BIOACTIVITY OF CRUDE ESSENTIAL OILS AND BLENDS OF *Artemisia afra, Ocimum kilimandscharicum* AND *Tagetes minuta* AGAINST *Anopheles gambiae* S.S.

BY

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DECLARATION

Declaration by the candidate

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DEDICATION

To my dear wife Linet and lovely children Lawrence, Fredrick and Carita.

ABSTRACT

Malaria causes heavy morbidity and high mortality in most parts of the world. Synthetic insecticides mostly employed to control the mosquito vector have detrimental effects to the environment, necessitating the search for ecofriendly alternatives. This study determined the larvicidal activity of crude essential oils (EOs) and blends of *Artemisia afra*, *Ocimum kilimandscharicum,* and *Tagetes minuta* against *Anopheles gambiae* s.s. using treatment solutions whose concentrations ranged from 25 to 200 parts per million (ppm). Chemical analysis of the crude EOs was done using gas chromatography- mass spectrometry (GC-MS) to determine their chemical composition. Synthetic blends of their major constituents were evaluated for their larvicidal, ovicidal, pupicidal, repellent and oviposition deterrent activities against *An. gambiae* s.s. in selective bioassays. Data obtained were subjected to probit analysis using STATSTICA 6.0 and means were compared using one-way analysis of variance (ANOVA). Among the crude EOs, *O. kilimandscharicum* was the most efficacious with LC_{50} and LC_{90} of 48.50 and 91.40 ppm after 24 hours, respectively. The crude EO of *T. minuta* had the least efficacy with an LC_{50} of 58.80 ppm. The larvicidal activity of selected blends of the plant extracts against *An. gambiae* s.s. was significantly lower ($P < 0.001$) than that of crude EOs with LC_{50} and LC_{90} values of blend OK-4 (derived from *O. kilimandscharicum*) being 59.50 and 93.80 ppm, after 24 hours under laboratory conditions. The LC_{50} (40 ppm) and LC_{90} (84.9 ppm) values obtained when larvicidal activity of the blends were tested under simulated field conditions (SFCs) were comparatively lower than those under laboratory conditions. Among the juvenile stages, ova were found to be the most susceptible while pupae were the most tolerant to the treatments. All the blends negatively influenced oviposition site selection by gravid female mosquitoes, and effectively repelled blood-starved female mosquitoes, with blend OK-4 being the most effective. From this study, it is evident that the three plants are potential sources of insecticidal compounds that can be used to control the malaria vector. Formulation and commercialization of products from these plants should, therefore, be explored.

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LIST OF ABBREVIATIONS

AA-	*Artemisia afra*
ACT-	Artemisinin combined therapy
ANOVA-	Analysis of variance
C (-)-	Negative control
C (+)-	Positive control
CA, USA-	California, United States of America
CDC-	Centres for Disease Control
CPT-	Complete protection time
CGHR-	Centre for Global Health Research
DEET-	N, N-diethyl-m-toluamide
DMRT-	Duncan's multiple range test
ED_{50}-	Effective dose causing 50% repellency
ED_{95}-	Effective dose causing 95% repellency
EI-	Electron impact
EOs-	Essential oils
GABA-	Gamma-aminobutyric acid
GC-MS-	Gas chromatography- mass spectrometry
GTS-	Global technical strategy
HIV-	Human immunodeficiency virus
IGR-	Insect growth regulator
IMM-	Integrated mosquito management
IRS-	Indoor residual spraying
ITIS-	Interagency Taxonomic Information System
ITN-	Insecticide-treated bed nets
IVM-	Integrated vector management
KEMRI-	Kenya Medical Research Institute
LC_{10} -	Lethal concentration causing 10% mortality
LC_{50} -	Lethal concentration causing 50% mortality
LC_{90} -	Lethal concentration causing 90% mortality
LLINs-	Long lasting insecticide treated bed nets
MU-	Moi University
MW-	Molecular weight
NACOSTI-	National Commission for Science, Technology and Innovation
NIST-	National Institute of Standards and Technology
NOEC-	No observable effects concentration
OAI-	Oviposition activity index
OK-	*Ocimum kilimandscharicum*
Orco-	Corereceptor
ORx-	Odorant receptors
PE-	Protection efficiency
PMD-	p-methane-3,8-diol
Ppm-	Parts per million
PTLC-	Preparatory thin layer chromatography
PTN-	Pyrethroid treated bed nets
®	Registered trademark
R_f –	Retention factor
RH-	Relative humidity
rtp-	Room temperature and pressure

SAR-	Structure- activity relationships
SFCs-	Simulated field conditions
s.s-	Sensu stricto
SSA-	Sub-Saharan Africa
TM-	*Tagetes minuta*
UOE-	University of Eldoret
UV-	Ultra violet
VN-	Voucher number
WHO-	World Health Organization
WHO-PQ-	World Health Organization pre-qualification
WP-	Wettable powder

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CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Globally, mosquitoes are the most significant vectors in the medical and veterinary spheres, transmitting both parasitic and viral infections to humans and domesticated animals (WHO, 2013; Weaver and Lecuit, 2015; Gaddaguti *et al*., 2016; Kindhauser *et al*., 2016). Vector-borne diseases transmitted by mosquitoes are still responsible for debilitating morbidity and mortality in their endemic countries (Benelli *et al*., 2016; Kroeger and Nathan, 2016; Benedict *et al*., 2017), with more than two billion people at risk. The most affected individuals live in tropical countries (Jones *et al*., 2017; Maduka, 2018). Malaria is the most significant among the mosquito-borne diseases because of its heavy socio-economic burden with an estimated annual expenditure of at least US$ 2.7 billion for control and elimination alone in endemic countries (WHO, 2017).

Anopheles gambiae mosquitoes are the prime vectors of malaria parasites in Africa (Robert *et al*., 2003; Rizzo *et al*., 2011; Pates and Curtis, 2015; Derua *et al.,* 2016; White, *et al.,* 2017), where the disease claims many lives particularly in low income countries (Warren *et al*., 2009, Archana *et al*., 2014). This species has been found to be the most prevalent in the Sub-Saharan Africa (SSA) countries including Kenya (Robert *et al*., 2003; Menge *et al*., 2006; Mutuku *et al*., 2006; Kelly-Hope *et al*., 2009; Kweka *et al*., 2011b). Three common mosquito species dominate the region, which are *An. gambiae*, *An. arabiensis* and *An. funestus. An. gambiae* sensu stricto is the most efficient and effective human malaria vector because of its anthropophilic and endophilic characteristics (Odiere *et al*., 2007; Bayoh *et al*., 2010; Molina-Cruz *et al*., 2012; Bekele, 2018).

Kenya is among the 15 countries in the world that contribute nearly 80% of deaths attributed to malaria (WHO, 2017). Unfortunately, the WHO (2017) reported that despite being one of the 41 high-burden countries whose fight against malaria mainly relies on international funding, Kenya, had suffered reduced funding of more than 50% in per capita population at risk, a factor that could reverse previous gains. These fears were confirmed with reports showing that progress had stalled in many countries, with the world unlikely to achieve the WHO global technical strategy (GTS) for malaria 2016-2030 (WHO, 2018). The knowledge that the poor natural setting and limited vector control strategies have aggravated malaria transmission in Kenya (Becker *et al*., 2003; Robert *et al*., 2003), has inspired the search for ambitious vector control measures, touted to play a central role in curbing the disease by most researchers (Enayati *et al*., 2009; Mendis *et al*., 2009).

Tremendous progress has been made in reducing malaria morbidity and mortality through integrated vector management (IVM), with chemical control as the main component (Gleiser and Zygadlo, 2007; White *et al*., 2017). This is founded upon an expanded coverage of insecticide-treated bed nets (ITNs), indoor residual spraying (IRS), and drug therapy (Greenwood, 2008; WHO, 2018). Most vector control programmes rely on the use of synthetic chemical insecticides formulated as larvicides, adulticides, baits or repellents (Fillinger *et al.,* 2009; Bayoh *et al.,* 2010; Bøgh *et al.,* 2016).

Despite the success of insecticide-based control programmes in reduction of malaria prevalence (Hemingway *et al*., 2006; Sadasivaiah *et al*., 2007; Reddy *et al*., 2011), there is growing interest due to negative effects of wide-scale and prolonged use of synthetic insecticides on human health and the environment (Fillinger *et al*., 2009;

Bøgh *et al*., 2016). Prolonged use of synthetic insecticides has led to numerous ecological problems such as residues toxic to both flora and fauna with bioaccumulation evident along the trophic level (Sadasivaiah *et al*., 2007; Pates and Curtis, 2015; Huang *et al*., 2018). The increasing mosquito resistance to most conventional chemical insecticides is also a matter of current concern, posing a threat to life (Asidi *et al*., 2005; Kawada *et al*., 2011; Ranson *et al*., 2011; Karunamoorthi and Sabesan, 2013; Uragaya *et al*., 2015; Huang *et al*., 2018).

There is re-emergence of vector-borne diseases coupled with an ever-increasing unreliability of synthetic insecticides for vector control (Koella *et al*., 2009; Jones *et al*., 2017). These have initiated spirited advocacy for continued search for eco-friendly alternatives that are biodegradable, with low toxicity to non-target organisms, and with low probability of generating resistance against them (Govindarajan *et al.,* 2013; Liu *et al*., 2013; Benelli, 2015; Madhiyazhagan *et al*., 2015; Arena *et al*., 2018). The alternatives include the use of secondary plant metabolites also called phytochemicals (Regnault-Roger *et al*., 2012; Pavela, 2015).

