table 3.14: Morphology of *Artemia franciscana* nauplii of the lethal eugenol and derivatives, compared to negative and positive controls, saltwater and potassium dichromate, respectively after 24 hours. Analysed microscopically using the Olympus SC50 microscope at a magnification of 20x (Scale bar: 200 µm). [KEY: A: Antennae; B: Abdomen; C: Eye]

Compound	Picture	Compound	Picture
Untreated		**Potassium dichromate**	
Eugenol		**Methyl-isoeugenol**	
σ-Eugenol		**Methyleugenol**	

3.3.1.2. Essential Oils

Of the five essential oils, black pepper essential oil and sweet basil essential oil demonstrated the lowest lethality against the *A. franciscana* nauplii, similar to propoxur (Table 3.15). While clove, oregano and thyme essential oils exhibited the highest lethality on the *Artemia* (100.00 ± 0.00%) at 24 hours.

Table 3.15. The lethality of the essential oils at a 100 µg/mL against the *Artemia franciscana* nauplii compared to positive control propoxur and DDT, at 24-, 48- and 72- hours.

Compound	% *Artemia* mortality (Average ± s.d.; *n*=3)		
	24 hours	48 hours	72 hours
Propoxur (10 µg/mL)	0.00 ± 0.00	0.00 ± 0.00	22.27 ± 10.10
Potassium dichromate (10 µg/mL)	5.63 ± 3.27	82.74 ± 3.32	95.53 ± 7.78
Black pepper	**0.00 ± 0.00**	**0.00 ± 0.00**	**12.32 ± 2.49**
Sweet basil	**8.65 ± 3.96**	**13.44 ± 3.16**	**28.59 ± 3.94**
Clove	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
Oregano	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
Thyme	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00

The LC_{50} values of lethal EOs (clove, oregano and thyme) were determined and compared to the standard control, potassium dichromate. After 24 hours, clove essential oil was safer than potassium dichromate, showing statistical significant difference (*p*-value of ≤ 0.01). In contrast, oregano and thyme essential oils were 2.9-fold and 2.1-fold more lethal than potassium dichromate, respectively. Where the oregano and thyme essential oils were significantly different (*p*-value: ≤ 0.001) to potassium dichromate (Figure 3.5).

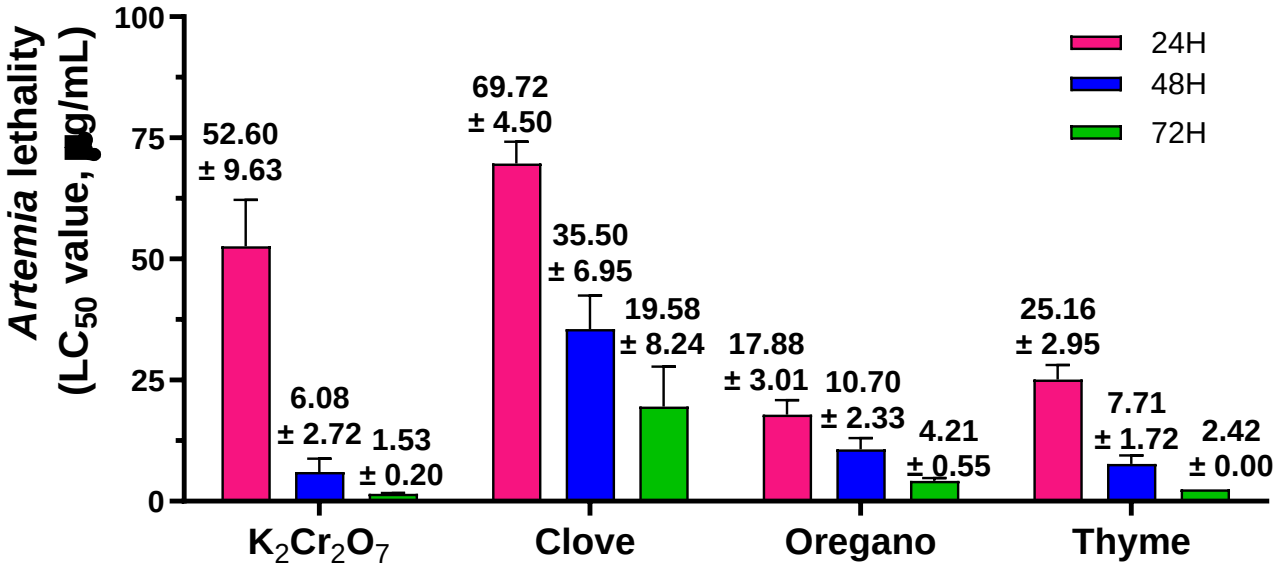
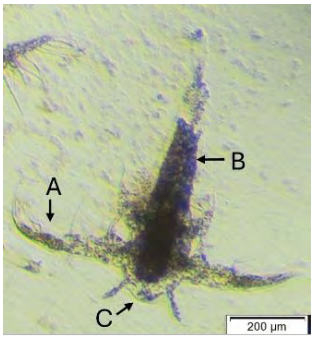
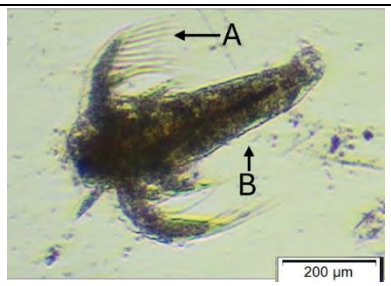


Figure 3.5. Lethal EOs (clove. oregano and thyme) bar graph of the LC_{50} values at 24-.48- and 72-hours on *Artemia franciscana* nauplii, compared to positive control potassium dichromate (*n*=3).

3.3.1.2.1. Effect of Essential Oils on *Artemia franciscana* Nauplii Morphology

After 24 hours, oregano and thyme essential oils both resulted in damaged appendages (A), as well as a decrease in size and degradation of nauplii (Table 3.16), as compared to the untreated control (Table 3.14). It also resulted in damage to the abdomen (B) and gut. Clove essential oil resulted in darkening of the abdomen (B). Oregano essential oil resulted in degradation of nauplii to the point where the eyes (C) were not seen.

Table 3.16: Morphology of *Artemia franciscana* nauplii of the lethal EOs, compared to saltwater and potassium dichromate controls after 24 hours. Analysed microscopically using the Olympus SC50 microscope at a magnification of 20x (Scale bar: 200 µm).

Compound	Picture	Compound	Picture
Clove		**Oregano**	
Thyme			**A: Antennae** **B: Abdomen** **C: Eye**

3.3.1.3. Main Constituents

Of the six main constituents, two constituents showed more than 60% mortality after 24 hours at a 100 µg/mL, namely carvacrol (100.00 ± 0.00%) and thymol (100.00 ± 0.00%) (Table 3.17). While γ-terpinene, *p*-cymene, linalool and β-caryophyllene showed favourable safety effects against the *A. franciscana* nauplii with percentage mortality range of 1.07 ± 0.32 to 31.29 ± 7.19%, after 24 hours (Table 3.17).

Table 3.17: The percentage mortality of *Artemia franciscana* nauplii of the main constituents at 100 µg/mL (average ± s.d.; *n*=3), compared to positive controls propoxur and DDT at 24-, 48- and 72-hours.

Compound	% *Artemia* mortality (Average ± s.d.; *n*=5)		
	24 hours	48 hours	72 hours
Propoxur (10 µg/mL)	0.00 ± 0.00	0.00 ± 0.00	22.27 ± 10.10
Potassium dichromate (10 µg/mL)	5.63 ± 3.27	82.74 ± 3.32	95.53 ± 7.78
β-Caryophyllene	**1.07 ± 0.32**	**11.42 ± 5.75**	**39.69 ± 11.27**
Linalool	**2.55 ± 1.85**	**9.03 ± 4.03**	**9.41 ± 5.24**
***p*-Cymene**	22.27 ± 2.76	31.42 ± 10.45	41.66 ± 6.91
γ-Terpinene	31.29 ± 7.19	36.02 ± 15.35	36.72 ± 9.96
Carvacrol	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
Thymol	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00

Thereafter the lethal main constituents (carvacrol and thymol) LC_{50} values were determined and compared to the standard control, potassium dichromate. After 24 hours, carvacrol and thymol were respectively, 6.2-fold and 8.5-fold, significantly (*p*-values: ≤ 0.001) more lethal than potassium dichromate (Figure 3.6). The lethality of carvacrol was comparable to potassium dichromate after 48 hour (*p*-value: > 0.05), whilst thymol was 3.1-fold more lethal than potassium dichromate after 48 hours (*p*-value: > 0.05). Carvacrol (LC_{50} value: 1.74 ± 0.37 µg/mL) was also comparable to potassium dichromate (LC_{50} value: 1.53 ± 0.20 µg/mL) after 72 hours (*p*-value: > 0.05), while thymol was 3.1-fold more lethal than potassium dichromate after 72 hours (*p*-value: ≤ 0.01) (Figure 3.6).

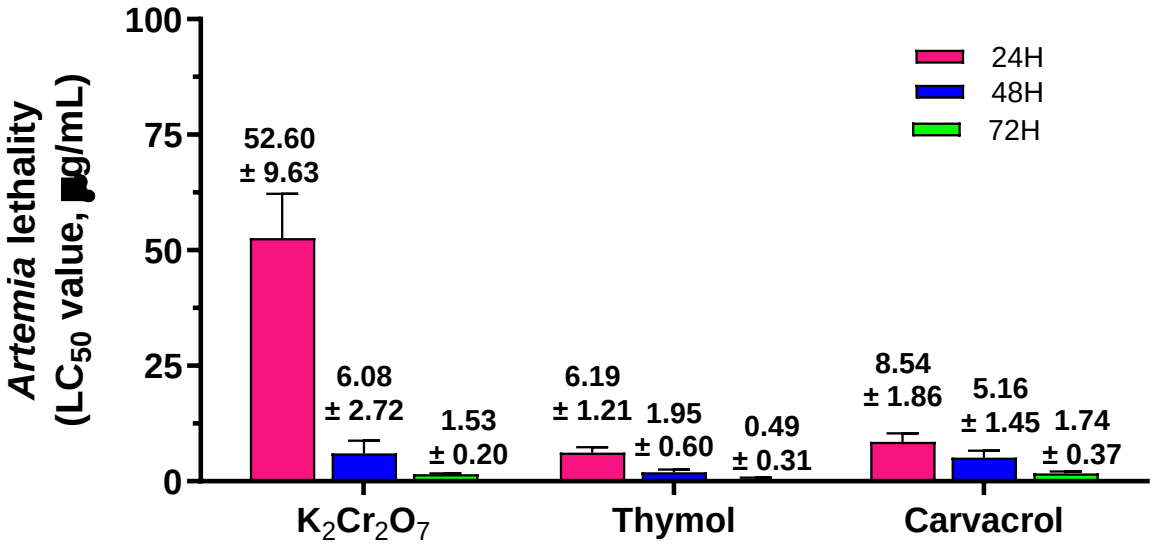
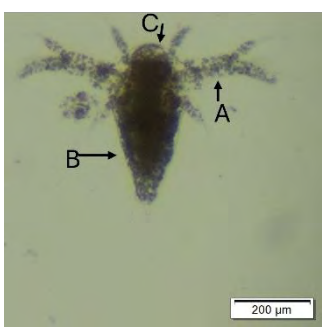
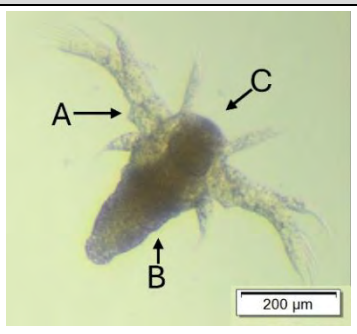


Figure 3.6. Lethality induced by the main constituents (thymol and carvacrol) represented by the LC_{50} values at 24- 48- and 72-hours on *A. franciscana* nauplii compared to positive control, potassium dichromate (*n*=3).

3.3.1.3.1. EOCs *Artemia franciscana* Nauplii Morphology

After 24 hours, carvacrol and thymol induced similar morphological changes in *Artemia franciscana* nauplii (Table 3.18). Compared to the untreated control (Table 3.14), both EOCs caused shrinkage and decomposition of appendages (A), and the abdomen (B) enlarged. Additionally, in the carvacrol morphological picture the eye (C) is not visible.

Table 3.18: Morphology of *Artemia franciscana* nauplii of the lethal main constituents compared to saltwater and potassium dichromate controls after 24 hours. Analysed microscopically using the Olympus SC50 microscope at a magnification of 20x (Scale bar: 200 µm). [A: Antennae; B: Abdomen; C: Eye]

Compound	Picture	Compound	Picture
Carvacrol		Thymol	

3.3.2. *Artemia franciscana* Nauplii Combinations

Overall, the *p*-cymene : propoxur combinations in a 1:1 ratio, did not show high mortality percentages after 24 hours. The combination of *p*-cymene 100 : propoxur 0.001 resulted in mortality rate of 28.36 ± 9.80%, while all other combinations exhibited low mortality in the range 0% to 10.6% (Figure 3.7). In contrast, the γ-terpinene 100: Propoxur 0.001 combination in a ratio of 1:1 resulted in 41.37 ± 8.49% mortality after 24 hours. The other combinations showed overall low mortality at 24 hours with a mortality range of 0% to 1.32% (Figure 3.8).

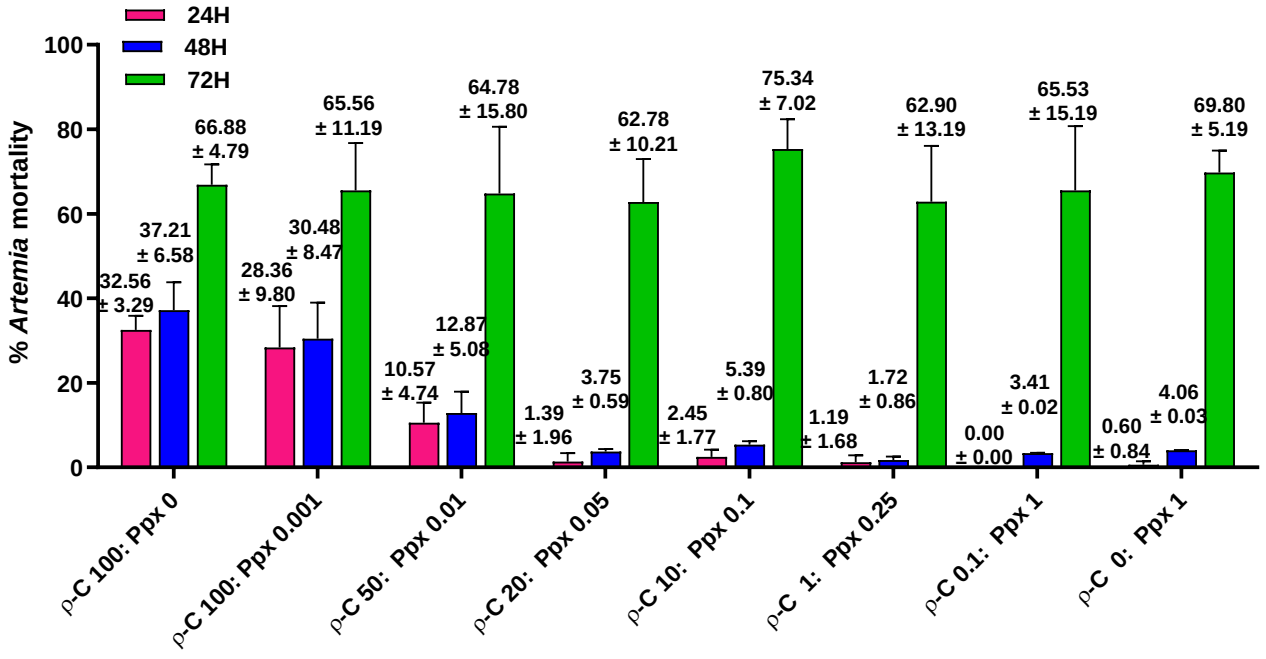


Figure 3.7. The % *Artemia* nauplii mortality as a result of ρ-cymene (ρ-C) : propoxur (Ppx) combinations at different concentrations in a 1:1 ratio after 24- 48- and 72- hours (*n*=2).