Phytochemicals derived from various plants have been found to be potent mosquito larvicides, insect growth regulators, repellents, and oviposition deterrents, hence they can play an important role in the interruption of disease transmission (Isman, 2006; Regnault-Roger *et al*., 2012). The advantages of phytochemicals include their eco-safety, and lack of development of resistance against them so far (Govindarajan, 2009; Regnault-Roger *et al*., 2012). Individual or combinations of phytochemicals with insecticidal properties have been tried for their efficacy on mosquito vectors (Pavela *et al*., 2019).

Essential oils (EOs), extracted by steam distillation (Bekele, 2018), are volatile oils that give distinctive scent or flavour to more than 17500 aromatic plant species (Regnault-Roger *et al*., 2012). Several studies have reported that EOs from some plants possess ovicidal, larvicidal, pupicidal, and adulticidal (Chowdhury *et al*., 2008; Hakil *et al*., 2017), repellent, antifeedant, and oviposition deterrent effects against many mosquito vectors of human diseases (Isman, 2006; Nerio *et al*., 2010; Govindarajan *et al*., 2013; Pavela 2015).

1.2 Statement of the Problem

Malaria is a parasitic disease and a leading cause of morbidity and mortality in many countries globally (WHO, 2013). Several countries, whose burden of disease is disproportionate, were reported to have had an increase in malaria cases in the year 2017 which was a setback compared to the year before (WHO, 2018), hence malaria is a truly re-emerging parasitic disease (Koella *et al*., 2009). The development of multidrug resistant *Plasmodium falciparum* to artemisinin combined therapy (ACT), the drug of choice for treatment of severe and uncomplicated *falciparum* malaria (Ashley *et al*., 2014; WHO, 2014; Blasco *et al*., 2017), leading to treatment failure with several ACTs, is both alarming and provoking (WHO, 2018).

An. gambiae s.s., the major Afrotropical vector predominantly associated with the most virulent malaria parasite *P. falciparum,* has developed insecticide resistance to commonly used pyrethroids, organochlorines and organophosphates (Himeidan *et al*., 2013; Bekele, 2018). Insecticide resistance notwithstanding, the detrimental effects of conventional insecticides to the environment is a course of concern. This has led to concerted efforts in the search for ecofriendly alternatives including plant-derived products such as EOs, which have been found to possess biocidal activities against

many pests and disease vectors including mosquitoes (Uragaya *et al*., 2015; WHO, 2018).

The efficacy of crude EOs of *A. afra, O. kilimandscharicum* and *T. minuta* and blends of their major compounds against all stages of *An. gambiae* s.s. has not been documented. The current study, therefore, sought to determine the biocidal efficacy of crude EOs of *A. afra, O. kilimandscharicum* and *T. minuta,* and blends of their major constituents on larvae, ova, pupae and the adult stages of *An. gambiae* s.s.

1.3 Justification of the Study

The continuing importance of malaria in many developing countries is undeniably disturbing, as does the spread of multiple resistances of *P. falciparum* to ACTs, and of *An. gambiae* s.s. to all classes of conventional insecticides (WHO, 2018). There is no single method of malaria control has been found effective enough to control the high transmission intensities in SSA (WHO, 2018). Even the most efficacious and the most widely used intervention, the pyrethroid treated bed nets (PTN), is not sufficiently accessed and utilized, besides their insecticide resistance driven loss of efficacy (Hemingway *et al*., 2006). It is obvious that in spite of control programmes in many countries, the situation has shown little improvement (Kweka *et al*., 2010; Sanei-Dehkordi *et al*., 2016), with setbacks reported in some countries (WHO, 2018).

Many researchers have advocated for plant-based alternatives because the likelihood that increasing resistance would reduce the efficacy of chemical-based interventions could not be ignored, their environmental degradation effects notwithstanding (Sanei-Dehkordi *et al*., 2016). Among botanical insecticides developed, EOs have been considered the most suitable options (Dias and Moraes, 2014).

The biocidal efficacy of crude EOs of *A. afra, O. kilimandscharicum* and *T. minuta* and blends of their major compounds had not been evaluated against larvae, ova, pupae and adults of *An. gambiae* s.s. as done in this study. In view of the fact that a comprehensive approach that includes vector control measures is required to conquer malaria, and considering documented evidence on studies done using the three plants, besides the multistage-target approach used against *An. gambiae* s.s. in the current study was thus justified.

1.4 Objectives of the Study

1.4.1 Broad Objective

The main objective of this study was to evaluate the insecticidal activities of EOs of *A. afra* Jacq., *O. kilimandscharicum* Baker ex Guerke and *T. minuta* and their blends against the malaria vector, *An. gambiae* s.s.

1.4.2 Specific Objectives

1) To determine the efficacy of crude EOs of *A. afra, O. kilimandscharicum* and *T. minuta* against third instar larvae of *An. gambiae* s.s.
2) To evaluate the efficacy of blends of compounds present in EOs of *A. afra, O. kilimandscharicum* and *T. minuta* against third instar larvae of *An. gambiae* s.s. under laboratory and simulated field conditions.
3) To determine the ovicidal and pupicidal efficacy of blends of compounds present in EOs of *A. afra, O. kilimandscharicum* and *T. minuta* against ova and pupae of *An. gambiae* s.s. under laboratory conditions.
4) To assess the oviposition deterrent and repellent activities of blends of compounds present in EOs of *A. afra, O. kilimandscharicum* and *T. minuta* against adult females of *An. gambiae* s.s.

1.5 Hypotheses

The hypotheses of this study were:

$H_0 1$: There is no significant difference in the larvicidal activities of crude EOs of *A. afra, O. kilimandscharicum* and *T. minuta* against third instar larvae of *An. gambiae* s.s.

$H_0 2$: There is no significant difference in the efficacy of different blends of compounds contained in EOs of *A. afra, O. kilimandscharicum* and *T. minuta* against third instar larvae of *An. gambiae* s.s. under laboratory and simulated field conditions.

$H_0 3$: The blends of compounds contained in EOs of *A. afra, O. kilimandscharicum* and *T. minuta* neither possess ovicidal nor pupicidal activities against ova and pupae of *An. gambiae* s.s.

$H_0 4$: The blends of compounds contained in EOs of *A. afra, O. kilimandscharicum* and *T. minuta* neither exhibit oviposition deterrent nor repellent activities against adult females of *An. gambiae* s.s.

1.6 Scope of the Study

The current study tested the efficacy of crude EOs extracted from *T. minuta*, *O. kilimandscharicum* and *A. afra* and blends of their major compounds with biocidal activity against third instar larvae of *An. gambiae* s.s. under laboratory and simulated field conditions. In a bioassay guided approach, the study also tested the efficacy of the blends against other aquatic mosquito stages; ova and pupae of *An. gambiae* s.s. Furthermore, repellency of the blends from EOs against blood-starved female *An. gambiae* s.s mosquitoes and oviposition deterrence to their gravid counterparts, were tested in this study.

1.7 Significance of the Study

The outcome of this study outlines the existence of ecofriendly interventions that can be made use of in targeting the malaria vector at all of its developmental stages. Such interventions are practically simple to apply, readily available, and equally effective and can be used in complementing already existing interventions, hence can be utilized at village levels. The success of this work also contributes to the body of knowledge, and can be useful for further research in related areas. This study, therefore, provides another possible tool that can be of focus in the concerted efforts to combat this deadly yet preventable and curable disease, malaria, as the world gears towards its elimination and possible eradication in the foreseeable future.

CHAPTER TWO

LITERATURE REVIEW

2.1 The Malaria Situation

The World Health Organization reported that an estimated 228 million malaria cases occurred worldwide in the year 2018 (WHO, 2019), an increase from the 219 million cases estimated by the same organization for the year 2017 (WHO, 2018). Africa still had the largest burden of malaria morbidity, with 213 million cases, accounting for 93% of the total infections in 2018 (WHO, 2019). Globally, 272000 malaria deaths were estimated to be in children aged below five years, 85% of which occurred in Africa and India (WHO, 2019). Kenya is among the countries reported to have registered an increase of more than 50,000 malaria cases (WHO, 2017).

Malaria is the most important parasitic disease caused by *Plasmodium* parasites transmitted through infective bites by female Anopheles mosquitoes (Himeidan *et al*., 2013; Bekele, 2018). Many approaches have been developed to control malaria (Sanei-Dehkordi *et al*., 2016) but despite intensive efforts, the disease incidence continues to proliferate and cause enormous mortality and debilitation rates (Cuthbert *et al*., 2019) with huge economic losses (Afolabi *et al*., 2018) in the resource-limited settings of Africa, Asia, Latin America, and beyond (Karunamoorthi *et al*., 2014).

2.2 The Malaria Vectors

There are about 490 known species of *Anopheles* mosquitoes with approximately sixty to seventy species capable of transmitting malaria in various parts of the world and about thirty species being vectors of major importance (Becker *et al*., 2003; WHO, 2013; Derua *et al*., 2016). Most of the important malaria vectors belong to sibling (cryptic) species complexes whose members are isomorphic with different

behavioural and ecological characteristics (Rizzo *et al*., 2011; WHO, 2013). The major malaria vectors in Africa belong to the *An. gambiae* complex and *An. funestus* group, which are by far the most studied mosquito species in the world given the great importance of the disease in the continent (Williams and Pinto, 2014).

In the sub Saharan Africa (SSA) region, transmission of *Plasmodium falciparum,* which is the deadliest malaria parasite, is primarily done by *An. gambiae* s.s. (Rizzo *et al*., 2011; WHO, 2013). It's a member of a species complex comprising of seven morphologically indistinguishable sibling species (Williams and Pinto, 2014; Derua *et al*., 2016; Foster and Walker, 2019) with varying levels of range overlap and reproductive isolation (Bernadini *et al*., 2019). Other members of the complex include *An. arabiensis, An. bwambae, An. melas, An. merus, An. quadriannulatus* A and B (Williams and Pinto, 2014). *An. gambiae* s.s. has a very high vectorial capacity and is considered one of the most efficient vectors of human malaria in the world because of its anthropophilic, endophilic, and endophagic behaviours (Reddy *et al*., 2011; Pates and Curtis, 2015).

2.1.1 Biology of the Malaria Vectors

Mosquitoes undergo a holometabolous lifecycle with four distinct stages: egg, larva, pupa and adult, completed in two (aquatic and terrestrial) different environments (WHO, 2013; Foster and Walker, 2019). The duration of various developmental stages depends on temperature and nutritional factors, with development being more rapid at higher temperatures (WHO, 2013). Female *Anopheles* mosquitoes typically feed on sugar, naturally found in nectar and honeydew, to obtain enough energy for sexual maturation and for the flight necessary for dispersal, mating, and finding a vertebrate blood-meal (Becker *et al*., 2003; Foster and Walker, 2019).

Female *Anopheles* mosquitoes normally mate once in their lifetime and mating usually occurs soon after adult emergence (WHO, 2013). Under normal circumstances the first blood-meal is taken after mating but can also be taken by young virgin females (WHO, 2013). Since female *Anopheles* mosquitoes are anautogenous, a blood-meal is required for egg development by stimulating a cascade of hormonal communication from the brain to the ovaries (Foster and Walker, 2019). The blood-meal is normally taken two to three days before ovipositing the eggs (WHO, 2013). Depending on species, one or two blood-meals are required to develop the first batch of eggs while only one blood-meal is required for development of successive batches of eggs (Becker *et al*., 2003; WHO, 2013).

During each oviposition, a batch of about 100 to 150 ova are laid on the water surface of the species-preferred habitat (WHO, 2013), based on availability of specific requisite structural and chemical properties (Foster and Walker, 2019). The average lifespan of female *Anopheles* mosquitoes is three to four weeks under the most favourable tropical conditions, a period within which most lay one to three batches of eggs, though some may lay up to seven batches (WHO, 2013; Williams and Pinto, 2014).

When laid, the eggs are white in colour but darken as the chorion tans (Foster and Walker, 2019). The embryonic development is completed faster in typical tropical and subtropical environments than in cold temperate ones (Foster and Walker, 2019). Under conducive conditions, the eggs hatch into first instar larvae after one to two days and generally float below and parallel to the water surface, from where they breathe air and feed by filtering food particles from water (WHO, 2013). There are four larval instars, which resemble one another save for the size (Foster and Walker,

2019), with each lasting about two days except the fourth instar which lasts one to two more days before pupation at normal tropical water temperatures, but take longer at lower temperatures (WHO, 2013). The larvae lack a respiratory siphon with the spiracles borne on a short spiracular plate (Williams and Pinto, 2014; Foster and Walker, 2019).

The pupa is a non-feeding, comma-shaped transitional stage with two body parts; a cephalothorax and abdomen. (Foster and Walker, 2019). A major transformation in the lifecycle of mosquitoes occurs during the pupal stage, from aquatic life to a flying adult (Williams and Pinto, 2014). This developmental stage lasts for two to three days after which the pupal case breaks allowing the adult to emerge. Immediately after emergence, the rests temporarily on the water surface before flying away (WHO, 2013). The lifecycle of *Anopheles* mosquitoes is shown in Figure 2.1.

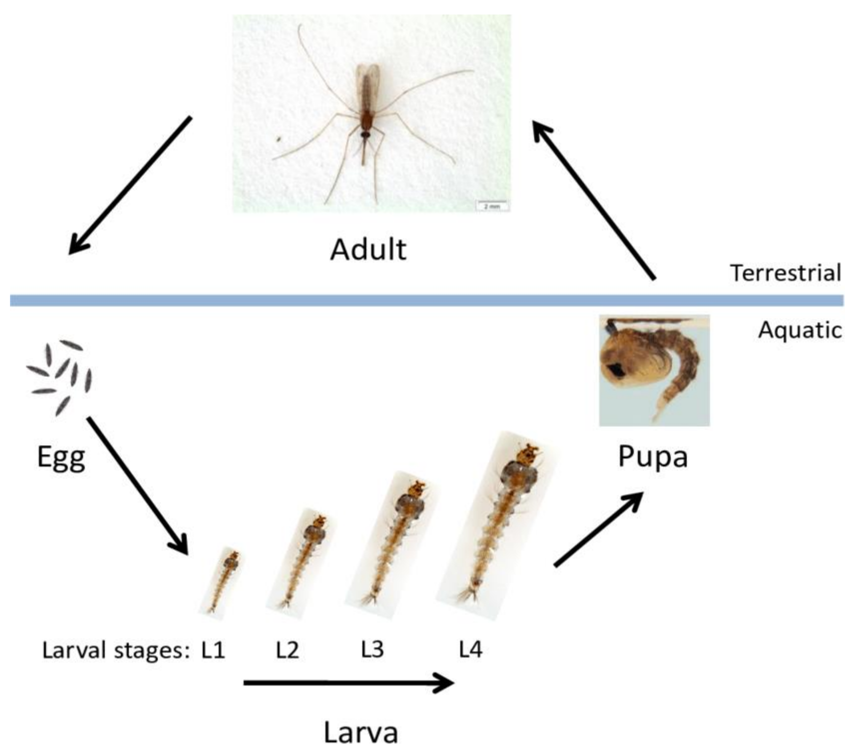


Figure 2. 1 Lifecycle of *Anopheles* mosquito species

(Source: Williams and Pinto, 2014)

2.1.2 Current Control Strategies of the Malaria Vectors

There are four overlapping aims in malaria vector control, which include: preventing mosquito bites, keeping mosquito populations at acceptable densities, minimizing mosquito-human contact, and reducing the longevity of female mosquitoes (Enayati *et al*., 2009; Foster and Walker, 2019). These effectively reduce the vectorial capacity of the mosquitoes (WHO, 2013). The main focus in malaria vector control is on adult female *Anopheles* mosquitoes because they are the only sex that take a blood-meal, hence of public concern (Bekele, 2018). However, the adult mosquito is very mobile and easily avoids intervention measures. This has led to an increased interest in targeting juvenile stages which include the egg, larval and pupal stages (Sukmar, *et al*., 1991; Kovendan *et al*., 2012; White *et al*., 2017).

Chemical control is still considered the most effective current vector control intervention and entails the extensive use of long-lasting insecticide treated bed nets (LLINs) and indoor residual spraying (IRS) (WHO, 2018). The chemicals used include pyrethroids, organophosphates and organochlorines, which have been successful in reducing the malaria disease burden (Becker *et al*., 2003; Mdoe *et al*., 2014; Sanei-Dehkordi *et al*., 2016). Out of the four classes of conventional insecticides, pyrethroids are the only ones recommended by WHO for use in LLINs (Kisinza *et al*., 2017; Zoh *et al*., 2018).

The extensive use of synthetic insecticides has resulted to the development of insecticide resistance by the vector, has multifarious detrimental effects to non-target organisms, and fostered environmental degradation on a global scale, bioaccumulation and non-biodegradability (Matasyoh *et al*., 2008; Misire *et al*., 2011; Ghosh *et al*., 2012; Maduka, 2018). The ever-changing climatic conditions and

complex social structures (Cuthbert *et al.*, 2019), and the development of multiple resistances by mosquito vector species to synthetic insecticides of different classes currently in use (Uragaya *et al.*, 2015; WHO, 2018), pose a bigger challenge in combating the disease (Lynd *et al.*, 2018; Okia *et al.*, 2018; Pavela *et al.*, 2019). The WHO (2018) reported that insecticide resistance existed in 68 of the 80 endemic countries with three countries reporting resistance for the first time, and the detected resistance to each class being; 82% for organochlorines, 82% for pyrethroids, 63% for carbamates, and 50% for organophosphates.

An. gambiae s.s., the major Afrotropical vector predominantly associated with the most virulent malaria parasite, *P. falciparum,* was found to have developed widespread and strong resistance to organochlorines and pyrethroids, and moderate level of resistance to organophosphates (Camara *et al.*, 2018; Zoh *et al.*, 2018). Additionally, Lynd *et al.*, (2018), and Okia *et al.*, (2018) detected a high frequency of phenotypic resistance to two pyrethroids; deltamethrin and permethrin, and an organochlorine, dichlorodiphenyltrichroethane (DDT) in *An. gambiae* s.s. Okia *et al.* (2018), also reported that a point mutation, L1014S, was responsible for insecticide resistance in *An. gambiae* s.s. This rapid evolution of insecticide resistance phenotypes was attributed to a selective pressure with multiple origins, the most significant being exposure to synthetic insecticides used in vector control (Camara *et al.*, 2018).

The spread and rise of insecticide resistance have jeopardized the efficacy of these tools (Mdoe *et al.*, 2014), necessitating search for alternative measures. The integrated mosquito management (IMM) emphasizes the application of eco-friendly alternatives, a central focus of mosquito control in lieu of the chemical insecticides (Ghosh *et al.*,

2012). Increasingly, vector control efforts targeting larval stages have been highly prioritized as larvae are relatively less mobile compared to adult mosquitoes (Misire *et al.*, 2011; Mdoe *et al.*, 2014; Vignesh *et al.*, 2016). Adulticiding, on the other hand, only reduces the population temporarily (Vignesh *et al.*, 2016).