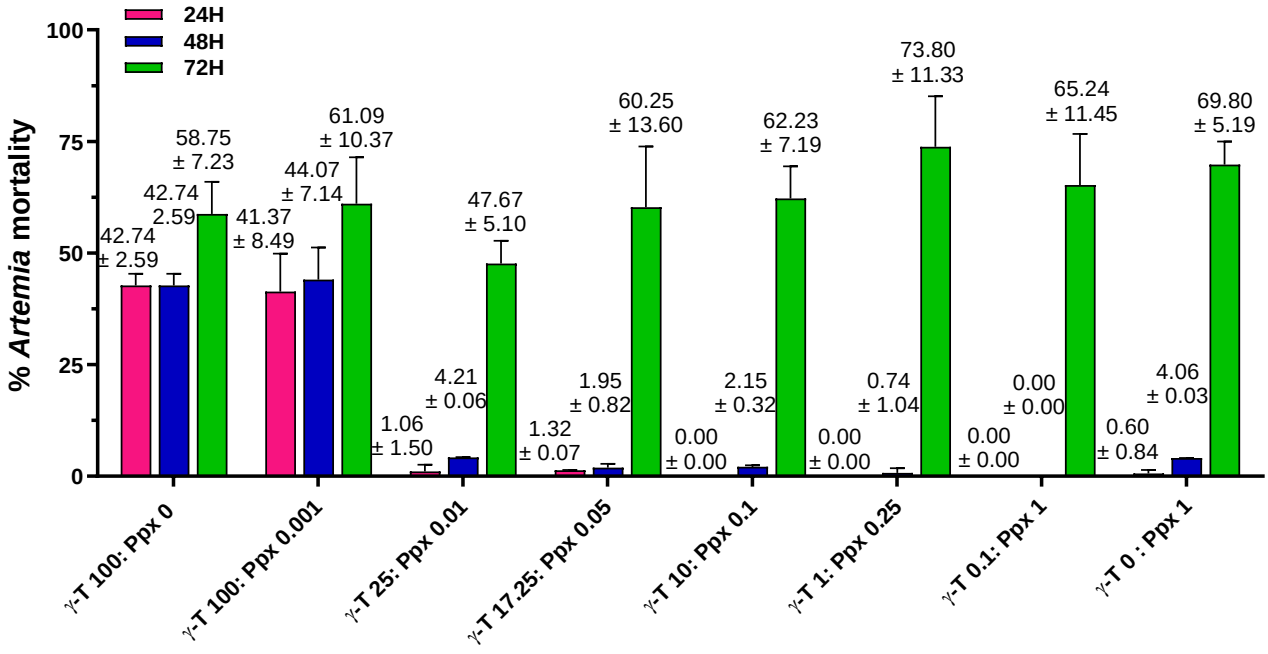


Figure 3.8. The % *Artemia* nauplii mortality as a result of γ-terpinene (γ-T): propoxur (Ppx) combinations at different concentrations in a 1:1 ratio after 24- 48- and 72- hours (*n*=2).

3.3.3 Selectivity Index

A selectivity index was calculated to determine the safety of the test compounds, and whether the compounds were more selectivity towards *Anopheles* larvae or *Artemia* nauplii. If a compound is more selective towards a species, it will have a decreased risk of being more lethal towards other organisms. The selectivity index is an indication of toxicity (*Anopheles*) relative to safety (*Artemia*) to determine if the EO/EOCs is organism specific or toxic in more organisms (Section 2.4.4) (Carlier *et al.*, 2008; Rants'o *et al.*, 2022). Of the EOs and EOCs evaluated, eugenol (0.75), clove essential oil, β-caryophyllene (0.84), carvacrol (0.34) and thymol (0.32) had the lowest selectivity indices over 24 hours. Of the eugenol and derivative group, eugenyl acetate (1.89) was the safest after 24 hours, being 28.6-fold more toxic than propoxur (54.05). The safest essential oil was black pepper essential oil (2.36), where it was 22.9-fold more toxic than propoxur. Of the essential constituents evaluated, γ-terpinene (10.21) was the safest, although it was 5.6-fold more toxic than propoxur**.** Compounds with selectivity indices above 10 were considered safe, such as γ-terpinene (10.21) (Table 3.19).

Table 3.19: The selectivity index (*Artemia* LC_{50} value or highest concentration where applicable/ larvae LC_{50} values) of the eugenols and derivatives, EOs and main constituents.

Compound		24 H	48H	72H
Positive controls	**Propoxur**	54.05	227.27	246.91
	DDT	579.71	581.30	19.54
Eugenol and Derivatives	**Eugenol**	0.75	0.40	0.10
	Isoeugenol	1.03	1.41	1.59
	Methylisoeugenol	1.03	1.33	0.32
	Eugenyl acetate	1.89	2.66	5.53
	σ-Eugenol	1.40	1.91	1.30
	Methyleugenol	1.53	1.26	0.92
Essential oils	**Black pepper**	2.36	3.47	4.40
	Sweet basil	2.21	2.38	2.84
	Clove	0.75	0.45	0.30
	Oregano	1.18	0.79	0.40
	Thyme	1.08	0.37	0.14
Main Constituents	**γ-Terpinene**	**10.21**	**10.99**	**11.55**
	***p*-Cymene**	4.64	5.18	7.89
	β-Caryophyllene	0.84	1.81	3.93
	Carvacrol	0.34	0.29	0.13
	Thymol	0.32	0.11	0.03

3.3.4. Haemolysis

None of the 6 eugenol derivatives caused notable haemolysis at 100 µg/mL. The haemolysis ranged from < 0.1% to 3.28% at a 100 µg/mL (Table 3.20). Of the EOs and EOCs evaluated, black pepper essential oil and β-caryophyllene had high haemolytic activity comparable to the positive control, Triton X-100. In comparison to clove essential oil, thyme essential oil, sweet basil essential oil and oregano essential oil only resulted in <0.1% to 10.70% haemolysis. The positive larvicidal control, propoxur, did not cause notable haemolysis at this concentration. This is in contrast with the positive control Triton X-100 that caused 100% haemolysis at only 2.14 µg/mL (Table 3.20).

Table 3.20: The percentage haemolysis of the compounds at a 100 µg/mL. Compared to positive control Triton X-100 and larvicidal control propoxur (Average ± s.d.; *n*=3).

Compound	% Haemolysis (Average ± s.d.; *n*=3)	Compound	% Haemolysis (Average ± s.d.; *n*=3)
Positive controls		**Essential oils**	
Triton X-100 (0.2%) (2.14 µg/mL)	100.00 ± 2.99	**Black pepper**	**100.00 ± 0.00**
Propoxur	<0.1 ± 0.01	**Sweet basil**	5.97 ± 0.05
Eugenol and Derivatives		**Clove**	10.70 ± 2.87
Eugenol	<0.1 ± 0.01	**Oregano**	<0.1 ± 0.01
Isoeugenol	1.29 ± 0.54	**Thyme**	9.74 ± 1.07
Methylisoeugenol	1.12 ± 0.47	**Main Constituents**	
Eugenyl Acetate	1.19 ± 0.56	**γ-Terpinene**	20.75 ± 6.51
σ-Eugenol	3.28 ± 0.65	***p*-Cymene**	21.67 ± 7.23
Methyleugenol	1.57 ± 0.38	**Linalool**	<0.1 ± 0.01
		β-Caryophyllene	**100.00 ± 0.00**
		Carvacrol	<0.1 ± 0.01
		Thymol	<0.1 ± 0.01

The most haemolytic compound was β-caryophyllene (IC_{50} value: 20.67 ± 1.23 µg/mL), 2.5-fold more toxic than the black pepper essential oil (IC_{50} value: 51.69 ± 4.51 µg/mL) (Figure 3.9). The haemolytic activity of black pepper essential oil and β-caryophyllene was not comparable to that of the Triton-X, a known haemolytic agent with IC_{50} value of 0.03 ± 0.00 µg/mL (*p*-value ≤ 0.001) (Figure 3.9).

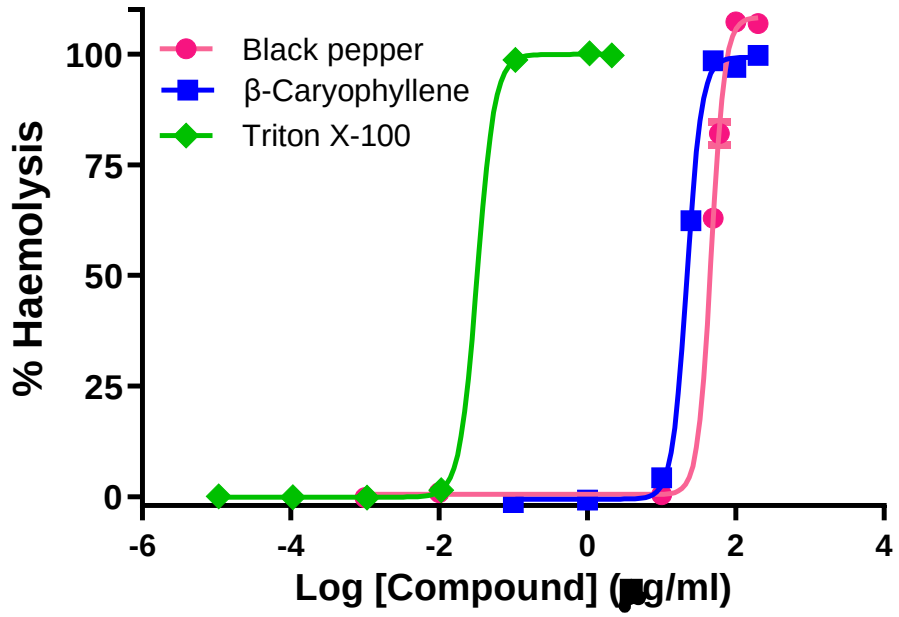


Figure 3.9. The log sigmoidal dose response curve with variable slope used to determine IC_{50} values of black pepper essential oil (IC_{50} value: 51.69 ± 4.51 µg/mL) and β-caryophyllene (IC_{50} value: 20.67 ± 1.23 µg/mL). Compared to positive control Triton X-100 (IC_{50} value: 0.03 ± 0.00 µg/mL) (*n*=3).

3.4. Mechanism of Action

3.4.1. Iron Chelation

The EOs and EOCs were tested for their possible ferrous iron chelation properties as a potential mechanism of action. From the eugenol derivatives, only isoeugenol (71.61 ± 2.35%) exhibited ferrous iron chelation at a 100 µg/mL. In contrast, eugenol, methylisoeugenol, eugenyl acetate, σ-eugenol, and methyleugenol showed minimal chelation activity, ranging from 12.45% to 36.71% (Table 3.21).

Among the EOs, black pepper essential oil (95.57 ± 1.05%) demonstrated the highest ferrous iron chelation activity at a 100 µg/mL as compared to known ferrous iron chelators EDTA (98.08 ± 3.14%) and 2,2-bipiridyl (90.18 ± 4.27%) (Table 3.21). In contrast, sweet basil, clove, oregano and thyme essential oils exhibited low iron chelation activity ranging from 10.43% to 18.95%. Similarly, the main constituents displayed negligible ferrous iron chelation, with values ranging from <0.1% to 0.60% (Table 3.21).

Isoeugenol (IC_{50} value: 40.71 ± 6.03 µg/mL) and black pepper essential oil (IC_{50} value: 18.29 ± 2.51 µg/mL) demonstrated the most chelation activity against ferrous iron; with black pepper essential oil chelation activity being comparable to that of the positive control, 2,2-bipiridyl (IC_{50} value: 16.45 ± 1.66 µg/mL) (*p*-value > 0.05) (Figure 3.10).

Table 3.21: The percentage ferrous ion chelation at a 100 µg/mL, as compared to the positive controls, EDTA and 2.2-bipridyl (Average ± s.d.; *n*=3).

Compound	% Iron chelation (Average ± s.d.; *n*=3)	Compound	% Iron chelation (Average ± s.d.; *n*=3)
Positive controls		**Essential oils**	
EDTA	98.08 ± 3.14	**Black pepper**	**95.57 ± 1.05**
2.2-Bipyridyl	90.18 ± 4.27	**Sweet basil**	17.86 ± 2.77
Eugenol and Derivatives		**Clove**	15.45 ± 2.45
Eugenol	12.45 ± 3.00	**Oregano**	18.95 ± 3.03
Isoeugenol	**71.61 ± 2.35**	**Thyme**	10.43 ± 2.18
Methylisoeugenol	20.20 ± 4.79	**Main Constituents**	
Eugenyl Acetate	21.75 ± 3.87	**γ-Terpinene**	0.60 ± 0.11
σ-Eugenol	36.71 ± 8.28	***p*-Cymene**	<0.1 ± 0.01
Methyleugenol	26.73 ± 4.78	**Linalool**	<0.1 ± 0.01
		β-Caryophyllene	<0.1 ± 0.01
		Carvacrol	<0.1 ± 0.01
		Thymol	<0.1 ± 0.01

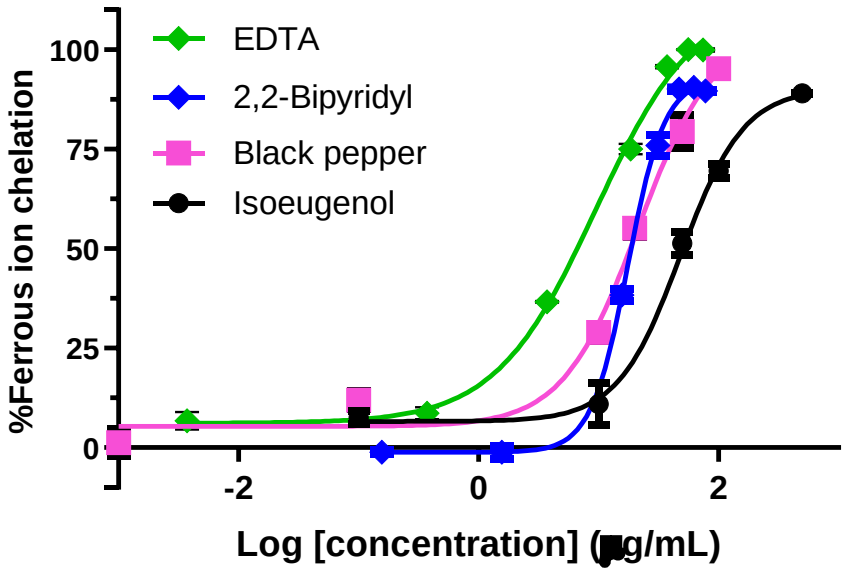


Figure 3.10. The log sigmoidal dose-response curves with variable slopes used to determination the IC_{50} values for black pepper essential oil (IC_{50} value: 40.71 ± 6.03 µg/mL. $p > 0.05$) and isoeugenol (IC_{50}: 18.29 ± 2.51 µg/mL. $p \leq 0.001$). Compared to the positive control 2,2'-bipyridyl (IC_{50} value: 16.45 ± 1.66 µg/mL. $n = 3$) and EDTA (9.45 ± 0.07 µg/mL. $n = 3$).

3.4.2. Copper (II) Chelation

The positive control, tetrathiomolybdate, a known copper (II) chelator exhibited high copper (II) chelation properties (90.59 ± 7.30%) at a 100 µg/mL (Table 3.22). At the same concentration, the eugenol derivatives demonstrated low copper (II) chelation properties with percentage chelation ranging from 3.65% to 20.79%. Similar to eugenols, the EOs exhibited very low copper (II) chelation properties ranging from <0.1% to 1.55%, with the EOCs also demonstrating low copper (II) chelation activity ranging from <0.1% to 3.55% (Table 3.22).

Table 3.22: The percentage copper (II) chelation at a 100 µg/mL as compared to the positive control, Tetrathiomolybdate (Average ± s.d.; *n*=3).