There is interest in the control of *An. gambiae* s.s. due to its role as the predominant vector of the deadly *P. falciparum*, which causes severe human malaria in the SSA region (Robert *et al.*, 2003). The emergence of insecticide resistant phenotypes in *An. gambiae* s.s. continues to pressure the scientific community to develop new and improved vector control strategies (Lynd *et al.*, 2018). Consequently, the most efficacious approach of minimizing the incidence of malaria is to control and eradicate mosquito vectors mainly by application of insecticides to larval habitats. Use of phytochemicals as larvicides has the advantage of being harmless to beneficial non-target organisms and environment when compared to synthetic ones (Sanei-Dehkordi *et al.*, 2016). The synthetic insecticides do not only affect non-target organisms but also constantly increase resistance to the insecticides by the vector (Kelly-Hope *et al.*, 2009; Madhiyazhagan *et al.*, 2015; Pates and Curtis, 2015).

Due to development of insecticide resistance, use of a combination of approaches is a good way to ensure the beneficial qualities of the various tools implemented (Uragaya *et al.*, 2015; Maduka, 2018). EOs are one such weapon for exploration (Sathantriphop *et al.*, 2014). Development of natural repellent formulations with long-lasting protection is necessary to be comparable to the effectiveness of the standard topical repellent DEET (Sathantriphop *et al.*, 2014).

In order to interfere with breeding in the wild, *An. gambie* s.s. mosquitoes can be genetically engineered to express endonucleases that recognize and cut DNA

sequences exclusively present on the X-chromosome (Bernadini *et al*., 2019). When the enzymes are restricted to spermatozoa development, X-carrying spermatozoa are targeted and a strong bias towards males is induced in the progeny. From their study, Bernadini *et al*. (2019) observed a 98% male sex bias such that when transgenic males of *An. gambiae* were crossed with 50 blood-fed females of *An. arabiensis*, only 1 female among them laid eggs that hatched into larvae.

2.1.3 Taxonomy of *An. gambiae* s.s.

Kingdom: Animalia
Phylum: Arthropoda
Class: Insecta
Order: Diptera
Superfamily: Culicoidea
Family: Culicidae
Subfamily: Anophelinae
Genus: *Anopheles*
Subgenus*: Cellia*
Series: Pyretophorus
Species: *Anopheles gambiae* complex
Subspecies: *An. gambiae* sensu stricto (s.s.) Giles (Foster and Walker, 2019)

2.2 Insecticidal Potential of Plant-derived Products

Prior to the discovery of modern insecticides, plant extracts containing pyrethrin and nicotine were widely used as insecticides (Rattan, 2010). The infinite ability of plants to synthesize aromatic products, especially so as secondary metabolites, is well documented. Some of these substances have exhibited immense potential as biocidal agents (Senthilkumar *et al*., 2012). Insecticidal activities of many plants against several insect pests have been demonstrated (Barakat, 2011; Bachrough *et al*., 2015; Bekele, 2018).

An analysis done to assess plant species whose products had been used against various mosquito vectors revealed that 429 plant species from 101 botanical families have exhibited potential biocidal activity against a variety of insects in a number of studies (Pavela *et al.*, 2019). According to the aforementioned authors, most of the plant extracts tested on mosquitoes belonged to a few families namely Asteraceae (49 species), Lamiaceae (41 species), Fabaceae (40 species), and Rutaceae (18 species). Some extracts have exhibited oviposition deterrence, repellency, attractiveness and being stimulants to other mosquito species (Kweka *et al.*, 2010).

There are quite a number of plants commonly used against insect pests in traditional and modern agricultural practices. Various plant products are widely used in developing countries, especially so in the tropics and subtropics, to control different types of pests (Isman, 2006). Several investigations have established that bioactive compounds are borne in different parts of the same plant albeit in different quantities, which accounts for dissimilar lethality of different parts of the same plant. The composition of bioactive compounds of plants of the same species have also been found to vary due to geographical and seasonal factors (Huang *et al.*, 2018).

Apart from pyrethroid insecticides, which have gained international and commercial acclaim due to their high effectiveness and broad-spectrum insecticidal activity in the past few decades, very few insecticides have been developed from natural products (Huang *et al.*, 2018). The harmful effects of plant extracts or pure compounds on insects can be manifested in several ways including toxicity, mortality, antifeedant, growth inhibition, suppression of reproductive behaviour, defense barriers, growth regulating and development modifying properties, and reduction of fecundity and fertility (Barakat, 2011, Senthilkumar *et al.*, 2012). Based on investigations done in

different parts of the world, many plants have been reported to possess broad-spectrum insecticidal activity with some studies revealing the mode of action of selected plant products based on structure-activity relationships (SAR) (Dias and Moraes, 2014).

2.2.1 Taxonomy of the three candidate plants

The classification of the three candidate plants, whose EOs were used in this study, is shown in Table 2.1.

Table 2.1: Classification of *A. afra, O. kilimandascharicum*, and *T. minuta*

Taxonomic unit	*A. afra*	*O. kilimanscharicum*	*T. minuta*
Domain	Eukaryota	Eukaryota	Eukaryota
Kingdom	Plantae	Plantae	Plantae
Subkingdom	Viridaeplantae	Viridaeplantae	Viridaeplantae
Division	Tracheophyta	Tracheophyta	Tracheophyta
Subdivision	Euphyllphytina	Euphyllphytina	Euphyllphytina
Infradivision	Radiatopses	Radiatopses	Radiatopses
Class	Magnoiopsida	Magnoiopsida	Magnoiopsida
Subclass	Asteridae	Asteridae	Asteridae
Superorder	Asteranae	Asteranae	Asteranae
Order	Asterales	Lamiales	Asterales
Family	Asteraceae	Lamiaceae	Asteraceae
Genus	*Artemisia*	*Ocimum*	*Tagetes*
Specific epithet	*aAfra* Jacq.	*kilimandscharicum* Baker ex Guerke	*minuta L.*
Botanical name	*Artemisia afra*	*Ocimum kilimandscharicum*	*Tagetes minuta*

(**Source: Interagency Taxonomic Information System (ITIS), 2018**)

2.2.2 *Artemisia afra*

The genus *Artemisia* is a member of the Asteraceae family and has hundreds of species, cultivars, and hybrids (Amidon *et al*., 2014). *Artemisia afra* (African wormwood) is an erect, clump-forming, perennial shrub which grows up to 2 metres tall (Patil *et al*., 2011). *A. afra* is the only species of *Artemisia* that is truly indigenous

to Africa, where it grows in thick, bushy clumps (Abad *et al.*, 2012). It has alternate oval-shaped leaves which are soft and finely divided, almost fern-like, with dark green adaxial and pale abaxial surfaces, respectively (Amidon *et al.*, 2014). Their stems are covered with small white hairs, which give the shrub a characteristic overall silver-grey colour. *A. afra* tolerates poor soils, but is susceptible to root rot in wet soils (Abad *et al.*, 2012). *A. afra* is one of the oldest and best-known medicinal plants and is still used effectively today in South Africa by people of all cultures (Patil *et al.*, 2011; Abad *et al.*, 2012; Amidon *et al.*, 2014). Extensive research on the phytochemistry of *Artemisia* species has revealed the presence of many compounds (Pirbalouti *et al.*, 2013; Mohiuddin *et al.*, 2018).

Studies have shown that the plant has been traditionally used to treat diabetes, cardiovascular disease, cancer and a number of respiratory diseases (Patil *et al.*, 2011), as an antihelminthic remedy against gastrointestinal nematodes (Molefe, *et al.*, 2012, Mohiuddin *et al.*, 2018), and as antidepressant as well as neuroprotective medicine (Bachrouch *et al.*, 2015). Methanolic extracts from *A. afra* have reportedly been found to exhibit antifungal activity, inhibiting growth of *Alternaria alternata,* a fungal pathogen of *Amaranthus hybridus* (Kena and Lepheana, 2016). Besides medicinal importance, *A. afra* has been used in culinary as herbal tea and brandy, as a fragrance in lotions and EOs, and as natural insecticidal spray and moth repellent agent (Amidon *et al.*, 2014).

2.2.3 *Ocimum kilimandscharicum*

O. kilimandscharicum, on the other hand, besides being considered a sacred and holy herb in India, has been reported to possess broad-spectrum bioactivity (Selvarani *et al.*, 2016). Studies from various parts of the world have shown that *O.*

kilimanscharicum has a high concentration of camphor, which has been reported to induce low mortalities in mosquitoes (Selvarani *et al*., 2016). The effect of camphor is attributed to toxicity of several terpenoids representing a range of functional groups including pulegone (ketone), linalool (alcohol), and 1,8-cieole (ether) (Kweka *et al*., 2008a). It has been documented that leaves of *O. kilimadscharicum* possess a number of compounds with a predominance of monoterpenes, with most studies reporting some of the major constituents as being camphor, linalool, and 1,8-cineole (Selvarani *et al*., 2016). EOs of *O. kilimandscharicum* have been found to possess larvicidal activity against *Cx. quinquefasciatus* with a median lethal concentration of 323.6 ppm (Runyoro *et al*., 2010). In the same study, 1000 µg of *O. kilimandscharicum* were found to cause an inhibition zone of 21.0 mm against *Candida albicans*.