Compound	% Copper (II) chelation (Average ± s.d.; *n*=3)	Compound	% Copper (II) chelation (Average ± s.d.; *n*=3)
Positive control		**Essential oils**	
Tetrathiomolybdate	90.59 ± 7.30	**Black pepper**	<0.1 ± 0.01
Eugenol and Derivatives		**Sweet basil**	<0.1 ± 0.01
Eugenol	<0.1 ± 0.01	**Clove**	<0.1 ± 0.01
Isoeugenol	<0.1 ± 0.01	**Oregano**	<0.1 ± 001
Methylisoeugenol	20.79 ± 3.10	**Thyme**	1.55 ± 0.16
Eugenyl Acetate	7.11 ± 2.67	**Main Constituents**	
σ-Eugenol	5.33 ± 0.15	**γ-Terpinene**	<0.1 ± 0.01
Methyleugenol	3.65 ± 0.81	***p*-Cymene**	<0.1 ± 0.01
		Linalool	3.55 ± 0.52
		β-Caryophyllene	<0.1 ± 0.01
		Carvacrol	1.62 ± 0.16
		Thymol	3.42 ± 0.10

3.4.3. DPPH• Free Radical Scavenging Assay

Three of the eugenol derivatives were potent free radical scavengers (>94%) at 100 µg/mL, namely eugenol, isoeugenol and σ-eugenol, being comparable to *N*-propyl gallate (Figure 3.11). Clove essential oil was the only essential oil with potent free radical scavenging activity at 100 µg/mL. Oregano and thyme essential oils demonstrated low free radical scavenging activity at 100 µg/mL (Figure 3.11). None of the main constituents of the EO showed free radical scavenging activity, except for thymol and carvacrol (Figure 3.11), where β-caryophyllene, *p*-cymene and linalool had <0.1 ± 0.01 activity.

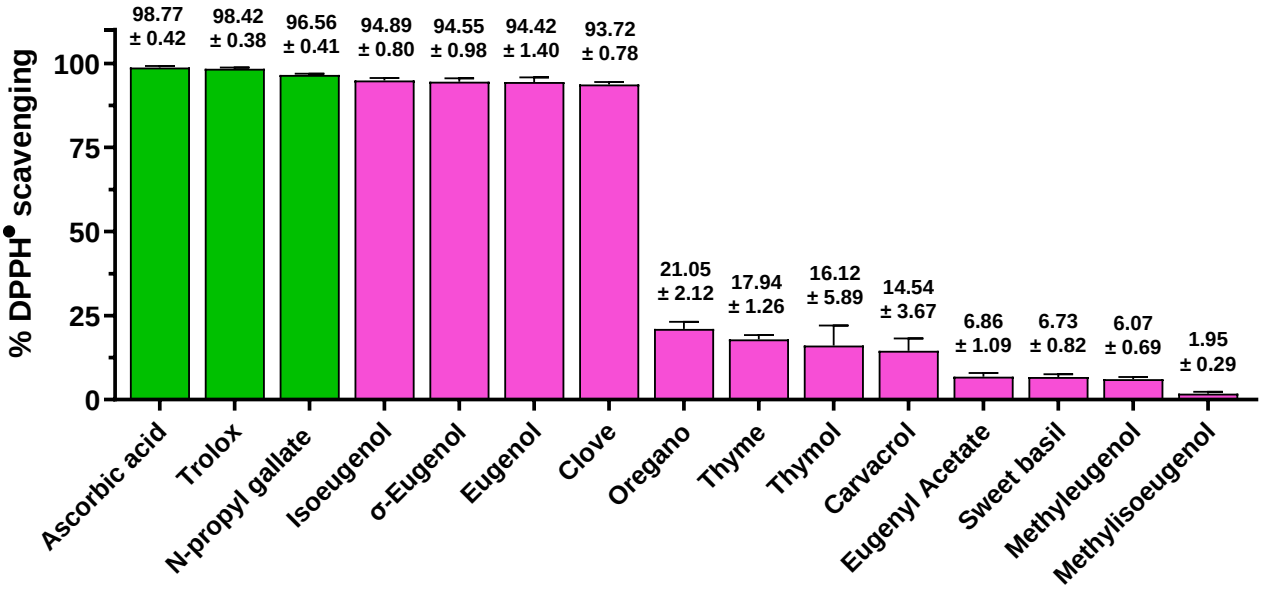


Figure 3.11. The percentage DPPH free radical scavenging (average ± s.d.; *n*=3) of eugenol and derivatives. EOs and main constituents, compared to Trolox, ascorbic acid and N-propyl gallate, at a 100 µg/mL.

The four potent free radical scavengers were further evaluated to determine IC_{50} values and compared to ascorbic acid and Trolox. The most potent free radical scavenger among all the compounds was σ-eugenol, followed closely by eugenol and were comparable to ascorbic acid (p > 0.05). Isoeugenol and clove essential oil were also potent free radical scavengers, but not comparable to ascorbic acid (Figure 3.12).

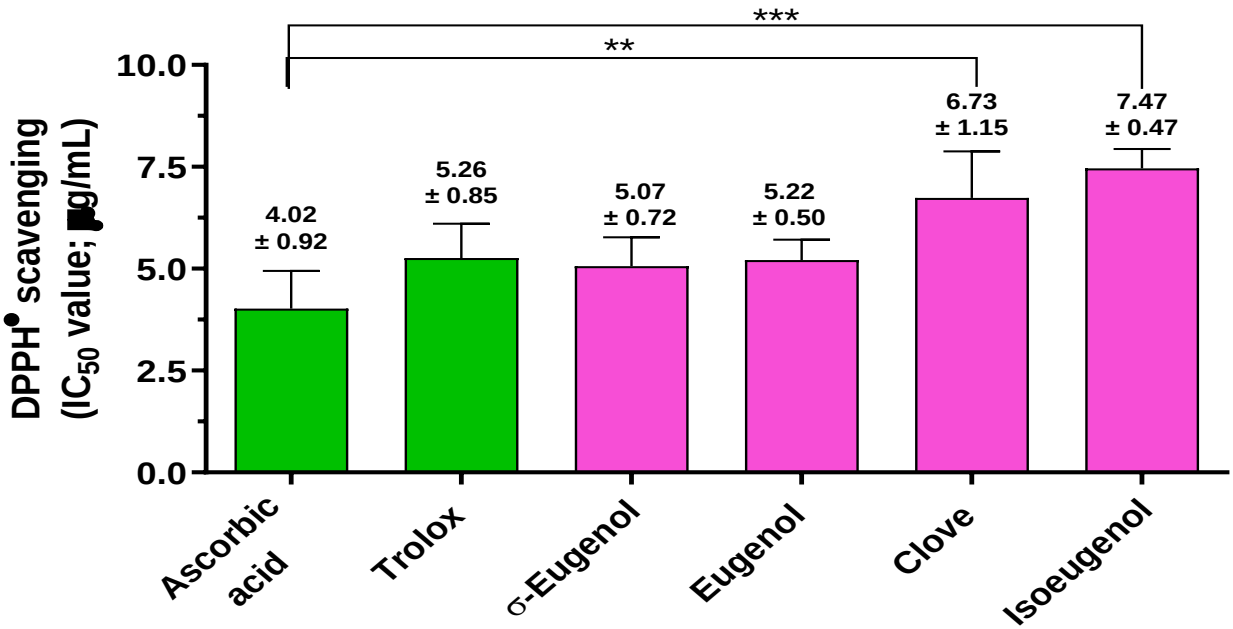


Figure 3.12. The free radical (DPPH•) scavenging of σ-eugenol (p-value: >0.05), eugenol (p-value: >0.05), clove essential oil (p-value: ≤ 0.01; **) and isoeugenol (p-value: ≤ 0.001; ***) IC_{50} values compared to ascorbic acid and Trolox.

Very low correlation between larvicidal properties and anti-oxidant properties were observed with a low r^2-value (0.06), as well as low correlation between *Artemia* mortality and anti-oxidant properties (r^2-value: 0.47).

3.5. *In silico* Pharmacokinetic Predictions

From the *in silico* pharmacokinetic studies, it was shown that all EOCs showed drug-like properties as they adhered to Lipinski's rule of 5 (Table 3.23). All EOCs were predicted to undergo cytochrome P450 metabolism (Table 3.24). Eugenol, eugenyl acetate, σ-eugenol and methyleugenol were predicted to all undergo O-dealkylation as the primary route of CytP450 metabolism, similar to propoxur. Thymol, carvacrol, γ-terpinene and *p*-cymene were predicted to undergo aliphatic hydroxylation, similar to DDT. The rest of the EOCs, isoeugenol, methylisoeugenol, linalool and β-caryophyllene were predicted to undergo epoxidation as the primary route of P450 metabolism.

Table 3.23: The Lipinski adherence and properties of the EOCs as compared to larvicidal control. Propoxur and DDT.

Compound	Lipinski Adherence	< 5-H bond donors	< 10 H-bond acceptors	MW < 500	Log *P* Value
Propoxur	Yes	1	3	209.24	1.79
DDT	yes (1 violation)	0	0	354.49	6.19
Eugenol and derivatives					
Eugenol	Yes	1	2	164.2	2.01
Isoeugenol	Yes	1	2	164.2	2.01
Methylisoeugenol	Yes	0	2	178.23	2.3
Eugenyl Acetate	Yes	0	3	206.24	2.43
σ-Eugenol	Yes	1	2	164.2	2.01
Methyleugenol	Yes	0	2	178.23	2.3
Main constituents					
γ-Terpinene	yes	0	0	136.23	3.27
ρ-Cymene	Yes (1 violation)	0	0	134.22	4.47
Linalool	Yes	1	1	154.25	2.59
β-Caryophyllene	yes (1 violation)	0	0	204.35	4.63
Carvacrol	Yes	1	1	150.22	2.76
Thymol	Yes	1	1	150.22	2.76

Except for *γ*-terpinene, *p*-cymene and β-caryophyllene, all EOCs had high gastrointestinal tract absorption. β-caryophyllene was the only compound predicted to not cross the blood brain barrier. All EOCs were classified as low class I toxic hazards, compounds that are simple in structure and an efficient mode of metabolism exists, that have a low order of oral toxicity. DDT and propoxur were classified as high class III toxic hazards, that is, compounds with chemical structures that cannot be presumed safe initially or may suggest significant toxicity or have reactive functional groups (Table 3.24).

Table 3.24: The Metabolism. primary metabolic reaction. enzyme inhibition predictions. toxic hazard. blood brain barrier crossing. GIT absorption and bioavailability predictions of the EOCs as compared to larvicidal control, Propoxur and DDT. (BBB: blood brain barrier; GIT: gastrointestinal tract; Cyp: cytochrome)

Compound	Cyt P450 metabolism	Primary P450 Metabolism	Cyp3a4 inhibitor	Inhibits	Toxic hazard	BBB penetration	GIT Absorption	Bioavail-ability Score
Propoxur	Yes	O-dealkylation	No	Cyp1A2	High (Class III)	Yes	High	0.55
DDT	Yes	Aliphatic hydroxylation	No	Cyp 2C9 & 2C19	High (Class III)	**No**	**Low**	0.55
Eugenol and Derivatives								
Eugenol	Yes	O-dealkylation	No	Cyp1A2	Low (Class I)	Yes	High	0.55
Isoeugenol	Yes	Epoxidation	No	Cyp1A2	Low (Class I)	Yes	High	0.55
Methylisoeugenol	Yes	Epoxidation	No	Cyp1A2	Low (Class I)	Yes	High	0.55
Eugenyl Acetate	Yes	O-dealkylation	No	Cyp1A2	Low (Class I)	Yes	High	0.55
σ-Eugenol	Yes	O-dealkylation	No	Cyp1A2	Low (Class I)	Yes	High	0.55
Methyleugenol	Yes	O-dealkylation	No	Cyp1A2	Low (Class I)	Yes	High	0.55
Main Constituents								
γ-Terpinene	Yes	Aliphatic hydroxylation	No	**None**	Low (Class I)	Yes	**Low**	0.55
ρ-Cymene	Yes	Aliphatic hydroxylation	No	CYP2d6	Low (Class I)	Yes	High	0.55
Linalool	Yes	Epoxidation	No	None	Low (Class I)	Yes	High	0.55
β-Caryophyllene	Yes	Epoxidation	No	CYP2C19 & 2C19	Low (Class I)	**No**	**Low**	0.55
Carvacrol	Yes	Aliphatic hydroxylation	No	Cyp1A2	Low (Class I)	Yes	High	0.55
Thymol	Yes	Aliphatic hydroxylation	No	Cyp1A2	Low (Class I)	Yes	High	0.55

3.6. *In silico* Target Predictions and ECOSAR Predictions

Using the SWISS target prediction program, the top two biological targets for each compound were identified along with the probability of the compound affecting these targets in humans. A probability closer to 0 indicated a lower likelihood of the compound to having an effect. At the concentrations used in this study, the EOCs were predicted to be relatively safe, with probabilities generally low except for eugenyl acetate, carvacrol and thymol which had probabilities greater than 0.9 for one of the targets. This contrasts with propoxur, which had a high probability of affecting acetylcholinesterase (0.99) (Table 3.25).

The ECOSAR program predicted LC_{50} values for fish at 96 hours and daphnids at 48 hours for the neutral organics groups of the EOCs, with γ-terpinene (LC_{50} value for fish: 0.12 µg/mL; LC_{50} value for daphnids: 0.09 µg/mL), *p*-cymene (LC_{50} value for fish: 1.78 µg/mL; LC_{50} value for daphnids: 1.21 µg/mL) and β-caryophyllene (LC_{50} value for fish: 0.02 µg/mL; LC_{50} value for daphnids: 0.02 µg/mL), displaying the lowest LC_{50} values for both fish and daphnids (Table 3.25). Compared to the predictions for propoxur (LC_{50} value for fish: 210.00 µg/mL; LC_{50} value for daphnids: 118.00 µg/mL), these LC_{50} values were very low. γ-Terpinene and β-caryophyllene were > 1000-fold more lethal towards daphnids than propoxur and *p*-cymene > 100-fold lethal.

Table 3.25: Eugenol and derivatives and main EOCs target predictions by SWISS target predictions, containing the potential targets in humans as well as ECOSAR predictions for LC_{50} values in Fish and Daphnids.

Compound	SWISS Target Predictions		ECOSAR Predictions		
	Target	Probability	Organism	Duration	LC_{50} (µg/mL)
Propoxur	Acetylcholinesterase	**0.99**	Fish	96H	210.00
	Melanin-concentrating hormone receptor 1	0.04	Daphnid	48H	118.00
Carvacrol	Cyclooxygenase-1	**0.94**	Fish	96H	5.37
	Transient receptor potential cation channel subfamily A member 1	0.25	Daphnid	48H	3.51
Thymol	Transient receptor potential cation channel subfamily A member 1	**0.94**	Fish	96H	5.37
	Cyclooxygenase-1	0.29	Daphnid	48H	3.51
Eugenyl acetate	Cyclooxygenase-1	**0.99**	Fish	96H	18.80
	Aldehyde dehydrogenase	0.04	Daphnid	48H	11.80
β-Caryophyllene	Peroxisome proliferator-activated receptor alpha	0.50	Fish	96H	**0.02**
	Cannabinoid receptor	0.50	Daphnid	48H	**0.02**
Isoeugenol	Tubulin beta-1 chain	0.23	Fish	96H	35.00
	Cytochrome P450 1A1	0.22	Daphnid	48H	21.10
Methylisoeugenol	Quinone reductase 2	0.27	Fish	96H	20.40
	Cytochrome P450 1A1	0.22	Daphnid	48H	12.70
Eugenol	Fatty acid desaturase 1	0.13	Fish	96H	29.80
	Histone deacetylase 6s	0.13	Daphnid	48H	18.10
σ-Eugenol	Carbonyl reductase [NADPH] 1	0.13	Fish	96H	29.80
	Adenosine A1 receptor	0.13	Daphnid	48H	18.10
Methyleugenol	Cyclooxygenase-1	0.08	Fish	96H	17.40
	Quinone reductase 1	0.08	Daphnid	48H	10.80
Linalool	Transient receptor potential cation channel subfamily V member 3	0.06	Fish	96H	7.28
	Carbonic anhydrase II	0.06	Daphnid	48H	4.70
γ-Terpinene	Vanilloid receptor	0.02	Fish	96H	**0.12**
	Serine/threonine-protein kinase PLK1	0.02	Daphnid	48H	**0.09**
ρ-Cymene	Cytochrome P450 2A6	0.04	Fish	96H	**1.78**
	Acetylcholinesterase	0.02	Daphnid	48H	**1.21**

Chapter 4 – Discussion

4.0. Overview

In this chapter the lethality activity against 3rd instar *An. arabiensis* larvae of eugenol and its derivatives, along with various essential oils and their main constituents are discussed in relation to their effect on mortality and morphology of the larvae, as well as their potential mechanism of action. Additionally, the interaction between the two EOCs that exhibited the highest larvicidal activity, namely γ-terpinene and *p*-cymene, and propoxur are discussed.

Beyond larval inhibition, the preliminary environmental safety profile of these compounds was assessed using *Artemia franciscana* nauplii and *in silico* ECOSAR predictions. The preliminary toxicity profile in humans was explored using haemolysis data, complemented by *in silico* predictions from Toxtree and SWISS ADME. Additionally, SWISS target predictions were incorporated to assess the likelihood of these compounds interacting with biological targets and whether such interactions were relevant at the tested concentrations. Finally, potential mechanisms of action are discussed based on experimental findings.

4.1. Eugenol and Derivatives

Eugenol and its derivatives are common constituents found in the essential oils from numerous plants that have diverse pharmacological properties (Section 1.6.1), including insecticidal properties (Huang *et al.*, 2002; Khalil *et al.*, 2017; Fernandes *et al.*, 2020). In the current study, the tested eugenol and derivatives, showed larvicidal properties against the 3rd instar *An. arabiensis* larvae (Section 3.2.1). Isoeugenol showed a time-dependant increase in mortality (Figure 3.1), whereas eugenol, methylisoeugenol, σ-eugenol, eugenyl acetate and methyleugenol had 86.67% to 100.00% larvae mortality at 24 hours (Table 3.2).

Similar to the current study, previous studies also demonstrated larvicidal activity of eugenol (LC_{50} value: 75.76 µg/mL), isoeugenol (LC_{50} value: 81.26 µg/mL) and σ-eugenol (LC_{50} value: 8.84 µg/mL) on *Aedes aegypti* at 24 hours (Wang *et al.*, 2019) and methyleugenol (LC_{50} value: 2.47 µM) against *An. arabiensis* (Rants'o, Koekemoer and Van Zyl, 2023). The larvicidal activity of eugenol in Wang *et al.* (2019), was comparable to the larvicidal activity in this study, yet isoeugenol and σ-eugenol was 1.2-fold and 3.4-fold less active on *An. arabiensis* in this study than *Aedes aegypti* (Table 3.3)(Wang *et al.*, 2019). Whilst eugenol produced a LC_{50} value of 25.45 µg/mL against *An. subpictus* at 24 hours, showing more activity than eugenol in this study after 24 hours (LC_{50} value: 72.02 ± 2.47 µg/mL)(Govindarajan *et al*., 2016b). The difference between activity might be due to *Anopheles* species differences, as well as the fact

that the current study was done on using commercially purchased eugenol and Govindarajan *et al*. (2016b), extracted eugenol from *Plectranthus barbatus* leaves.

Eugenyl acetate (LC_{50} value: 18.09 ± 6.83 µg/mL) exhibited the most promising larvicidal activity after 72 hours, however it was not comparable to that of propoxur (LC_{50} value: 0.04 ± 0.01), with eugenyl acetate being 452.3-fold less active than propoxur (*p*-value ≤ 0.01)(Table 3.3). The order of eugenol derivatives larvicide inhibitory activity ranked as follows: eugenyl acetate (LC_{50}: 18.09 ± 6.83) > methylisoeugenol (LC_{50}: 18.52 ± 5.60) > σ-eugenol (LC_{50}: 21.47 ± 2.13) > methyleugenol (LC_{50}: 31.96 ± 2.10) > eugenol (LC_{50}: 36.21 ± 2.79) >> isoeugenol (LC_{50}: 62.92 ± 9.82)(Table 3.3), with isoeugenol exhibiting the lowest larvicidal activity. Eugenyl acetate caused similar morphological changes to propoxur in enlarging larvae thorax, whereas the other eugenol derivates caused elongated heads (Table 3.4.A and Table 3.4.B). Looking at the chemical structures, eugenol and isoeugenol only differ at one group where eugenol has a methyl (CH_3) group, whilst isoeugenol has a methylene (CH_2) group. Where eugenol has hydroxyl group, eugenyl acetate has an acetyl group, and is formed by esterification of eugenol (Vanin *et al.*, 2014).

Eugenyl acetate demonstrated to be the safest eugenol derivative against *Artemia franciscana* nauplii after 24 hours with percentage mortality of 14.99 ± 5.36% at a 100 µg/mL (Table 3.13), showing the derivatives safety on environmental settings in case the larvicide enters water sources. However, the mortality rate was time-dependent as it increased after 48-hours (40.46 ± 14.53%) and 72-hours (97.97 ± 1.96%) showing cumulative toxicity. This toxic effect was consistent throughout the eugenol and derivatives, with the exception of σ-eugenol being the most lethal from 24-hours. Methyleugenol demonstrated the safest toxicity profile against the *Artemia franciscana* nauplii with 64.43 ± 8.32% mortality after 72 hours at 100 µg/mL. Eugenol, isoeugenol, methyl isoeugenol and σ-eugenol was lethal toward *Artemia franciscana* nauplii (LD_{50} range: 3.66 µg/mL to 29.54 µg/mL after 72 hours).

Eugenyl acetate exhibited a selectivity index of 5.53 at 72-hours and showing non-selectivity towards the *Anopheles* larvae and *Artemia*, though it was a better selectivity index at 72 hours as compared to other eugenols derivatives (SI range: 0.1 to 1.6) and not comparable to that of propoxur (SI: 246.91). *In silico* studies further predicted that the eugenyl acetate is toxic towards other water animals including fish and daphnids (Table 3.25). Similar to the current study, it has been shown that eugenyl acetate can be toxic to microcrustaceans like *Artemia salina* (Cansian *et al*., 2016). *In silico* studies also further predicted that the other eugenol derivatives are also toxic towards other water animals where isoeugenol had the highest LC_{50} values predicted *in silico* towards fish at 96 hours (35.00 µg/mL) and daphnids at 48 hours (21.10 µg/mL). Even though *in silico* studies predicted lethal LC_{50} values, it has been shown

that when trout were exposed to 75 mg/L eugenol for 15 minutes that it had a half-life of 12.14 hours with no tissue anomalies or serious adverse effects. More research is needed to determine toxicity profile with repeat or prolonged exposures (Guénette *et al.*, 2007b). These results suggest that these compounds are suitable for use as larvicides in aquatic environments.

Considering the need for more potent insecticides with a safe toxicological profile for mammalian organisms, following possible ingestion of the insecticide or from water sources, the effects of the derivatives on human cells were assessed. As per the haemolysis assay, eugenol and the derivatives resulted in very low red blood cell lysis at 100 µg/mL (< 0.1% to 3.28%), as compared to Triton X-100 that showed 100% haemolysis at 2.14 µg/mL (Table 3.20). Eugenol was comparable to propoxur both with <0.1 ± 0.01% haemolysis at 100 µg/mL. This is supported by Hidalgo and De la Rosa (2009) that determined eugenyl acetate to exhibit low haemolytic properties at a 100 ppm (0.74%).

This preliminary toxicity of eugenol and derivatives towards humans shows that when spraying this compound as a larvicide, there is limited toxicity exposure towards humans, as concentrations would be much lower. This is reiterated by *in silico* predictions, predicting that eugenol and the derivatives were low class I toxic hazards, even though it is predicted to have high GIT absorption and crosses the blood brain barrier which may lead to causing CNS related adverse effects (Table 3.24). A study done by Guénette *et al.* (2007a) on male Sprague-Dawley rats, determined pharmacokinetics of eugenol in the red blood cells and plasma after an oral 40 mg/kg eugenol dose. Mean pharmacokinetic parameters revealed maximum plasma concentration to be 80.5% in the plasma and 25.7% in the blood. Terminal elimination half-life was 14.0 hours in the plasma and 18.3 hours in the blood. Over 24 hours eugenol plasma concentrations peaked at 0.25 hours and 4 hours yet declined at a slow rate. Blood concentrations also declined slowly after declining fast initially. Mean oral clearance was 86.8 L/h/kg in the plasma (Guénette *et al.*, 2007a). In humans the plasma concentration of eugenol is 5 µM from 150mg, 2 hours earlier (Ito, Murakami and Yoshino, 2005). It has been shown that eugenol, when used in small doses, seems to cause minimal side effects, primarily limited to local irritation, infrequent allergic reactions, and contact dermatitis (National Institute of Diabetes and Digestive and Kidney Diseases, 2012). However, exposure to or ingestion of large quantities, such as in the case of an overdose, may lead to tissue damage and the sudden onset of symptoms including seizures, coma, and harm to the liver and kidneys (National Institute of Diabetes and Digestive and Kidney Diseases, 2012). In the one-electron pathway it is possible for eugenol to be turned into eugenol quinonemethide, that can be cytotoxic (Fujisawa *et al.*, 2002a). *In silico* results predicted eugenyl acetate (0.99) and

methyleugenol (0.08) both showed a probability to affect Cyclooxygenas-1 (COX-1) as predicted by SWISS targets (Table 3.25). Cyclooxygenase is an enzyme responsible to catalyse prostaglandin synthesis. Inhibition of COX reduces pain and inflammation (Landa *et al.*, 2009).

As for the possible mechanism of action in killing the larvae, only isoeugenol demonstrated promising ferrous iron chelation properties that was half as potent as the standard ferrous iron chelating agent, 2,2'-bipyridyl (Section 3.4.1). In comparison to eugenol, methylisoeugenol, eugenyl acetate, σ-eugenol and methyleugenol that displayed low chelating abilities (12.45% to 36.71%)(Table 3.21). However, in relation to the larvicidal activity, isoeugenol had the lowest activity which indicates that ferrous iron chelation is unlikely the mechanism of action of the eugenol derivatives. To support this was the inverse relation of larvicidal activity and iron chelating properties of eugenyl acetate which was the most potent larvicidal eugenol derivative (Table 3.3, 3.21) but was a weak (21.75± 3.87%) ferrous iron chelator. In the presence of isocitrate it inhibits oxidation of ferrous ions by keeping iron in the reduced form (Ito, Murakami and Yoshino, 2005).

Methylisoeugenol demonstrated the highest copper (II) chelation properties, with only 20.79 ± 3.10% at a 100 µg/mL, while other derivatives demonstrated less than 7.11% activity (Table 3.22). Eugenol has been found to attach to copper chloride through the copper complexation mechanism (Elgushe *et al*., 2024).

Eugenol (LC_{50} value: 5.22 ± 0.50 µg/mL), isoeugenol (LC_{50} value: 7.47 ± 0.47 µg/mL) and σ-eugenol (LC_{50} value: 5.07 ± 0.72 µg/mL) demonstrated potent free radical scavenging properties (Figure 3.12), whilst eugenol acetate, methyleugenol and methylisoeugenol had little (1.95% to 6.86%) anti-oxidant properties at a 100 µg/mL (Figure 3.11). Eugenol is a known anti-oxidant, through which it traps chain-carrying peroxy radicals, but can also be a prooxidant under different conditions. Eugenol has the ability to scavenge superoxide radicals as well as 2,2-diphenyl-1-picrahydrazyl ($DPPH^{\bullet}$) and nitric oxide (NO) radicals (Fujisawa *et al*., 2002b). Eugenol compounds are capable of inhibiting iron-mediated lipid peroxidation as well as copper-dependant low density lipoprotein oxidation. It inhibits copper-dependant low density lipoprotein oxidation, through reducing the metal and forming an inactive complex, and has oxygen radical scavenging activities. Isoeugenol has also been reported to inhibit the oxidation of ferrous ions in the presence of isocitrate more effectively than eugenol (Ito, Murakami and Yoshino, 2005).

Eugenol has several properties that pose as mechanism of action against mosquitoes. Rants'o *et al.* (2022a) demonstrated that eugenol exhibits inhibitory activity against *Anopheles* acetylcholinesterase. Dalai *et al*. (2014) determined that eugenol has acetylcholinesterase

inhibitory activity (IC_{50} value: 42.44 ± 1.21 µg/mL). It can be neurotoxic by the inhibition of GABA-gated chlorine channels and agonist to octopamine that leads to an increase in cyclic adenosine monophosphate (Demirak and Canpolat, 2022). Eugenol can affect tyramine receptors and transient receptor potential channels, which will all target insect nervous systems. Monoterpenes can possible also target the mitochondria and affect the respiratory systems of insects, leading to energy depletion (Qasim *et al.*, 2024). As eugenol is classified as a monoterpene these potential mechanisms of action cannot be excluded.

Overall, eugenol and its derivatives exhibited moderate to low inhibitory activity against *An. arabiensis* larvae, with selective toxicity towards *Artemia* and other aquatic organisms such as fish and daphnids (Table 3.24). As a result, these compounds do not merit further investigation as potential insecticides, especially considering that propoxur exhibits superior activity and a better safety profile.

4.2. Essential Oils and Their Main Constituents

The use of essential oils as insect repellents and insecticides has been around for many years (Koul, Walia and Dhaliwal, 2008). Essential oils derived from plants contain many different constituents with different reactive groups that possess different properties (Başer and Demirci, 2007). In the present study, all five evaluated essential oils and their main constituents showed larvicidal properties, except for main constituent linalool (Table 3.8). Of the essential oils, oregano and thyme were the most potent larvicides, and of the main constituents γ-terpinene and *p*-cymene were the most potent larvicides, and present in both oregano and thyme.

4.2.1. Oregano Essential Oil (*Origanum vulgare*) and Main Constituents

Oregano and thyme essential oils were the most potent essential oils when testing their larvicidal activity against *An. arabiensis* 3rd instar larvae (Table3.6). According to the GC-MS data, oregano consisted of four constituents namely γ-terpinene, *p*-cymene, thymol and carvacrol (Table 3.1.D). Thymol, γ-terpinene and *p*-cymene, although in lesser amounts in oregano EO, these constituents also had potent larvae inhibitory properties with 100% mortality at a 100 µg/mL at 24 hours. Oregano possessed the most larvicidal activity, with an LC_{50} value of 15.16 ± 2.22 µg/mL, 13.47 ± 2.87 µg/mL and 10.42 ± 1.56 µg/mL at 24-, 48- and 72- hours (Table 3.6). Carvacrol that made up 88.1% of the oregano oil (Table 3.1.D), had an LC_{50} value of 25.10 ± 6.05 µg/mL against *An. arabiensis* 3rd instar larvae at 24 hours, that

decreased to 13.88 ± 3.08 µg/mL at 72 hours (Figure 4.1). Oregano and carvacrol have been shown to possess potent larvicidal activity against *An. stephensi* (LC_{50} value: 67.00 µg/mL and 21.15 µg/mL) and *An. subpictus* (LC_{50} value: 74.14 µg/mL and 24.06 µg/mL) at 24 hours, respectively (Govindarajan *et al*., 2016a). Carvacrol also demonstrated potent larvicidal activity against *Aedes aegypti* (LC_{50} value: 20.58 µg/mL), *Culex quinquefasciatus* (LC_{50} value: 36.67 µg/mL) and *An. stephensi* (LC_{50} value: 40.55 µg/mL) (Govindaraju, Karthik and Arulselvi, 2016). These studies show similar results to the current study of carvacrol, but not oregano EO. EOs purchased commercially for this study were analysed by GC-MS and indicated that 88.1% carvacrol was present in the oregano EO (table 3.1.D). The oregano EO from the Govindarajan *et al.* (2016a) study, was collected in India and extracted by hydro-distillation, with 38.3% carvacrol within the EO as determined by GC-MS. The exact mechanism of action of oregano EO and carvacrol in *Anopheles* remains elusive as this study failed to show that the mechanism was due to iron (II) or copper (II) chelation (Table 3.21 and Table 3.22). However it has been reported that oregano EO possesses some anticholinesterase activity (25%) at larvicidal concentrations (López *et al*., 2019).