2.2.4 *Tagetes minuta*

T. minuta is an aromatic annual herb naturalized in mild temperate zones (Wanzala and Ogoma, 2013). The plant has various uses ranging from in food and beverages, in perfumery, in cultural practices, in insecticides, to medicinal decoctions (Wanzala and Ogoma, 2013). *T. minuta* is an erect widespread herbaceous plant found in places ranging from temperate to tropical regions, with a long history of use as an insecticidal agent (Wanzala and Ogoma, 2013). Members of the genus *Tagetes* are rich in thiophenes which have been found to be potential sources of larvicidal extracts (Pavela *et al*., 2019).

Studies have shown that the main components of *T. minuta* EO vary according to location, growth stage, the part of the plant harvested, and prevailing environmental conditions (Arena *et al*., 2018). However, compounds that have been widely reported

albeit in different concentrations include dihydrotagetone, *cis*-ocimenone, *trans*-tagetone and *trans-β*-ocimene (Arena *et al*., 2018).

2.2.5 Phytochemicals in Plant EOs

Phytochemicals can be grouped into three main chemical classes: alkaloids, phenolics and terpenes (Rattan, 2010). Alkaloids exhibit insecticidal activity at low concentrations and their mode of action varies but many affect acetylcholine receptors in the nervous system (Sukumar *et al*., 1991). Alkaloids are not volatile and may be used as repellents by burning the plant material (Rattan, 2010). Phenolic compounds possess a hydroxyl (-OH) group attached to a benzene ring or to other complex aromatic ring structures. Phenolics negatively influence insect growth such as the hydrolysable tannins of oak (Rattan, 2010).

Terpenes (terpenoids) are the largest group of natural products from plants comprising EOs, flavourings, fragrances, and lipid-soluble plant pigments (Rattan, 2010). They are hydrophobic and comprise of 5-carbon isoprene units such as C_5 hemiterpenes, C_{10} monoterpenes, C_{15} sesquiterpenes, C_{20} diterpenes, C_{25} sesterpenes, C_{30} triterpenes, C_{40} tetraterpenes, and C_{50} and over polyterpenes Rattan, 2010).

Based on previous studies, plant derived chemicals have been found to possess remarkable insecticidal properties, hence touted as potentially useful weapons that can be employed in the control programmes of the malaria vector (Sukumar *et al*., 1991; Nerio *et al*., 2010). Generally, plant EOs or their products are mostly nontoxic to vertebrates with the exception of a few purified terpenoids which are moderately toxic to mammals (Govindarajan *et al*., 2016).

A study done by Vignesh *et al*. (2016) on volatile metabolites of wild-growing *Mentha spicata*, *M. longifolia*, *M. suaveolens*, *Melissa officinalis*, *Salvia fruticose*, *S. pomifera calycina* and *S. pomifera pomifera* from Greece, demonstrate the presence of 1,8-cineole and piperitenone oxide among the plants. Larvicidal activity of *Glycosmis pentaphylla* attributed to caryophyllene oxides, terpinene and beta pinene compounds (Vignesh *et al*., 2016). Caryophyllene present in EOs of different plants possesses significant insecticidal activities against different species of mosquitoes (Vignesh *et al*., 2016). For instance, β-caryophyllene and α-pinene have also shown high toxicity as well as demonstrated a strong repellent effect against the cotton aphid, *Aphis gossypii* (Barakat, 2011). Gamma terpinene from EOs of *Blumea martiniana* possesses potent larvicidal activity against *Ae. anthropophagus*, *Ae. aegypti* and *Ae. albopictus* (Vignesh *et al*., 2016). High sesquiterpene content of EOs may be responsible for larvicidal activity (Vignesh *et al*., 2016).

Thujone whose molecular formula is $C_{10}H_{16}O$ and structurally has two diastereomers namely *α*- and *β*-thujone. α-Thujone, a monoterpenoid, is generally considered to be the principal active ingredient of wormwood oil and the toxic component in absinthe (Hold *et al*, 2000). The percentage composition of *β*-thujone has often been found to exceed that of *α*-thujone. However, the content depends on the source of the plant. Generally, the *β*-diastereomer has lower toxicity than the *α*-diastereomer (Hold *et al*, 2000). Medically, α-thujone is an active ingredient in some other herbal medicines and is reported to have antinociceptive, insecticidal, and antihelminthic activities and according to Hold *et al*. (2000), records of use of wormwood extracts to control gastrointestinal worms date back to ancient Egyptian times.

α- Terpineol is another active monoterpenoid compound found in *Artemisia* species, with attributable insecticidal activity (Abad *et al*., 2012). Liu *et al*., (2013), identified the compound as one of the repellent and insecticidal constituents of *A. rupestris,* in which it accounted for 10% composition.

2.3 Insecticidal activity of Plant Essential Oils

EOs are complex natural mixtures of volatile, lipophilic and odiferous substances from the secondary metabolism of plants (Pandey *et al*., 2014). They are mainly composed of monoterpenes, sesquiterpenes and their oxygenated derivatives (alcohols, aldehydes, esters, ketones, phenols and oxides) (Da Silva *et al*., 2012). EOs contain about 20-60 components at different concentrations and are characterized by two or three major components at fairly high concentration (20-70%) as compared to other components present in trace amounts (Pandey *et al*., 2014). EOs are obtained by steam distillation and have historically been utilized in the food industry as flavours, in the perfumery industry as fragrances, as herbal medicine and in aromatherapy (Da Silva *et al*., 2012).

Generally, EOs are complex mixtures of various chemical groups comprised of biogenetically related phenols, monoterpenes, and sesquiterpenes (Pandey *et al*., 2014). Examples include 1, 8-cineole, the major constituent of EOs from rosemary (*Rosmarinus officinale*) and eucalyptus (*Eucalyptus globus*); eugenol from clove oil (*Syzygium aromaticum*); thymol from garden thyme (*Thymus vulgaris*); and menthol from various species of mint (*Mentha* species) (Govindarajan *et al*., 2013).

Even though numerous source plants have traditionally been used for protection of stored products, renewed interest in EOs emerged with the demonstration of their insect repellency (Lee, 2018), contact and fumigant toxicities to a wide range of pests

as well as their possession of antifeedant and harmful effects on the reproductive systems of insects (Liu *et al*., 2013). EOs have a broad spectrum of insecticidal activity due to presence of several modes of action, one of the features that delay development of resistance against them (Zoubiri and Baaliouamer, 2014).

The EOs are rich in bioactive compounds that are biodegradable into nontoxic products and are potentially suitable for use in IVM programmes (Gleiser and Zygadlo, 2007; Aba-Toumnou *et al*., 2016; Vignesh *et al*., 2016). The high volatility of EOs and their constituents is an indicator that the residue problem is minimal when applied in the field (Pavela, 2015). However, both natural enemies and pollinators are vulnerable to poisoning by broad-spectrum insecticide products based on EOs (Vignesh *et al*., 2016). Therefore, despite the susceptibility of natural enemies via direct contact, there is no likelihood of residual contact poisoning of parasitoids and predators reinvading a treated crop one or more days after treatment as is the case with conventional insecticides (Gleiser and Zygadlo, 2007; Barakat, 2011).

EOs of *Juniperus macropoda* and *Pimpinella anisum*, have been reported to possess larvicidal, adulticidal, ovicidal, repellent and oviposition deterrent activities against *Cx. quinquefasciatus*, *Ae. aegypti* and *An. stephensi* (Vignesh *et al*., 2016). Their lipophilic nature interferes with the basic metabolic, biochemical, physiological and behavioural functions of insects (Vignesh *et al*., 2016). Bioinsecticidal control of postharvest insects, using indigenous plants has been studied extensively and developed commercially as alternatives to chemical control (Aba-Toumnou *et al*., 2016). Vignesh *et al*., (2016) reported that the rapid action of EOs against some pests was due to neurotoxicity and attributed it to interference with the neuromodulator, octopamine, by some EOs, and with GABA-gated chloride channels by others.

Biological activity of EOs is related to chemical composition and bioactivity of EOs may be attributed to their active and inactive compounds (Pandey, 2014). Inactive compounds may influence resorption, rate of reaction and bioavailability of the active compounds, hence their interaction may be synergistic or antagonistic in the activity of the tested oil (Regnault-Roger *et al.*, 2012; Pandey, 2014). The composition of the EOs is determined by a number of factors which include agroclimatic conditions, stage of maturity, adaptive metabolism of source plants, distillation conditions and the part of the plant used (Mohiuddin *et al.*, 2018).

2.4 Oviposition Site Selection

Reproductive success, as evident in many organisms, strongly depends on a number of characteristics found on a specific breeding site, which vary in regard to space and time (Himeidan *et al*., 2013). The spatial distribution, and by extension the population dynamics, of a given species is strongly influenced by its oviposition site selection behaviour (Wong *et al*, 2011). The attractiveness of an oviposition site to the malaria vector is dependent upon a number of physical and chemical factors (Kweka *et al.*, 2010). Many aspects of mosquito behaviour, including host and oviposition are mediated by volatile semiochemicals (Himeidan *et al.*, 2013). *An. gambiae* mosquitoes actively select habitats for enhancing survivorship and development of their immature stages (Wong *et al*, 2011).

Gravid mosquitoes use chemosensory (olfactory, gustatory, or both) cues to select oviposition sites (Afify and Galizia, 2015; Asmare *et al.*, 2017). Natural cues include plant infusions, microbes, mosquito immature stages, and predators. Odour-based oviposition site selection preference is one of the potential mechanisms regulating the differential oviposition (Asmare *et al.*, 2017). Attractant and stimulant cues signify

availability of food and suitable conditions, while repellents and deterrents indicate the risk of predation, infection by pathogens, or strong competition (Afify and Galizia, 2015). The oviposition process requires complex integration of physical and chemical cues by gravid mosquitoes (Himeidan *et al*., 2013) to locate and evaluate suitable sites (Rattan, 2010), and their preference for conspecific-conditioned water attributed to semiochemicals produced by larval associated bacteria which act as attractants to help females locate cryptic sites (Wong *et al*., 2011).