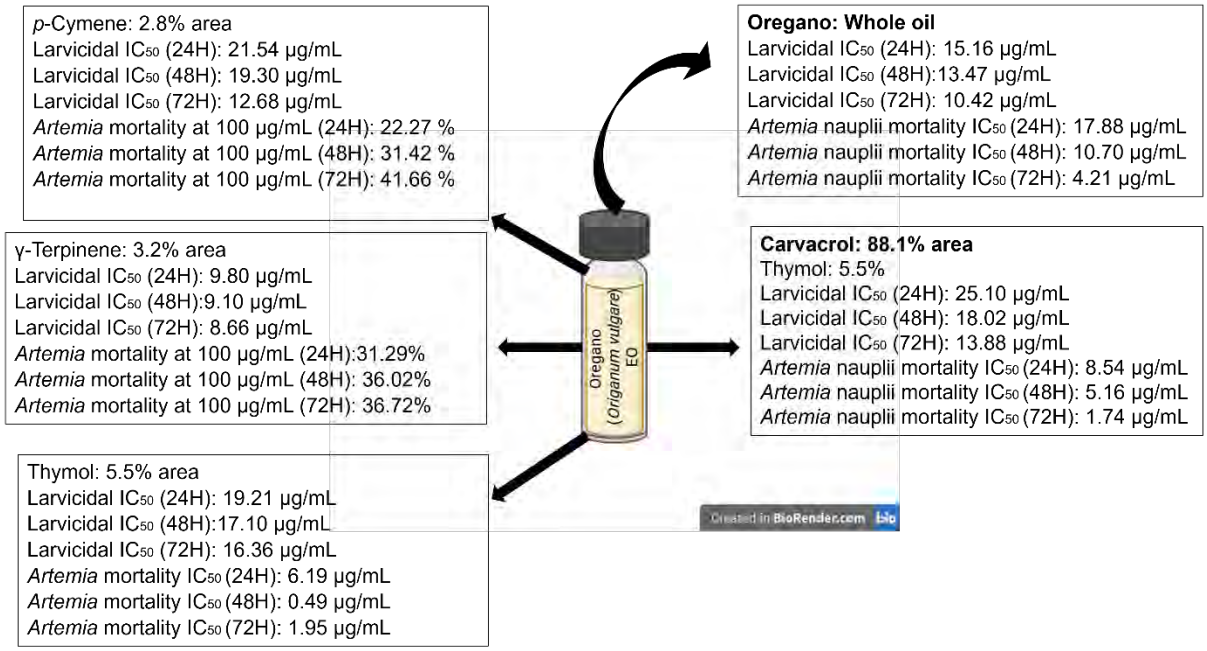


Figure 4.1. A summary of oregano EO and the main constituents with their larvicidal and *Artemia* mortality.

Oregano and the main constituent, carvacrol both showed toxicity towards *Artemia fransiscana* nauplii from 24 hours with a 100% mortality rate (Table 3.15 and Table 3.17). In a previous study 10% (500 µL per 5000 µL) oregano extract demonstrated 100% mortality within 24 hours (Manzanares *et al*., 2015). Oregano was lethal at 24 hours with an LC_{50} value of

17.88 ± 3.01 µg/mL and carvacrol extremely lethal an LC_{50} value of 8.54 ± 1.86 µg/mL. Thymol of which made up 5.5% of the oregano oil also had a low LC_{50} value of 6.19 ± 1.21 µg/mL. The rest of the main constituents (*p*-cymene, γ-terpinene) only resulted in 22.27% to 31.29% mortality after 24 hours at 100 µg/mL, which can account for oregano as whole oil being less lethal than carvacrol. The selectivity indices of oregano EO and carvacrol reiterates that the concentrations needed to kill larvae, is lethal towards *Artemia franciscana* nauplii. Oregano EO has a selectivity index of 1.18 and carvacrol 0.34 after 24 hours, however after 72 hours both was well below 1 and suggests it is unsafe to other non-target organisms. The ECOSAR program predicted carvacrol to be toxic in aquatic environments with regards to fish and daphnids.

To ascertain if oregano and carvacrol were to ends up in a water resource after spraying, not only would wildlife be at risk but also human in their drinking or bathing water. As such a preliminary safety profile towards humans was untaken using human red blood cells and *in silico* predictions. Antoniadou *et al.* (2024) reported low haemolysis with 5.45% haemolysis of oregano aqueous extract at 100 µg/mL after 1 hour; whilst in this study, oregano EO and carvacrol showed minimal <0.1% haemolysis at a 100 µg/mL, which was comparable to propoxur (0.1%) (Table 3.20). This suggested that oregano EO and carvacrol do not pose a potential risk to humans at the concentration needed to kill larvae which is well below 100 µg/mL. Furthermore, carvacrol was predicted by the SWISS ADME program to have a low class I toxic hazard that adhere to Lipinski rule of 5 (Table 3.23 and Table 3.24). Yet *in silico* predictions proposed a high probability of affecting cyclooxygenase-1 (COX) (Table 3.25), which is supported by *in vitro* data where carvacrol inhibited prostaglandin E_2 production by potently inhibiting COX-1 (IC_{50} value: 0.7 µM) and COX-2 (IC_{50} value: 0.8 µM) (Landa *et al.,* 2009). Prostaglandin synthesis is dependent on COX enzymes to catalyse the initial reaction in inflammatory sites. This mechanism of carvacrol mimics that of NSAIDs which are clinically used to reduce inflammation and for their analgesic properties (Landa *et al.*, 2009). A potential clinical benefit of oregano EO and carvacrol is the anti-oxidant or specifically the free radical scavenging activity (<25%) (Figure 3.11) at a 100 µg/mL. This has been verified by Han *et al.* (2017), where variable $DPPH^{\bullet}$ free radical scavenging activity (20% to ± 27%) at 100 µg/mL.

4.2.2. Thyme (*Thymus vulgaris*) Essential Oil and Main Constituent

Thyme oil had four main constituents namely, linalool, γ-terpinene, *p*-cymene and thymol, where thymol made up 61.6% of the oil; with carvacrol and β-caryophyllene in lesser amounts (Table 3.1.E and Figure 4.2). Thyme oil showed inhibitory activity against *An. arabiensis* 3rd instar larvae with an LC_{50} value of 23.40 ± 2.22 µg/mL at 24 hours, which was found to be

similar to that reported for *Culex quinquefasciatus* (LC_{50} value: 32.9 to 71.1 µg/mL) and *An. stephensi* (LC_{50} value: 48.88 µg/mL) larvae (Pavela, Vrchotová and Tříska, 2009; Pandey, Upadhyay and Tripathi, 2009). The main constituent in thyme EO, namely thymol (61.6% composition; Table 3.1 and Table 3.9) contributed 82.1% of the overall larvicidal activity of the whole oil, indicating that there was an additive/synergistic interaction with other minor constituents to kill 100% of the larvae, where all but linalool had larvicidal properties.

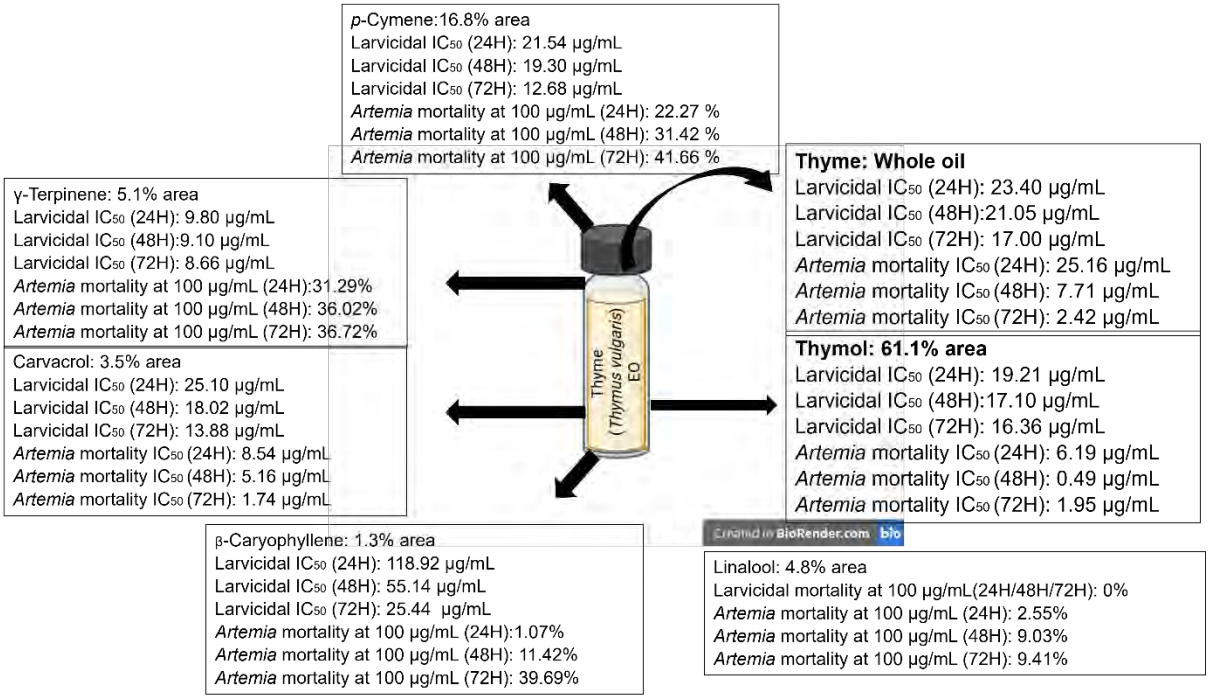


Figure 4.2. The larvicidal and *Artemia* mortality summary of thyme EO and the main constituents.

A comparable lethal concentration of thyme was noted to kill both the *Anopheles* larvae and *Artemia franciscana* nauplii after 24-hours (LC_{50} value of 23.40 ± 2.22 µg/mL; 25.16 ± 2.95 µg/mL, respectively)(Figure 4.2). This was pattern was also reported against *Artemia salina* where an LC_{50} value of 37.99 µg/mL against *Artemia salina* at 24 hours was noted (Niksic *et al*., 2021). Although thymol displayed good larvicidal activity it appears to have decreased the safety profile of the thyme oil, where thymol was 3.62-fold more toxic than the whole oil to *Artemia* (Figure 3.5 and Figure 3.6). The rest of the main constituents and some lesser constituents proved safer thus making the whole oil less lethal towards *Artemia franciscana.* Thyme and the main constituent thymol have very low selectivity indices of 0.14 and 0.03, respectively, suggesting that they are not environmentally safe. Furthermore, thymol was predicted to be toxic towards fish (LC_{50} value: 5.37 µg/mL) and daphnids (LC_{50} value: 3.51 µg/mL) by the ECOSAR program. The mechanism by which thymol is toxic is poorly understood, but it has been proposed to be a neurotoxic inhibiting acetylcholinesterase as

demonstrated in female guppy fish (Bullangpoti *et al*., 2018). Acetylcholinesterase is responsible for breakdown of the a neurotransmitter, acetylcholine and by inhibiting the enzyme could possibly lead to excess acetylcholine, resulting in overstimulation of cholinergic receptors, which can cause a range of effects, including muscle paralysis, respiratory failure, and potentially death (Rants'o, Koekemoer and Van Zyl, 2023). Further *in vitro* studies are needed to determine the effect on crustaceans such as *Artemia* and *Daphnia*.

The preliminary toxicity profile for thyme EO indicated that it had some haemolytic activity (9.74 ± 1.07%) at a 100 µg/mL, but at the lethal concentration for *Artemia* and *Anopheles* would not pose a risk to induce lysis in humans (Figure 4.2). As in this study, thymol has previously been reported to have minimal haemolytic properties in rats (<0.1% haemolysis at 100 µg/mL) (Elshopakey *et al*., 2021). In addition, thyme EO has been reported to have hepatoprotective properties against carbon tetrachloride-induced toxicity in male albino rats (Elshopakey *et al*., 2021); while thymol a protective effect against 2,2-azo-bis(2-amidinopropane) dihydrochloride-induced toxicity of red blood cells (Aman *et al.,* 2013). The protective effects were not attributed to reduction of oxidative stress by scavenging free radicals or chelating iron (II) or copper (II) (Table 3.21, Table 3.22 and Figure 3.12). This was supported by Aman *et al.* (2013) where it was reported that the $DPPH^{\bullet}$ free radical scavenging activity of thymol had an IC_{50} value of 393.3 ± 11.54 µg/mL, that is three times the concentration evaluated in this study.

The favourable safety profile was reiterated by SWISS ADME predictions that thymol is a low class I toxic hazard and adhered to Lipinski rule of 5 (Table 3.23 and Table 3.24). SWISS target predictions indicated that there was a high probability that thymol could affect the transient receptor potential cation channel subfamily A member 1 (TRPA1); where the transient receptor potential channels are sensors necessary for detecting changes in stimuli, cellular as well as environmental factors (Zhang *et al*., 2023). Lee *et al.* (2008) determined that thymol potently activates human TRPA1, by depolarising hTRPA1-HEK293 cells with an IC_{50} value of 6 µM. This activation resulted in changes in mitogen activated protein kinase, transforming growth factor-β, nuclear factor kappa-β and AMP-activated protein kinase pathways. Activation of TRPA1 by thymol can thus increase intracellular Ca^{2+}. The latter ion increase can result in induced skeletal muscle contraction, prolonged muscle paralysis and inability to move in the water to acquire oxygen at the water surface (Zhang *et al*., 2023). An increase in intracellular Ca^{2+} concentration could also activate the endonuclease and initiate programmed cell death cascade (Pinton *et al*., 2008) in larvae and nauplii, but this would need *in vivo* verification.

4.2.3. Black pepper (*Piper nigrum*) Essential Oil

After 72 hours, black pepper EO had some larvicidal properties with an LC_{50} value of 22.73 ± 1.73 µg/mL, but had little *Artemia franciscana* nauplii mortality (12.32 ± 2.49% at 100 µg/mL (Figure 4.3); indicating a favourable safety index. Amer and Mehlhorn (2006) support the latter finding reporting that black pepper EO had some larvicidal activity against *C. quenquefasciatus* (LC_{50} value: 50 ppm), but less so against *An. stephensi* (LC_{50} value:100 ppm) and *Aedes aegypti* (LC_{50} value: 100 ppm) at 24 hours. Whilst, Bajracharya and Tuladhar (2011) reported slightly more lethality against *Artemia salina* (36% at 100 µg/mL) that was observed in this study. Of the main constitutes in black pepper EO, β-caryophyllene (29.7%; Table 3.1.B), showed similar tendencies towards larvae (LC_{50} value: 25.44 ± 0.86 µg/mL) and *Artemia franciscana* nauplii (100 µg/mL: 39.69 ± 11.27%). The larvicidal activity of β-caryophyllene was also reported towards *C. quenquefasciatus* (LC_{50} value: 45.6 µg/mL; at 24hrs)(Andrade-Ochoa *et al*. 2018). After 72 hours, black pepper EO (SI: 4.40) and β-caryophyllene (3.93) had similar selective indices (Table 3.19). These selective indices compared to propoxur (SI: 246.91) are very low and not safe for water animals due to non-selectivity (Indrayanto, Putra and Suhud, 2021).