2.5 Mosquito Repellents

Repellents are substances that discourage arthropods from landing or biting human skin (WHO, 2009). Specific scents are detected by insects via odorant receptors (ORx), which form complexes with corereceptors (Orco) that act as ion channels (Lee, 2018). An open odorant binds to an ORx, the Orco ion opens, ultimately activating a sensory neuron that detects the odour (Lee, 2018). A number of synthetic chemicals have been tested for their repellent activity against mosquitoes but the prohibitive retail cost of proprietary formulations such as DEET restricts their usage (Pitasawat, 2003; Amerasan *et al*., 2012). Natural products are preferred because of their affordability and benign effects to the environment. (Nerio *et al*., 2010; Amerasan *et al*., 2012).

Seyoum *et al*. (2002), studied the repellency of live potted plants against *An. gambiae* from human baits in semi field experimental huts. *Ocimum americanum* (Lamiaceae), *Lantana camara* (Verbenaceae), and *Lippia uckambensis* (Verbenaceae) repelled at an average of 39.7%, 32.4%, and 33.0% of the mosquitoes, respectively (Seyoum *et al*. (2002). The authors also reported that a combination of *O. americanum* with either *L. camara* or *L. uckambensis* repelled 31.6 % and 45.2 % of mosquitoes, respectively.

The ethanol extract of *Curcuma aeruginosa*, *C. aromatica*, and *C. xanthorrhiza* have also been tested for repellent activity against *Ae. togoi*, *Armigeres subalbatus*, *Cx. quinquefasciatus*, and *Cx. tritaeniorhynchus* (Pitasawat *et al*., 2003).

Choochote *et al*., (2006) reported that the crude seed extract of celery, *Apium graveolens* showed repellency against *Ae. aegypti* adult females with ED_{50} and ED_{95} values of 2.03 and 28.12 mg/cm^2, respectively. The authors further reported that the crude extract also provided a biting protection time of 3 hours at a concentration of

25%, and its ethanol extract did not induce dermal irritation. Kayedi *et al*. (2014) evaluated the repellent activity of EOs of *Satureja khuzestanica*, *Myrtus communis*, *Lavendula officinalis*, and *Salvia Sclarea* against *An. stephensi* and found that the EOs exhibited mosquito repellency in a concentration depended response. From the foregoing study, it was found that the EOs of *M. communis*, *L. officinalis*, and *S. Sclarea* offered protection against mosquito bites for up to 5 hours, and compared to DEET.

Repellent properties of EOs extracted by steam distillation from flowers of *L. camara* and of their different fractions obtained by solvent partition and through chromatographic methods, were evaluated by Dua *et al.* (2003) against Aedes mosquito species. The authors (Dua *et al.,* 2003) found the complete protection time (CPT) conferred by a chloroform eluted fraction from a silica gel column to be 3.45 hours. From the same study, the above authors reported that one application of the same fraction recorded 100% protection against bites by *Aedes* mosquito species for a duration of two hours and registered 75.8% protection after seven hours.

Closely related to the insecticidal agents, and sometimes used in combination with insecticides in pest management strategies, are some classes of insecticides with interesting and peculiar biological activities (Gaddaguti *et al*., 2016). It has also been found that sometimes, a given insecticide may act as an insecticide or as a repellent depending on the concentration and according to Gaddaguti *et al*., (2016), the major difference between the two is that a repellent does not kill insects but keeps them away by exuding pungent vapour or exhibits slightly toxic effects. By these activities a repellent prevents insects from perching or landing on the surfaces of targets, thus prevent and control the outbreak malaria (Pitasawat *et al*., 2003). The use of plant materials as insect repellents is increasingly receiving attention, particularly in the developing countries (Kweka *et al*., 2010). Subsequently, some studies on repellent plants against mosquitoes have led to the isolation and characterization of some active components (Gaddaguti *et al*., 2016).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Research Design

This study employed the experimental research design and followed the post-test only assessment. Two groups of various mosquito stages (larvae, ova, pupae, and gravid and blood-starved adult females) were randomly selected as the experimental and control groups, with the latter comprising of positive and negative controls except for oviposition deterrent tests where only the negative control was included in the study. The positive controls were subjected to known insecticides, BiLarv^{TM} 25 WP for larvicidal, ovicidal and pupicidal tests, and DEET for repellency tests, while the negative controls were not subjected to any treatment. The independent variables were the treatment doses ranging from 25 ppm to 200 ppm to which the experimental groups were subjected with each test done in triplicates and each setup repeated five times while the depended variables were similar conditions under which the experimental and control groups were evaluated.

3.2 Collection and Processing of Plant Materials

3.2.1 Harvesting and Identification of Plant Materials

Fresh aerial parts of full-bloom *T. minuta, O. kilimandscharicum,* and *A. afra,* were collected early in the morning before sunrise from the confines of the University of Eldoret (UoE) grounds. Samples of the plant materials were deposited at the UoE's herbarium for identification and issuance of voucher numbers. The three plants were identified as *Artemisia afra*, VN: MU/0107/87, *Ocimum kilimandscharicum*, VN: MU/0033/89, and *Tagetes minuta*, voucher number (VN): MU/0003/87. The images of *A. afra, O. kilimandscharicum*, and *T. minuta* are shown in Plate 3.1, Plate 3.2 and

Plate 3.3, respectively. The collected plant materials were taken to the laboratory for processing. The fresh aerial parts of each plant were separately chopped into small pieces.

Plate 3.1: Picture showing aerial parts of *A. afra*

(Source: Author, 2018)

Plate 3.2: Picture showing aerial parts of *O. kilimadscharicum*

(Source: Author, 2018)

Plate 3.3: Picture showing aerial parts of *T. minuta*

(Source: Author, 2018)

3.2.2 Extraction of Essential Oils by Steam Distillation

EOs of each plant species were extracted separately by steam distillation using the method described by Pavia *et al*., (1982), and Baser and Buchbauer, (2010). For each plant species, 250 grams of chopped fresh plant material were stuffed separately into a 500 mL conical flask which was tightly corked with a two-delivery tubes fitted cork, one connected to 1000 mL round bottomed flask containing boiling water (source of steam), and the other connected to a din and stack connected to a Liebig condenser as shown in Appendix I (a) and (b).

Steam from the boiling water in the round bottomed flask, heated using a Bunsen burner, was passed through the plant material in order to obtain the vaporizing EOs. During the distillation process, a continuous stream of cold water was run through the condenser to liquefy the hot vaporized EOs. The EOs obtained were dried using anhydrous calcium chloride and stored in tightly corked glass vials at 4^0C before further use. The colours and odours of the EOs were described and their percentage yield determined.

3.3 Chemical Analysis of Essential Oils by GC–MS

3.3.1 Sample Preparation

A stock solution of 1mg/ml of each sample was prepared and to it 1µl (100pg/µl) of internal standard, 1-heptene was added followed by 50 mg of Na_2SO_4 (drying agent). The essential oils samples were spun in a vortex for one minute before being extracted by ultrasonication for ten minutes. The sonication bath used was a Branson 2510 model made in Danbury, Connecticut (CT), United States of America (USA). The samples were then centrifuged at 13,000 revolutions per minute (rpm) for five

minutes at an ambient temperature of 5° C. The supernatant of each sample was filtered by separately passing each through a glass wool. The residue was reconstituted by toluene to obtain 1 mL solution, which was shaken for three minutes before a 1 μL aliquot was injected into the gas chromatograph for chemical analysis. Three replicates of the chemical analysis were done.

3.3.2 Chemical Analysis by Gas Chromatography-Mass Spectrometry (GC-MS)

The compounds present in the essential oils were detected and profiled using a GC-MS machine set in full scan mode. The GC-MS machine used for the analysis of the EOs comprised of a 7890A gas chromatograph (GC) model from Agilent Technologies, Incorporated (Inc.), made in Santa Clara, California (CA), USA. The GC was linked to a 5975C mass selective detector model from Agilent Technologies, Inc. Santa Clara, CA, USA.

The GC-MS analysis of the EOs was don under the following conditions: the inlet temperature set at 270° C, the transfer line temperature set at 280° C, and the column oven temperature that was programmed to run from 40° C to 285° C. The initial temperature was maintained for five minutes after which the temperature was gradually increased by 10° C per minute up to 280° C, at which it was maintained for ten and a half minutes. The temperature was then finally raised at 5° C per minute to 285° C, at which it was maintained for thirty one point nine minutes.

The column used in the analysis of the EOs was a low bleed HP-5MS capillary column fitted to the GC. The column length was 30 m, its internal diameter was 0.25 mm, and had a thickness of 0.25 μm (J & W, Folsom, CA, USA). The carrier gas was helium which flew at a rate of 1.25mL per minute. For the mass selective detector, an ion source temperature was maintained at 230° C with a quadruple temperature of

180° C. Mass spectra of the electron impact (EI) were obtained at an acceleration energy of 70 eV. The autosampler used to aliquot 1.0 µL of the EO that was injected in the split/splitless mode, was a 7683 model from Agilent Technologies Inc., made in Beijing, China. The analysis of fragment ions was done over a 40-550 m/z mass range held in full scan mode, with the filament delay set at five minutes.