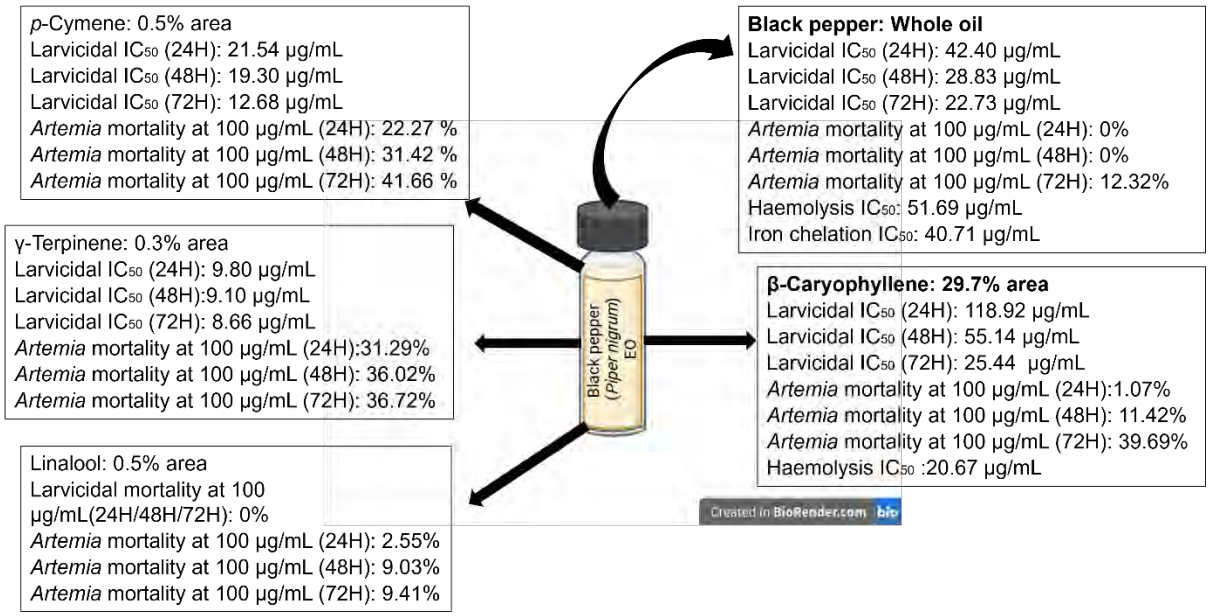


Figure 4.3. Black pepper EO and the main constituents summary of larvicidal and *Artemia* properties along with the main findings of the haemolytic properties of black pepper EO and β-caryophyllene.

Pharmacokinetic properties of β-caryophyllene indicated that it was the only constituent that was unable to cross the blood brain barrier similar to DDT, indicating a more peripheral

mechanism of action of β-caryophyllene. One possible mechanism of action could be interference with iron homeostasis in the larvae where black pepper EO was found to be a good ferrous iron chelator (IC_{50} value of 18.29 ± 2.51 µg/mL), comparable to the positive control 2,2-bipiridyl (IC_{50} value: 16.45 ± 1.66 µg/mL) (*p*-value > 0.05). In contrast, the main constituent β-caryophyllene, had no ferrous iron chelation properties at 100 µg/mL and thus is not responsible for the ferrous iron chelation properties of black pepper EO.

Black pepper EO (IC_{50}: 51.69 ± 4.51 µg/mL) and the main constituent β-caryophyllene (IC_{50}: 20.67 ± 1.23 µg/mL) both had high haemolytic properties (Figure3.9), with β-caryophyllene being the most haemolytic; which possess a safety concern for humans. Although β-caryophyllene has high haemolytic tendencies, *in silico* prediction program Toxtree, predicted the compound to be a low class I toxic hazard. SWISS targets did not predict any interaction with red blood cell targets; but did predict that β-caryophyllene to have a 50% probability to affect human peroxisome proliferator-activated receptor *alpha* and cannabinoid receptors. Cannabinoid receptors are located in the brain and β-caryophyllene is a cannabinoid receptor 2 agonist, that promotes cell death of glioblastomas and also modulates peroxisome proliferator-activated receptor-*gamma* (Irrera *et al.*, 2020). But this is an unlikely target as insects are one of the few animal groups that do not possess canonical cannabinoid receptors (McPartland *et al.*, 2001).

Peroxisome proliferator-activated receptors (PPARs) are a family of nuclear receptors that regulate gene expression involved in lipid metabolism, including fatty acid synthesis, oxidation, and fat storage and are essential in the control of gene transcription encoding proteins for lipid and glucose metabolism (Zieleniak, Wójcik and Woźniak, 2008). A main energy source in mosquitoes as well as larvae development is the accumulation of fat body lipids (Pinch *et al.*, 2021). Compared to the positive control, propoxur, black pepper led to emaciated larvae yet not seen with β-caryophyllene. As such inhibition of this biochemical process by black pepper EO or β-caryophyllene could result in death of the larvae or failure of adult development.

4.2.4. Sweet basil (*Oscimum basilicum*) Essential Oil

Sweet basil had good larvicidal inhibitory properties with LC_{50} values reaching 35.21 ± 7.08 µg/mL after 72 hours, while starting with a LC_{50} value of 45.22 ± 4.58 µg/mL at 24 hours (Table 3.6; Figure 4.4). Sweet basil was reported to have good larvicidal properties at 24 hours against 3rd instar *An. stephensi* (LC_{50} value:45.73 ppm)(Murugan *et al*., 2015). Sweet basil's main constituent was determined to be estragole and linalool (Table 3.1.C). For this study only linalool making up 17.4% of the oil was tested, but did not show larvicidal properties (Table

3.8; Figure 4.4). However estragole isolated from *Zanthoxylum acanthopodium* EO, has been shown to possess larvicidal activity against fourth instar larvae of *An. sinensis* (LC_{50} value: 41.67 µg/mL (mg/L)) and *An. anthropophagus* (LC_{50} value: 38.56 µg/mL (mg/L)) at 24 hours (He, Wang and Zhu, 2018). Estragole has been demonstrated to possess larvicidal activity against *An. stephensi* (LC_{50} value: 11.01 ppm) and anticholinesterase activity (IC_{50} value: 0.337 µM to 12.6 mM) (López *et al.*, 2015; Farag *et al.*, 2016; Spinozzi *et al.*, 2021; Rants'o *et al.*, 2022a).

Sweet basil EO was relatively safe against *Artemia franciscana* nauplii. At 24 hours sweet basil EO had 8.65 ± 3.96% mortality at a 100 µg/mL, increasing to 28.59 ± 3.94% mortality at 72 hours, and had 17.56 ± 3.89% mortality at 10 µg/mL, comparable to propoxur at 10 µg/mL with 22.27 ± 10.10% at 72 hours (Appendix B). A similar result at 24 hours was obtained against *Artemia salina* with sweet basil extracts (root, stem, leaves and flower methanol and ethanol extracts) resulting in mortality ranging from 0% to 35% (Laraib *et al.*, 2018). Sweet basil EO showed better selective indices above 2 for all three time points compared to clove, oregano and thyme EO (≤ 1.18)(Table 3.19). Sweet basil EO also produced minimal haemolysis with linalool not inducing any haemolysis at 100 µg/mL (Table3.20). The latter was supported with the lack of haemolysis using sheep red blood cells, as well as the 53.3 ± 11.6% mortality of the *Artemia salina* L. nauplii at 24 hours (Fujiwara *et al*., 2017). The percentage mortality against *Artemia salina* L. nauplii was 20.9-fold higher than the mortality against *Artemia franciscana* nauplii (2.55 ± 1.85%) at 100 µg/mL at 24 hours. The reported difference in lethality could be due to difference in species or difference in methodologies. In this current study ~20 nauplii were tested per well (60 at a time), whereas Fujiwara *et al.* (2017) exposed the EOs to half the number of nauplii (10) at a time. This could have resulted in higher mortalities.

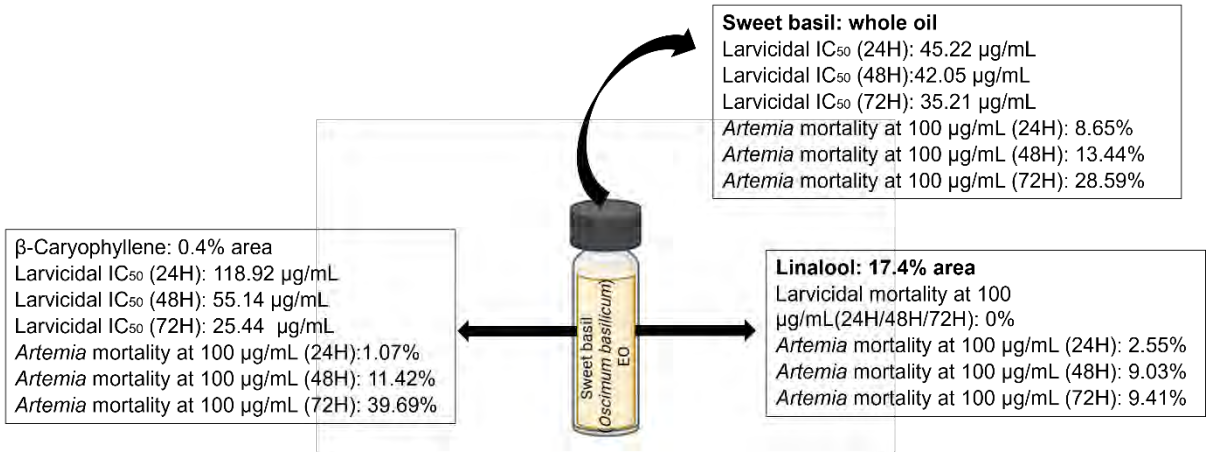


Figure 4.4. The summary of sweet basil EO and the main constituents' effect of *Anopheles* and *Artemia*. (Created in BioRender.com).

4.2.5. Clove (*syzygium aromaticum*) Essential Oil

Eugenol was the main constituent in the clove EO making up 85.7% of the oil, with β-caryophyllene making up 11.7% of the oil (Table 3.1.A). Clove exhibited moderate *Anopheles* larvae inhibitory properties (IC_{50} value: 66.11 ± 10.38 µg/mL) after 72 hours, however it was the least potent of all the tested essential oils (LC_{50} value: 93.36 ± 5.14 µg/mL; 24 hours) (Table 3.6). Conversely, eugenol, the main constituent, exhibited a time-dependent reduction in the concentration required for larvicidal activity at 24 hours, where it had a relatively high LC_{50} value of 72.02 ± 2.47 µg/mL, indicating lower larvicidal potential compared to the whole essential oil (Figure 4.5). However, after 72 hours, the LC_{50} value decreased to 36.21 ± 2.79 µg/mL, suggesting increased potency over time. Thomas *et al.* (2017) similarly reported a time-dependant decrease in LC_{50} value on *An. gambiae* at 24-hours (LC_{50} value:17.53 µg/mL); 48-hours (LC_{50} value: 19.52 µg/mL) and 72-hours (LC_{50} value: 8.61 µg/mL*)*. The latter inhibitory activity was confirmed by Osanloo *et al.* (2018) who reported that the clove EO (LC_{50} value: 57.49 ppm) and eugenol (LC_{50} value : 93.14 ppm) displayed larvicidal properties against *An. stephensi*. The whole essential oil, clove displayed better larvicidal properties against the larvae, similar to the results obtained in this current study. The mechanism of action of clove and the main constituent eugenol could not be correlated to ferrous iron or copper (II) chelation properties (Table 3.21 and Table 3.22).

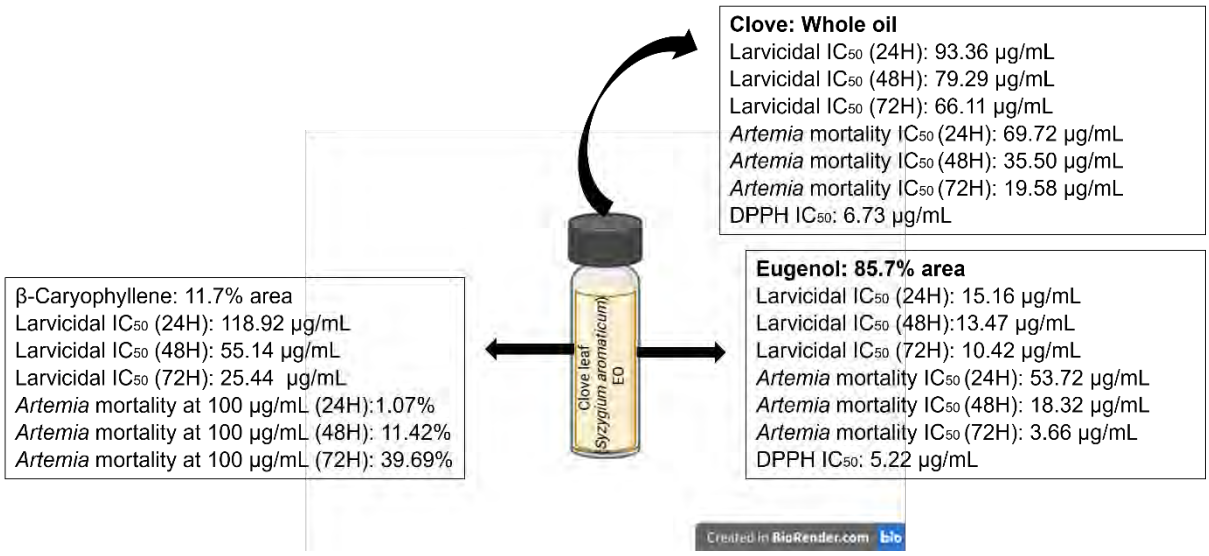


Figure 4.5. Clove EO and the main constituents summary of larvicidal and *Artemia* mortality along with potent anti-oxidant properties reported in this study.

In contrast clove was lethal towards *Artemia franciscana* nauplii with an LC_{50} value of 67.70 ± 4.50 µg/mL after 24 hours, decreasing to LC_{50} value of 19.58 ± 8.24 µg/mL. Bajracharya and

Tuladhar (2011) reported that clove oil was also lethal towards *Artemia salina* at 24 hours of exposure with an LC_{50} value of 42.16 µg/mL. Comparing the two studies, clove EO was 1.6-fold more toxic towards *Artemia salina* than *Artemia franciscana*. Unfortunately, eugenol was also lethal towards *Artemia* nauplii with an IC_{50} value of 3.66 ± 0.92 µg/mL at 72 hours, which led to an extremely low selectivity index of 0.10 at 72 hours, showing non-selective lethality towards non-targeted organisms.

With regards to a preliminary toxicity profile towards humans, clove and eugenol had little haemolytic properties with 10.70 ± 2.87% and <0.1 ± 0.01% haemolysis, respectively at a 100 µg/mL. In an earlier study by He *et al.* (2007), eugenol similarly produced low haemolytic percentages (4.4% haemolysis at 2000 µg/mL), whereas clove resulted in high haemolytic percentages (20% haemolysis at 625 µg/mL) at much higher concentrations used in this study and that by Uchôa Lopes *et al.* (2020). In conjunction with this data, *in silico* Toxtree predicted eugenol to be a low class I toxic hazard adhering to Lipinski rule of 5, showing drug-like properties (Table 3.23 and Table 3.24). The low toxicity of both clove and eugenol could be attribute in part to the potent free radical scavenging activity that was comparable to ascorbic acid (Figure 3.11).

4.3. Most Active Compounds Overall Against *An. arabiensis* Larvae

After determining the inhibitory effect of eugenol and the five derivatives, essential oils and the main constituents, it was found that γ-terpinene and *p*-cymene had the best inhibitory properties against the 3rd instar *An. arabiensis* larvae and demonstrated a better preliminary safety profile (Table 3.9 and Table 3.17).

4.3.1. γ-Terpinene

γ-Terpinene was present in three of the five oils of the essential oils in the following percentages: black pepper (0.3%), oregano (3.2%) and thyme (5.1%). Against the *An. arabiensis* 3rd instar larvae γ-terpinene produced the lowest LC_{50} values of 9.80 ± 1.60 µg/mL (24 hours), 9.10 ± 2.21 µg/mL (48 hours) and 8.66 ± 2.35 µg/mL (72 hours). A study done on 3rd instar *C. quenquefasciatus* larvae showed γ-terpinene also exhibiting some larvicidal activity (LC_{50} values: 45.4 µg/mL) at 24 hours (Andrade-Ochoa *et al*., 2018). γ-Terpinene was more active against *An. arabiensis* than C. *quenquefasciatus,* which could be due to different enzyme targets between species. When combined with propoxur, the combination produced

an additive effect at higher concentrations of γ-terpinene, and what looks like an antagonistic effect the lower concentration of γ-terpinene 10 and high concentrations of propoxur 0.1 at 24 hours (18.75 ± 8.54%). Although after 72 hours the percentage mortality for this combination increased to 80.00 ± 5.00% (Figure 3.3). γ-Terpinene resulted in decomposition of the larvae and even more so in combination with propoxur, this was in contrast with propoxur that did not result in much decomposition at the highest concentration (Table 4.4.A, Table 3.10 and Table 3.12). γ-Terpinene lead to the enlargement of larvae thorax, similar to that of the change observed in propoxur (Table 3.4.A and Table 3.10). A possible mechanism of action of γ-terpinene was not related to its ability to chelate ferrous iron or copper (II). It also had no free radical scavenging properties. Most pesticides induce oxidative stress, increasing free radicals (Abdollahi *et al*., 2004; Mahendran and Vimolmangkang, 2023). This could open the discussion as to whether the absence of anti-oxidant activity proportionately decreased larvicidal activity by scavenging free radicals and decreasing oxidative stress on the larvae. γ-Terpinene, a monoterpene has been shown to inhibit acetylcholinesterase, a key target of insecticides, but would need to be confirmed *in vivo* (Qasim *et al.*, 2024). γ-Terpinene have been reported as competitive acetylcholinesterase inhibitors (IC_{50} values: 5.8 mM) with larvicidal activity against *An. anthropophagus* (LC_{50} values: 29.21 mg/L) and *An. sinensis* (LC_{50} values: 36.42 µg/mL) (López *et al.*, 2015; Rants'o *et al.*, 2022a).

Furthermore γ-terpinene produced relatively low mortality rates against *Artemia franciscana* nauplii. After 24 hours at a 100 µg/mL, 31.29 ± 7.19% of the nauplii died and percentage mortality only increased to 36.72 ± 9.96% after 72 hours. When combined with propoxur, the preliminary study suggested the combination to be safe for aquatic environments. The combination with the lowest γ-terpinene concentration to produce 100% *An. arabiensis* mortality (γ-terpinene 17.25 µg/mL ; propoxur 0.05 µg/mL), produced low mortality rates against *Artemia franciscana* at 24 hours (1.32 ± 0.07%) and increased (60.25 ± 13.60%) at 72 hours (Figure 3.3 and Figure 3.8). The selectivity index for γ-terpinene was the highest for all the compounds tested in this study and remained above 10 for all three time periods recorded. This possibly indicated that γ-terpinene is more selective towards *Anopheles* than *Artemia* (Rants'o *et al.*, 2022b). The ECOSAR program predicted that γ-terpinene was quite toxic towards fish (LC_{50} value: 0.12 µg/mL) and daphnids (LC_{50} value: 0.09 µg/mL). The predicted toxicity for propoxur against fish (LC_{50} value: 210.00 µg/mL) and daphnids (LC_{50} value: 118.00 µg/mL), which is well over a 1000-fold safer against both fish and daphnids than γ-terpinene. For the exception of the ECOSAR predictions, this compound alone or in combination with propoxur demonstrated to be relatively safe for the environment.

To assess for potential human toxicity, γ-terpinene was found to induce low degree of haemolysis at a 100 µg/mL (20.75 ± 6.51%)(Table 3.20) and was classified as a low class I toxic hazard that adheres to Lipinski rule of 5 according to the *in silico* predictions (Table 3.23 and Table 3.24). According to SWISS target predictions, γ-terpinene had a low probability to have an effect on human biological systems and targets (Table 3.25). High γ-terpinene concentrations in rats produce oral toxicity (LC_{50} value: 1680 mg/kg), whilst a low γ-terpinene concentration is needed for *An. arabiensis* mortality (LC_{50} value after 72 hours: 8.66 ± 2.35 µg/mL; Table 3.9)(Spinozzi *et al.*, 2021; Rants'o *et al.*, 2022a). This suggested that this compound had a good safety profile towards humans in the concentrations used in this study and warrant further investigation as possible insecticides.

4.3.2. *p*-Cymene

p-Cymene was the second most potent larvicide of the tested compounds producing an LC_{50} value of 12.68 ± 1.28 µg/mL. In combination with propoxur, at the higher concentrations of *p*-cymene, an additive effect was observed in larvae mortality. Unfortunately, at the concentration closest to the LC_{50} value of *p*-cymene, and the propoxur concentration well above the LC_{50} (*p*-cymene 10 µg/mL: propoxur 0.1 µg/mL), negligible larvae mortality was recorded even at 72 hours (5.00 ± 0.00%). This suggests that at this concentration there is an antagonistic interaction on the effectiveness of the compounds, however further studies are required to confirm this interaction. *p*-Cymene especially when in combination with propoxur led to decomposition of larvae, altered the morphology observed of propoxur as a single compound (Table 3.4.A, Table 3.10 and Table 3.11). In contrast with propoxur, *p*-cymene caused shrinkage of the larval thorax (Table 3.4.A and Table 3.10).

Against *Artemia franciscana* nauplii, *p*-cymene showed low mortality, with 41.66 ± 6.91% mortality at a 100 µg/mL after 72 hours (Table 3.17). The preliminary toxicology data of the *p*-cymene: propoxur combinations against *Artemia franciscana* nauplii suggest that the combinations are relatively safe at 24 and 48 hours after exposure, although mortality increased after 72 hours of exposure. Even though mortality increased at 72 hours of exposure, the combinations needed to kill 100% *An. arabiensis*, did not exceed 65% mortality towards *Artemia* nauplii (Figure 3.7). *p*-Cymene also had the second most favourable selectivity indices of 4.64 (24 hours), 5.18 (48 hours) and 7.89 (72 hours) of the tested compounds (Table 3.19). The environmental safety of *p*-cymene was further elucidated by the ECOSAR predictions, demonstrating high mortality rates against fish (LC_{50} value: 1.78 µg/mL) and daphnids (LC_{50} value: 1.21 µg/mL) as compared to propoxur (LC_{50} value against fish: 210.00 µg/mL; LC_{50} value against daphnids: 118.00 µg/mL)(Table 3.25).

Similarly, potential low toxicity to humans was noted for *p*-cymene where low haemolytic properties were detected at 100 µg/mL for *p*-cymene (Table 3.20). Along with i*n silico* predictions indicating that this compound was a low class I toxic hazard that adheres to Lipinski rule of 5 with 1 violation, A LogP value of 4.47 that could indicate poor absorbance, yet is predicted to have high gastrointestinal absorption (Table 3.23 and Table 3.24).

p-Cymene also showed negligible $DPPH^{\bullet}$ free radical scavenging, copper (II) chelation and ferrous iron chelation properties (Table 3.21 and Table 3.22). SWISS targets identified acetylcholinesterase as a possible target in humans, similar to propoxur. The known mechanism of action of propoxur is acetylcholinesterase inhibition, yet there is no difference of the selectivity of propoxur to human and *Anopheles* acetylcholinesterase (Carlier *et al.*, 2008). *p*-Cymene, a monoterpene, has been reported to possess inhibitory properties of acetylcholinesterase, which acts on the central nervous system and can be seen as possible target for the mechanism of action as insecticide (Qasim *et al.*, 2024).

Chapter 5 – Conclusion, Limitations and Recommendations

5.1. Conclusions

The need for new bioinsecticides is crucial in preventing malaria transmission, particularly by targeting *Anopheles* vectors at their early life stages. With rising resistance to currently available synthetic insecticides, it is essential to explore alternative bioinsecticidal approaches to curb the spread of malaria. This study evaluated the larvicidal properties of eugenol, five of its derivatives, five essential oils, and their main constituents. Additionally, a preliminary toxicity profile was established, assessing potential environmental and human health risks, including *in silico* predictions. Furthermore, various properties were analysed to investigate possible mechanisms of action against *An. arabiensis* larvae.

Eugenol and its five derivatives exhibited larvicidal activity, with eugenyl acetate demonstrating the highest potency. Among these derivatives, only eugenyl acetate, methyleugenol and isoeugenol showed low mortality rates on *Artemia franciscana* nauplii. All tested compounds induced minimal to no haemolysis in human red blood cells. Isoeugenol was the only derivative that acted as a potent ferrous iron chelator and DPPH free radical scavenger, while eugenol and σ-eugenol also displayed potent free radical scavenging properties.

All five EOs demonstrated larvicidal properties, with oregano and thyme being the most active. Black pepper and sweet basil produced low mortality rates towards *Artemia* nauplii, whereas oregano, thyme and clove were lethal. These oils were relatively safe towards human erythrocytes with minimal haemolysis, yet black pepper was extremely haemolytic. Black pepper was the only EO with potent ferrous iron chelation properties and clove the only EO with potent free radical scavenging properties. Although oregano and thyme showed some free radical scavenging potential, their activity was insufficient to suggest a definitive mechanism of action.

The larvicidal effects of the main constituents largely aligned with those of the oils they were derived from. With the exception of linalool, all the main constituents had larvicidal properties, especially γ-terpinene and *p*-cymene. γ-Terpinene, *p*-cymene, linalool and β-caryophyllene were relatively safe towards *Artemia* nauplii, whereas carvacrol and thymol were lethal at low concentrations. Only β-caryophyllene had high haemolytic properties correlating to that of black pepper where it makes up the majority of the oil. None of the main constituents displayed notable DPPH free radical scavenging activity or copper (II) and ferrous iron chelation.

In conclusion, γ-terpinene and *p*-cymene emerged as the most potent larvicides in this study, with favourable safety profiles for both aquatic ecosystems and human exposure. *In silico* predictions further supported these findings. When combined with propoxur, their larvicidal efficacy was enhanced without compromising safety towards *Artemia* nauplii. These findings suggest that γ-terpinene and *p*-cymene hold promise for further testing and optimization as potential larvicides in malaria vector control efforts.

5.2. Limitations

- This study was limited to testing only five eugenol derivatives, despite the existence of numerous other derivatives that may have potential larvicidal activity.
- Only the main constituents of the EOs were evaluated, leaving out other potentially active EOCs and possible synergistic interactions between the major and minor constituents
- Given the vast number of potential biological targets, a definitive mechanism of action could not be determined.

5.3 Further Studies and Recommendations

- Explore a broader range of EOCs and EOs to better assess their larvicidal efficacy against various species and resistant strains.
- Evaluate the impact of these compounds on different life stages of *Anopheles*, particularly their adulticidal properties.
- More comprehensive cytotoxicity studies should be conducted to assess the safety of these compounds in greater detail.
- As γ-terpinene and *p*-cymene were the most active larvicides in this study, further research is needed to determine a complete toxicity profile on human cell lines.
- More research is needed to determine environmental toxicity in water animals and to clarify *in vitro* and *in silico* data.
- Many different mechanisms of action and enzymes are proposed for monoterpenes. As such for the two most active EOCs require further studies to determine an exact mechanism of action on *Anopheles.* As such, the effectivity and safety can further be increased by understanding the role of the EOCs on larvae mortality.

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Appendices

Appendix A- Ethics Documents

Appendix A.1 - Waiver Animal Research Ethics Committee for use of *Anopheles* and *Artemia franciscana* nauplii

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

ANIMAL RESEARCH ETHICS COMMITTEE
Registration number: AREC-101210-002

Date: 27/08/2024

Certificate reference: Waiver 27-08-2024-O
Category: O
Applicant: Professor Robyn van Zyl
Department: School of Therapeutic Sciences
Tel: 011 717 2271 **Email:** Robyn.VanZyl@wits.ac.za

Re: Waiver from the Animal Ethics Research Committee of the University of the Witwatersrand

This letter is to confirm that Professor Robyn van Zyl (Wits Research Institute for Malaria (WRIM), in the School of Therapeutic Sciences, Faculty of Health Sciences (WITS) does not require a full animal ethics applications for studies investigating the insecticidal or toxicity potential of novel synthetic or natural compounds against *Anopheles* mosquitoes species or *Artemia franciscana* nauplii species respectively.

Reason for waiver
Anopheles larvae and/or adults and *Artemia franciscana* nauplii constitute lower order arthropods, insects and crustaceans respectively, and thus do not require a full animal ethics application.

Details of the study
The proposed studies assess the insecticidal and toxicity potential of synthetic or naturally derived compounds against *Anopheles* larvae and/or adults or the *Artmeia franciscana,* respectively using an *in vitro* dose response, bioassay format.

Please contact me should you require further information.

Yours sincerely,

Dr Mark Killick
Deputy Chair: Animal Research Ethics Committee
University of the Witwatersrand.
Mail: Mark.Killick@wits.ac.za Office number: 011 717 2362

ANIMAL RESEARCH ETHICS COMMITTEE
Registration number: AREC-101210-002

Date: 18/02/2025

Certificate reference: Waiver 18-02-2025-O
Category: O
Applicant: Professor Robyn van Zyl
Department: School of Therapeutic Sciences
Tel: 011 717 2271 **Email:** Robyn.VanZyl@wits.ac.za

Re: Waiver from the Animal Ethics Research Committee of the University of the Witwatersrand

This letter is to confirm that the following studies to be undertaken in Professor Robyn van Zyl's research unit (Wit Research Institute for Malaria (WRIM), in the School of Therapeutic Sciences, Faculty of Health Sciences (WITS) does not require full Animal Ethics Research Committee clearance.

S.Malan 18-02-2025-O
Student details: Sune Malan (1849730)
Project title: Larvicidal and Toxicological Properties of Insect-Repellent Essential Oils and Their Main Constituents.

T. Chihamba 18-02-2025-O
Student details: Tatenda Faith Chihamba (879896)
Project title: The use of essential oil constituents in the control of insecticide-resistant malaria vectors

Reason for waiver
Anopheles larvae and/or adults and *Artemia franciscana* nauplii constitute lower order arthropods, insects and crustaceans respectively, and thus do not require a full animal ethics application

Details of the study
The proposed studies will assess the insecticidal and toxicity potential of synthetic or naturally derived compounds against *Anopheles* larvae and/or adults or the *Artmeia franciscana,* respectively using an *in vitro* dose response, bioassay format.

The individuals covered by this waiver include students, Sune Malan and Tatenda Chihamba and their supervisors Professor Robyn van Zyl and Professor Lizette Koekemoer (School of Therapeutic Sciences (WITS)).

Please contact me should you require further information.

Yours sincerely,

Dr Mark Killick
Chair: Animal Research Ethics Committee
University of the Witwatersrand.
Mail: Mark.Killick@wits.ac.za Office number: 011 717 2362

Appendix A.2.1 - HREC Waiver for using human blood

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG	HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research and Innovation)

TO: Professor R van Zyl
School of Therapeutic Sciences
Department of Pharmacy and Pharmacology
Faculty of Health Sciences

E-mail: Robyn.vanZyl @wits.ac.za

CC: Supervisor: Not applicable
<>
and <HREC-Medical Research Office@wits.ac.za>

FROM: Mr Iain Burns
Human Research Ethics Committee (Medical)
Tel: 011 717 1252

E-mail: Iain.Burns@wits.ac.za

DATE: 2024/11/20

REF: R14/49

PROTOCOL NO: **M240945** (This is your ethics application reference number. Please quote it in all enquiries, oral or written, relating to this study.)

PROJECT TITLE: *The chemotherapeutic properties of novel synthetic and natural antimalarial compounds*

Please find attached the Clearance Certificate for the above project. I hope it goes well and that an article in a recognized publication comes out of it. This will reflect well on your professional standing and contribute to Government funding of the University.

MSWorks2000/Iain0007/Clearscan.wps

Appendix A.2.2 - Self-administered questionnaire for volunteers complied with the inclusion and exclusion criteria to donate blood

Pharmacology Division / WRIM
Blood Donation Log book

Surname	
First name	
Age	
Assigned Donor #	

To assess if you meet all the inclusion and exclusion criteria for donating blood, please complete the following two sheet e time you volunteer to donate blood.