The identification of compounds was done by comparing the fragmentation pattern and retention time of the gas chromatographs obtained with those of authentic standards. When corresponding reference compounds were not found, chemical structures were proposed based on their general fragmentation and in relation to spectra published by library-MS databases: National Institute of Standards and Technology (NIST). The peak area of the internal standard, 1-heptene was used for quantification.

3.4 Laboratory Rearing of *An. gambiae* s.s.

An. gambiae s.s. Kisumu strain were obtained from the insectary at the CGHR CDC-KEMRI, Kisumu. Three-day old female mosquitoes were provided with a blood meal from a laboratory rabbit that was sheared on the back. Twenty fully engorged blood fed females were put into rearing cages measuring 30 cm x 30 cm x 30 cm. 30 mL plastic cups containing oviposition substrate were provided in the cages. Laid eggs were transferred to 500 mL white plastic trays half-filled with spring water.

Freshly hatched larvae were fed on a sprinkle of larval food and the ration was gradually increased as the larvae grew bigger. The rearing water was exchanged after every 48 hours to prevent fouling and larval mortalities. Pupae (up to 100) were transferred to 100 mL plastic cups containing rearing water and put into the rearing cages for adult emergence.

For colony maintenance, adult males and females were kept together and provided with ad libitum 10% sucrose solution, while females were provided with blood meal from a sheared rabbit following the method described by Amerasan *et al*., (2012). All mosquito stages used in the experiments in this study were obtained from the reared colony, which was maintained at a temperature of 27±2°C, relative humidity (RH) of 75±5%, and a 12L:12D (Light: Dark) photoperiod. The experiments were done in a room enclosed with a mosquito netting material to prevent escape of adult mosquitoes to the surroundings where they could cause havoc.

3.5 Bioassays

3.5.1 Larvicidal Bioassays using Crude Essential Oils against Third Instar Larvae of *An. gambiae* s.s.

Bioassays were done following the WHO 2005 standard protocol using crude EOs of *T. minuta, O. kilimandscharicum,* and *A. afra*. Homogenous batches of third instar *An. gambiae* s.s. mosquito larvae were used to determine the activity and biopotency of the crude EOs from *T. minuta*, *O. kilimandscharicum,* and *A. afra*. One percent stock solutions of each of the EOs were prepared following the WHO protocol (WHO, 2005) using acetone as the diluent, from which serial dilutions were done to make eight solutions of each, ranging from 25 ppm to 200 ppm at 25 ppm intervals. A 10-100 µL micropipette was used to aliquot the stock solutions of the compounds to make the ppm solutions.

The setups comprised of the experimental trials, positive and negative controls, respectively. The tests were done in triplicates with five repeats of the same concentration of treatment in volumes of 250 mL solutions contained in 500 mL white plastic trays. Into each tray, a batch of 30 freshly moulted third instar larvae of *An.*

gambiae s.s. was introduced and larval food added. Each positive control contained the recommended 1 g/L concentration of Bi-Larv^{TM} 25% wettable powder (25 WP), in 250 mL solution while the negative control contained 1 mL of acetone added to 249 mL water following the methods of AhbiRami, (2014). Bi-Larv^{TM} 25 WP is a registered trademark product of Bayer Pharmaceutics Company containing 25% diflubenzuron, an insect growth regulator (IGR) as the active ingredient, and is listed under WHO Pre-Qualification (WHO-PQ) scheme. Larval food, which comprised of a mixture of ground dog biscuits and brewer's yeast, was sprinkled on each tray every morning. Mortalities were recorded after every 24 hours up to 48 hours post-treatment.

Mortalities of larvae were determined by probing the larvae using a needle at the cervical region. Nonresponsive and moribund larvae were considered dead and used to calculate percentage mortality (WHO, 2005, Chowdhury *et al,* 2008). Macroscopic morphological changes were observed on dead larvae and developmental durations post-exposure recorded for larvae that withstood treatment. The test and control experiments were done at room temperature with a photoperiod of 12 h light followed by 12 h dark (12L:12D) as stipulated by WHO (2005).

3.5.2 Preliminary Larvicidal Bioassays using Blends of Major Constituents in the EOs of *A. afra*, *O. kilimandscharicum* and *T. minuta* under Laboratory Conditions

Larvicidal bioassays were also done using synthetic blends of some of the major constituents (Aldrich) of EOs based on GC-MS. The blends were constituted in their natural proportions following the method of Bekele and Hassanali (2001), and used in equivalent doses (25 - 200 ppm) as those of the crude EOs. Compounds whose

percentage composition were at least 2%. Twenty three synthetic compounds (six identified from the EO of *A. afra*, seven from the EO of *O. kilimansdscharicum* and ten from the EO of *T. minuta*) were used in formulating different blends. The blends formulated in approximate relative amounts of major constituents (2 – 58%) present in the EOs following the method of Omolo *et al*. (2004), and were as follows:

I. *A. afra* – 1,8-cineole, *cis*-thujone, *trans*-thujone, iso-3-thujanol, neoiso-3-thujanol, and terpinen-4-ol (4:55:10:4:16:3)

II. *O. kilimandscharicum* – 1,8-cineole, beta-(*Z*)-ocimene, beta-(*E*)-ocimene, eugenol, methyl eugenol, (*E*)-caryophyllene, and germacrene D (2:8:5:5:58:4:10)

III. *T. minuta* – (*Z*)-*β*-Ocimene, dihydrotagetone, 3-methyl-2-hexanol, *cis*-thujone, (*Z*)-tagetone, (*E*)-tagetone, 3-thujanol, car-3-en-2-one, (*Z*)-ocimenone, (10:16:3:13:13:7:2:12:15).

Each component was subtracted from the blend in turn to determine its relative contribution to the overall toxicity of the crude EO following the method of Bekele and Hassanali (2001). Thirty third instar larvae of *An. Gambiae* s.s. were exposed to 200 ppm solutions of various blend compositions for 48 hours to eliminate less potent compounds. The test solutions were prepared following WHO (2005) protocol in 250 mL volumes contained in 500 mL white plastic trays. Larval food was added to the treatment solutions every morning. Three most potent blends (one from each EO) were selected and used in subsequent bioassays.

The blend selected from the EO of *A. afra* was coded AA-3 and comprised of 1,8-cineole, *cis*-thujone, *trans*-thujone, iso-3-thujanol, neoiso-3-thujanol, and terpinen-4-ol in the ratio of 4:55:10:3, respectively. The blend selected from the EO of *O.*

kilimandscharicum was coded OK-4 that comprised of 1,8-cineole, eugenol, methyl eugenol, and (*E*)-caryophyllene in the ratio of 2:8:58:4, respectively. The blend selected from the EO of *T. minuta* was coded TM-4 and comprised of (*Z*)-*β*-ocimene, dihydrotagetone, *cis*-thujone, (*Z*)-tagetone, car-3-en-2-one, (*Z*)-ocimenone in the ratio of 10:16:13:13:12:15, respectively.

Different ppm concentrations of AA-3, OK-4 and TM-4 were prepared following WHO protocol and their efficacy against third instar larvae of *An. gambiae* s.s. tested alongside positive (1 g/L Bi-Larv™ 25 WP) and negative (1 mL acetone) controls in 250 mL solutions, under laboratory conditions. Thirty third instar larvae were exposed to each treatment. As done while using crude EOs, macroscopic morphological changes were also observed on dead larvae, and developmental durations post-exposure recorded for larvae that survived treatment.

3.5.3 Larvicidal Bioassays using Blends AA-3, OK-4 and TM-4 under Simulated Field Conditions

Simulated field conditions (SFC) trials were done, following the WHO protocol on pesticide evaluation (WHO, 2005), using blends of compounds that had been selected under laboratory conditions. Sixty 5L basins were placed in rows that were one by one metre apart (Appendix IV). The basins were smeared with clay soil in the inside to near brim and filled with 1000 mL of dechlorinated water, marked and left overnight. In the day that followed, dechlorinated water was filled to mark and left to settle before a batch of fifty laboratory-bred larvae was released into the test solutions. All the larvae of *An. gambiae* s.s. that were subjected to treatments under SFCs were in the early third instar stage. A sprinkle of larval food was added to each treatment

solution. After 3 hours of larval acclimation, the set-ups were treated with selected doses of the test compounds in a randomized manner using micro-pipettes.

The containers were covered with a mosquito netting material to prevent other mosquitoes or other insects from laying eggs on the set up and to protect the water from falling debris. Live larvae were counted after every 24 hours to score post-treatment larval mortality. The water level in the containers was maintained to the mark for 72 hours. The remaining larvae and/or pupae were destroyed after 72 hours and the smeared mud discarded and replaced with fresh mud before the next trial. The test trials were repeated five times. The positive control contained the recommended 1 g/L concentration of Bi-Larv™ 25 WP while the negative control contained 4 mL of acetone in 1000 mL water.

3.5.4 Ovicidal Bioassays using Blends AA-3, OK-4 and TM-4 under Laboratory Conditions

One hundred freshly laid eggs of *An. gambiae* s.s. were used in each setup to determine their hatchability when exposed to different solutions of selected blends at concentrations ranging from 25 ppm to 200 ppm. To synchronize and promote hatching, larval food was added to the treatment solutions and to dechlorinated water 24 hours prior to introducing the eggs (WHO, 2005). The larval food was meant to encourage bacterial growth, hence de-oxygenating the water, consequently triggering egg hatching within 12 hours of hydration. The setups were kept at a temperature maintained at $25\pm2^{\circ}$C and observed after 24 hours post exposure to a maximum of 48 hours. Upon being observed, first instar larvae were removed from the test solutions, counted and their number recorded. The larvae were discarded thereafter. After 48 hours, eggs that had not hatched were considered dead.