Donor #	Donation Date	Blood type	In good health today?	Eaten in the last 4 hours?	Donated blood to blood bank	Approved to donate	Number of tubes drawn	Consent to donate blood	Senior Technician
		Y/N/Specify	Y/N	Y/N	N/Y. When	Y/N	max 8	signature	signature

Pharmacology Division / WRIM
Blood Donation Log book

To assess if you meet all the inclusion and exclusion criteria for donating blood, please complete the following two sheet each time you volunteer to donate blood.											
Donor #	Females only: Are you pregnant ?	Are you currently on any of the following medications or taken any within the last 2 weeks:						Anaemia/ blood disorders?	Diabetes, thyroid, epilepsy?	Cardio-vascular or lung disorders?	Other chronic illnesses?
		Antimicrobial (antibiotic, antifungal, antiparasitic, antiviral)	Coagulation drugs: aspirin, heparin, indomethacin, warfarin	Anti-epileptics	Vitamins/anti-oxidants	Natural remedies or traditional medicines	Other				
	Y/N/NA	Y/N	Y/N	Y/N	Y/N	Y/N	Y/N/Specify	Y/N	Y/N	Y/N	Y/N/Specify

Appendix A.2.3 - Consent form for volunteers to donate blood

PHARMACOLOGY

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

Department of Pharmacy and Pharmacology
School of Therapeutic Sciences, Faculty of Health Sciences · Private Bag 3, Wits, 2050, South Africa
Tel: +27 11 717 2271 · E-mail: Robyn.vanZyl@wits.ac.za · www.wits.ac.za

CONSENT TO DONATE BLOOD

I have discussed the above points with volunteers; it is my opinion that the participant understands the risks, benefit and obligations involved in participating in this project.

Researcher's signature **Date**

I understand that my participation is voluntary and that I may refuse to give permission or can withdraw my consent and stop taking part at any time without penalty.

I hereby freely agree to supply the necessary information such as to donate blood and understand that there is no financial benefit to volunteering.

Volunteer's signature **Date**

Appendix A.3 - Institutional Biosafety committee

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Research Office

INSTITUTIONAL BIOSAFETY COMMITTEE (IBC)
(R 14/16)

CLEARANCE CERTIFICATE **PROTOCOL NUMBER: 20190404**

BRIEF DESCRIPTION OF APPLICATION:

The chemotherapeutic properties of novel synthetic and natural compounds

APPLICANT: Prof Robyn Van Zyl

SUPERVISOR: N/A

SCHOOL/DEPARTMENT: Pharmacology

DATE CONSIDERED: 24 February 2025

EXPIRY DATE: 07 March 2030

DECISION OF COMMITTEE: Approved unconditionally

1. This clearance certificate expires on 07 March 2030 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
4. Unreported changes to the application may invalidate the clearance given by the IBC

DATE: 07 March 2025

CHAIRPERSON:
(Professor J Larkin)

DECLARATION OF APPLICANT:

To be completed and emailed to mmatshepo.taunyane@wits.ac.za and HREC-Medical.ResearchOffice@wits.ac.za.

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:** _____

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix B- Supplementary data

Table B.1: The screening data from the larvicidal assay against *An. arabiensis* 3rd instar larvae at 24 hours (Screening concentration: 100, 10, 1, 0.1 µg/mL; *n=5*).

Percentage (%) larvae mortality (Average ± s.d.) at 24 hours								
Compounds	**100 µg/mL**		**10 µg/mL**		**1 µg/mL**		**0.1 µg/mL**	
	Average	**s.d.**	**Average**	**s.d.**	**Average**	**s.d.**	**Average**	**s.d.**
Propoxur	n.d.	n.d.	n.d.	n.d.	100.00	0.00	72.50	24.75
DDT	n.d.	n.d.	n.d.	n.d.	100.00	0.00	77.50	11.09
Eugenol	86.67	11.55	0.00	0.00	0.00	0.00	0.00	0.00
Isoeugenol	32.00	2.50	0.00	0.00	0.00	0.00	0.00	0.00
Methylisoeugenol	96.67	6.06	0.00	0.00	0.00	0.00	0.00	0.00
Eugenyl acetate	91.67	7.64	0.00	0.00	0.00	0.00	0.00	0.00
σ-eugenol	100.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Methyleugenol	100.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Black pepper	83.00	6.29	0.00	0.00	0.00	0.00	0.00	0.00
Sweet basil	99.00	2.24	0.00	0.00	0.00	0.00	0.00	0.00
Clove	95.00	8.66	0.00	0.00	0.00	0.00	0.00	0.00
Oregano	100.00	0.00	10.00	5.00	0.00	0.00	0.00	0.00
Thyme	100.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
γ-Terpinene	100.00	0.00	71.67	15.28	0.00	0.00	0.00	0.00
***p*-Cymene**	100.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Linalool	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
β-Caryophyllene	83.33	7.64	0.00	0.00	0.00	0.00	0.00	0.00
Carvacrol	100.00	0.00	5.00	0.00	0.00	0.00	0.00	0.00
Thymol	100.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

(n.d.) not determined

Table B.2: The screening data from the larvicidal assay against *An. arabiensis* 3rd instar larvae at 48 hours (Screening concentration: 100, 10, 1, 0.1 µg/mL; *n=5*).

Percentage (%) larvae mortality (Average ± s.d.) at 48 hours								
Compounds	**100 µg/mL**		**10 µg/mL**		**1 µg/mL**		**0.1 µg/mL**	
	Average	**s.d.**	**Average**	**s.d.**	**Average**	**s.d.**	**Average**	**s.d.**
Propoxur	n.d.	n.d.	n.d.	n.d.	100.00	0.00	96.67	7.64
DDT	n.d.	n.d.	n.d.	n.d.	100.00	0.00	87.50	8.66
Eugenol	95.00	7.07	5.00	2.89	0.00	0.00	0.00	0.00
Isoeugenol	78.33	2.89	0.00	0.00	0.00	0.00	0.00	0.00
Methylisoeugenol	100.00	0.00	1.67	2.58	0.00	0.00	0.00	0.00
Eugenyl acetate	96.00	4.18	0.00	0.00	0.00	0.00	0.00	0.00
σ-eugenol	100.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Methyleugenol	100.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Black pepper	98.00	2.74	0.00	0.00	0.00	0.00	0.00	0.00
Sweet basil	99.00	2.24	0.00	0.00	0.00	0.00	0.00	0.00
Clove	95.00	10.00	0.00	0.00	0.00	0.00	0.00	0.00
Oregano	100.00	0.00	15.00	7.64	0.00	0.00	0.00	0.00
Thyme	100.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
γ-Terpinene	100.00	0.00	75.00	15.00	0.00	0.00	0.00	0.00
***p*-Cymene**	100	0.00	5.00	0.00	0.00	0.00	0.00	0.00
Linalool	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
β-Caryophyllene	95.00	8.66	0.00	0.00	0.00	0.00	0.00	0.00
Carvacrol	100.00	0.00	5.00	0.00	0.00	0.00	0.00	0.00
Thymol	100.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

(n.d.) not determined

Table B.3: The screening data from the larvicidal assay against *An. arabiensis* 3rd instar larvae at 72 hours (Screening concentration: 100, 10, 1, 0.1 µg/mL; *n=5*).

Percentage (%) larvae mortality (Average ± s.d.) at 72 hours								
Compounds	**100 µg/mL**		**10 µg/mL**		**1 µg/mL**		**0.1 µg/mL**	
	Average	**s.d.**	**Average**	**s.d.**	**Average**	**s.d.**	**Average**	**s.d.**
Propoxur	n.d.	n.d.	n.d.	n.d.	100.00	0.00	96.67	5.77
DDT	n.d.	n.d.	n.d.	n.d.	100.00	0.00	98.33	2.89
Eugenol	100.00	0.00	13.75	4.79	0.00	0.00	0.00	0.00
Isoeugenol	86.25	8.54	0.00	0.00	0.00	0.00	0.00	0.00
Methylisoeugenol	100.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Eugenyl acetate	99.00	2.24	0.00	0.00	0.00	0.00	0.00	0.00
σ-eugenol	100.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Methyleugenol	100.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Black pepper	98.00	2.74	0.00	0.00	0.00	0.00	0.00	0.00
Sweet basil	99.00	2.24	0.00	0.00	0.00	0.00	0.00	0.00
Clove	97.50	5.00	0.00	0.00	0.00	0.00	0.00	0.00
Oregano	100.00	0.00	18.33	5.77	0.00	0.00	0.00	0.00
Thyme	100.00	0.00	3.00	2.74	0.00	0.00	0.00	0.00
γ-Terpinene	100.00	0.00	81.67	16.07	0.00	0.00	0.00	0.00
***p*-Cymene**	100	0.00	5.00	0.00	0.00	0.00	0.00	0.00
Linalool	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
β-Caryophyllene	97.50	5.00	0.00	0.00	0.00	0.00	0.00	0.00
Carvacrol	100.00	0.00	15.00	5.77	0.00	0.00	0.00	0.00
Thymol	100.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

(n.d.) not determined

Table B.4: *Artemia* mortality screening data at 10 µg/mL for EO/EOCs at 24-, 48-, 72-hours (Average ± s.d.; *n=3*).

Percentage (%) *Artemia*	24 hours		48 hours		72 hours	
Compounds	10 µg/mL		10 µg/mL		10 µg/mL	
	Average	s.d.	Average	s.d.	Average	s.d.
$K_2Cr_2O_7$	5.63	3.27	82.74	3.32	95.53	7.78
Propoxur	0.00	0.00	0.00	0.00	22.26	10.10
DDT	0.00	0.00	2.72	3.85	91.44	14.82
Eugenol	0.00	0.00	4.37	2.43	65.60	8.80
Isoeugenol	0.00	0.00	0.77	1.09	4.69	0.10
Methylisoeugenol	0.00	0.00	1.80	0.94	32.63	7.05
Eugenyl acetate	0.00	0.00	0.19	0.34	5.81	4.39
σ-eugenol	0.00	0.00	0.69	0.68	15.59	1.85
Methyleugenol	0.00	0.00	0.00	0.00	7.91	4.26
Black pepper	0.49	0.69	0.98	0.98	8.61	7.04
Sweet basil	0.00	0.00	0.00	0.00	13.73	9.31
Clove	0.00	0.00	0.00	0.00	16.43	6.60
Oregano	0.30	0.43	56.52	0.00	94.18	10.09
Thyme	0.00	0.00	81.82	0.00	84.76	3.57
γ-Terpinene	1.31	0.33	1.12	0.50	6.10	2.10
p-Cymene	0.00	0.00	2.84	0.58	13.48	8.61
Linalool	0.00	0.00	1.23	1.08	5.35	2.33
β-Caryophyllene	0.00	0.00	0.00	0.00	12.22	9.20
Carvacrol	95.63	7.18	100.00	0.00	100.00	0.00
Thymol	96.91	3.60	100.00	0.00	100.00	0.00

(n.d.) not determined

Table B.5: The IC_{50} values (µg/mL) of the compounds that exhibited more than 60% ferrous iron chelation at 100 µg/mL, compared to know iron chelators: EDTA and 2,2 Bipiridyl.

Compound	IC_{50}: µg/mL (Average ± s.d.; *n=3*)
EDTA	9.10 ± 0.64
2,2-Bipiridyl	16.45 ± 1.66
Isoeugenol	40.71 ± 6.03 (***)
Black pepper	18.29 ± 2.51 (ns)

Significance as compared to 2,2-bipiridyl. (ns) not significant, *p*-value > 0.05; (*) significant, *p*-value ≤ 0.05; (**) Significant, *p*-value ≤ 0.01(***) very significant, *p*-value ≤ 0.001.

Table B.6: The data of the potent DPPH• free radical scavenging EO/EOCs at 100, 10, 1 and 0.1 µg/mL.

Percentage DPPH• free radical scavenging (%) (Average ± s.d.; *n=3*)								
Compounds	**100 µg/mL**		**10 µg/mL**		**1 µg/mL**		**0.1 µg/mL**	
	Average	**s.d.**	**Average**	**s.d.**	**Average**	**s.d.**	**Average**	**s.d.**
Trolox	98.42	0.38	94.40	1.77	11.37	0.52	1.06	0.70
Ascorbic acid	98.77	0.42	97.23	0.20	2.21	0.87	<0.1	0.01
Butylhydroxyanisole	96.50	0.52	78.42	0.54	8.79	1.74	0.03	1.02
N-propyl gallate	96.56	0.41	96.19	0.52	37.80	2.70	0.89	1.39
Eugenol	94.42	1.40	75.71	16.71	10.60	2.12	<0.1	0.01
Isoeugenol	94.89	0.80	57.51	8.57	4.20	1.40	<0.1	0.01
σ-eugenol	94.55	0.98	63.45	9.39	8.53	4.82	<0.1	0.01
Clove	93.72	0.78	75.71	16.71	8.49	2.88	<0.1	0.01

(n.d.) not determined

Dose Response Curves for DPPH results:

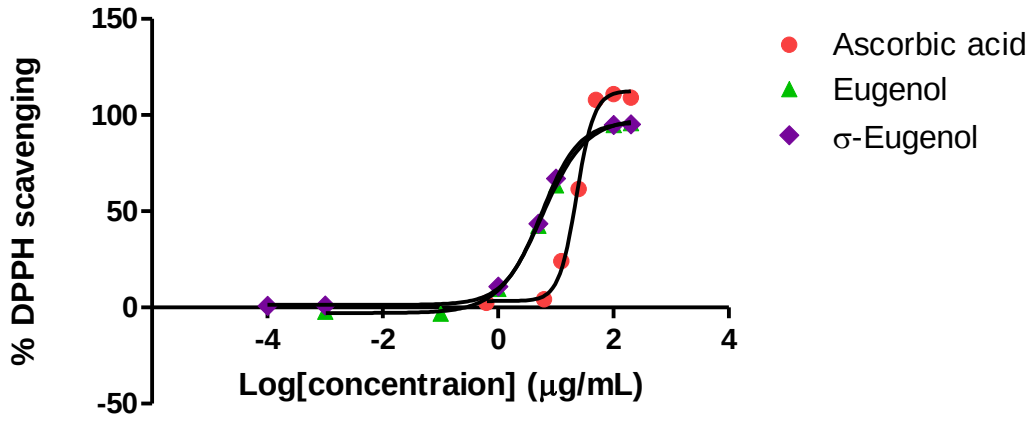

Figure B.1: The sigmoidal dose-response curve of eugenol and σ-eugenol, compared to Ascorbic acid standard controls in the DPPH• free radical scavenging assay. Ascorbic acid (IC_{50} value: 5.26 ± 0.85 µg/mL); Eugenol (IC_{50} value: 5.22 ± 0.50 µg/mL; *p*-value > 0.05); σ-eugenol (IC_{50} value: 5.07 ± 0.72 µg/mL; *p*-value > 0.05).

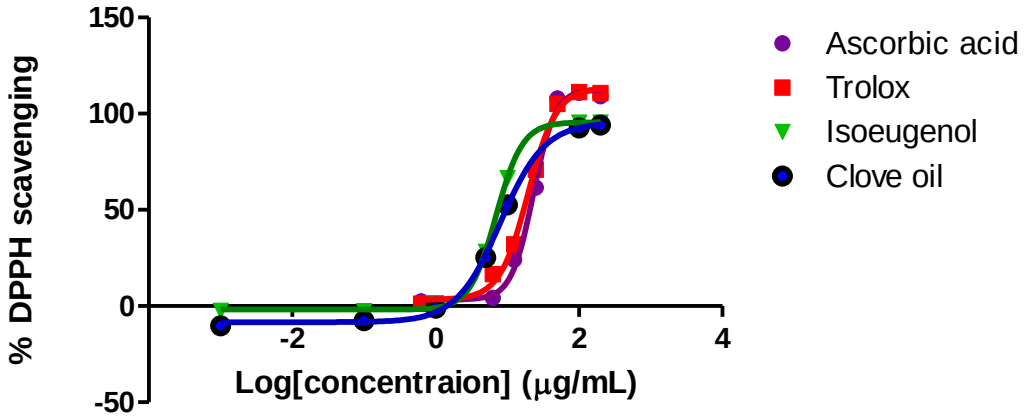

Figure B.2: The sigmoidal dose-response curve of isoeugenol and clove EO, compared to ascorbic acid and Trolox, standard controls in the DPPH• free radical scavenging assay. Ascorbic acid (IC_{50} value: 5.26 ± 0.85 µg/mL); Trolox (IC_{50} value: 4.02 ± 0.92µg/mL); Isoeugenol (IC_{50} value: 7.47 ± 0.47 µg/mL; *p*-value ≤ 0.001); Clove (IC_{50} value: 6.73 ± 1.15 µg/mL; *p*-value ≤ 0.01).