3.5.5 Pupicidal Bioassays using Blends AA-3, OK-4 and TM-4 under Laboratory Conditions

Thirty freshly formed pupae were put in treatment solutions (25 – 200 ppm) of selected blends in 100 mL paper cups covered with a mosquito netting material to prevent escape of adults in case they emerged. The number of dead pupae was recorded 24 hours post exposure. Live pupae and emerged adults were summed up and used to calculate the percentage of pupae unaffected by the treatment solutions. The setups were left to stand for 48 hours after which the contents were discarded.

3.5.6 Oviposition Deterrence Bioassays using Blends AA-3, OK-4 and TM-4 against Gravid Female *An. gambiae* s.s.

Five-choice experiments were conducted for treatments and controls using the methods described by Yap *et al*., (1995) and Asmare *et al*. (2017). Twenty 48-hours post-blood meal fed gravid laboratory reared *An. gambiae* s.s were released into cages with five paper cups of 30 mL capacity containing 10 mL of different test and control solutions. Four cups containing equimolar treatment solutions of selected blends from EOs of *T. minuta*, *O. kilimandscharicum* and *A. afra* were placed diagonally opposite in the cage. The fifth cup, which was centrally placed, contained spring water with a drop of acetone (negative control) as shown in Appendix V (a) and (b). Whatman 1 filter papers were carefully folded into a cone shape and placed in an inverted position on the cups with the tip of the cone partially immersed.

The number of eggs in treatment and control cups was counted after 48 hours. To avoid positional bias inside the cages, the cups were shifted one position in a clockwise rotation each day. The oviposition deterrence was determined using the

oviposition activity index (OAI) as described by Kweka *et al*., (2010) and Asmare *et al*., (2017).

Results of oviposition were explicated as the average number of eggs laid per unit in five days following one blood meal and OAI calculated as shown below:

OAI = (T-C)/(T+C); where,

T denotes the number of eggs laid in the test cups, and

C denotes the number of eggs laid in control cups.

Positive index values meant that more ova were oviposited in the test cups than in the negative control (spring water), whereas negative index values meant that more ova were oviposited in negative controls than in test cups. Total eggs laid in both treatment and control cups were tallied daily for five consecutive days, using a camel hair brush. Each experiment had three replicates and was repeated five times.

3.5.7 Repellency Bioassays using Blends AA-3, OK-4 and TM-4 against Blood-starved Female *An. gambiae* s.s.

Serial dilutions of solutions of selected blends of constituents of EOs from *T. minuta*, *O. kilimandscharicum*, and *A. afra*, were made using ethanol as diluent. The solutions were tested to determine the effective repellent dose range as stipulated in the WHO standard protocol for repellency tests (WHO, 2009) and following procedures described in previous studies by Amerasan, *et al*. (2012), and Afolabi *et al*. (2018), with some modifications.

Dosages giving responses between 10% and 90% were used for trials, and comprised of 3 dosages with less than 50% and 3 dosages with more than 50% repellent responses. Each volunteer used incremental dosages of the treatment on the test forearm for five successive applications of increasing dosage. A single test comprised

of continuous use of the same laboratory reared uninfected female *An. gambiae* s.s. mosquitoes by the same volunteer and was completed within a day. Five repeat tests were done using different batches of mosquitoes on different subsequent days using the same volunteers.

Prior to each repellency bioassay, forearms and hands of each volunteer were washed using neutral unscented soap. The washed arms were then rinsed thoroughly with plenty of clean water then allowed to air dry for ten minutes. The test solution was then applied to the washed area as described by Amerasan *et al*., (2012). To begin the test, 1 mL of ethanol diluent was applied evenly using a pipette to cover approximately 600 cm^2 of the left (test) forearm skin between the wrist and elbow and allowed to dry for one minute before testing for repellency.

The forearm was put into the cage and the number of mosquitoes that landed on and/or probed the skin during a thirty-seconds period tallied and recorded as negative control. The arm was held steady during testing to avoid bias in tallying. The forearm was withdrawn and then treated with 1 mL of the lowest dose (100 ppm) of repellent solution and allowed to dry. The treated arm was returned into the cage for another 30-seconds period and observed for mosquito landings and/or probing (Appendix VI). This procedure was repeated for each additional incremental repellent dose of 125, 150, 175, and 200 ppm solutions, respectively.

Five successive tests were carried out one after the other without delay and the repellent dose at each test calculated as the sum of the doses applied to arrive at the cumulative dose for each test. The right forearm was applied with the ethanol diluent and used to verify the negative control tally recorded at the beginning of the experiment. To determine the duration of protection for each blend, the hands treated

with the highest dose of the test solution used in this study (200 ppm) were continuously exposed until at least two mosquitoes landed and bit the treated area. The time between application of the test repellent and the second successive bite was recorded as the complete protection time (CPT) of each treatment solution.

To evaluate the efficacy of treatments used in this study, the volunteers applied 1mL of the recommended 20% DEET in ethanol on the control forearm and the highest dose (200 ppm) of the treatment on the test forearm and counts of each recorded for comparison. All the hands were protected from mosquito bites using rubber gloves. Each cage contained 100 blood-starved uninfected female *An. gambiae* s.s. mosquitoes.

Percentage repellency was determined using the formula:
Percentage repellency = [(Ta - Tb) / Ta] X 100; Where,
Ta = number of mosquitoes in the control group
Tb =number of mosquitoes in the treated group

3.6 Data Analyses

The bioassays performed against *An. gambiae* s.s. during this study consisted of mortality, survival, hatching, oviposition, and landing, which were all considered as response variables against varying concentrations of the crude EOs and their selected blends. All the response variables were binary in nature (died/lived, survived/did not survive, hatched/ did not hatch, oviposited/did not oviposit, landed/did not land) and naturally followed a binomial distribution (Agresti, 1990). Therefore, the relationship between the response variables and factors were modeled using Probit analysis using STASTICA 6.0 statistical software.

Maximum likelihood was used to estimate the regression coefficient (R square) because it gives more precise estimation of necessary parameters for correct evaluation of the results (Finney, 1952). During Probit analysis, all data were transformed to log base 10 to linearize the relationship between the response variables and factors. For each analysis, the response frequency was observed as response variables from a total observation of 30 or 50 larvae, 100 ova, 30 pupae, and 100 females of *An. gambiae* s.s. Meanwhile the concentrations of EOs and selected blends of their constituents were covariate.

The resulting probability outcomes were multiplied by 100 to determine the expected percentage of the response frequency. To test for the significance of the Probit plots, Z statistics was calculated; the larger the Z statistics the larger the differences from the smaller sized Z. Nevertheless, the differences were verified using P-value of ≤ 0.05. The modeled fit was confirmed using chi-square goodness of fit test between the observed response values and predicted probability of response values. The resultant graph plotted consisted of Probits of response variables in Y-axis and $\log_{10}$ concentration in X-axis.

The LC_{50} and LC_{90} were determined by projecting the Y axis for a probit of 5.00 and 9.00 and taking the inverse $\log_{10}(X)$ of the concentration of the crude EOs and their blends. The LC_{50} and LC_{90} were then compared using One Way Analysis of variance (One-Way ANOVA) to test which EO or blend was the most efficacious. Where significant differences existed following ANOVA, means were separated using Duncan’s Multiple Range Test (DMRT). In all analyses, significant differences were declared at $P \leq 0.05$.

3.7 Ethical Approval

Ethical clearance, reference number REC: UEAB/01/6/2018, was granted by the Ethics Committee of the University of Eastern Africa, Baraton (UEAB) after review of the study protocol. A copy of the clearance certificate is shown in Appendix X. Informed written consents, sample shown in Appendix XI, were signed by volunteers before participating in the tests for repellency. Participation was voluntary.

CHAPTER FOUR

RESULTS

4.1. Characteristics and Yield of Extracted Essential Oils

The essential oil (EO) obtained from aerial parts of *A. afra* was straw coloured with a slightly camphoric odour with an average yield of 0.82 ± 0.12 mL/kg wet plant material. The EO obtained from aerial parts of *O. kilimandscharicum* was colourless as shown in Appendix I (b) with a characteristic odour of camphor with an average yield of 0.68 ± 0.15 mL/kg of wet plant material. On the other hand, EO obtained from aerial parts of *T. minuta* was pale yellow with a sharp citrus-like odour with an average yield of 1.65 ± 0.2 mL/kg of wet plant material.

4.2. Results from GC-MS Analysis of Essential Oils

Results from the GC-MS analysis of the EO of *A. afra,* are presented in Table 4.1. A total of 27 compounds, constituting about 97.17% of the compounds present in the EO, were identified. A section of the GC-MS spectrum for compounds present in the EO of *A. afra* is shown in Appendix VII. Some of the major compounds identified in the EO were 1,8-cineole (3.74%), cis-thujone (55.10%), trans-thujone (9.71%), iso-3-thujanol (4.12%), neoiso-3-thujanol (16.36%), and terpinen-4-ol (3.20%).

Table 4. 1: Compounds identified in the EO of *A. afra*

RT	Compound name	Area	Abundance (%)
10.6741	Sabinene	3405394	1.06
10.9608	1-Hexen-3-ol	362325	0.11
11.0719	Myrcene	147596	0.05
11.0945	3-Octanone	544698	0.17
11.2640	δ-3-Carene	107291	0.03
11.5341	α-Terpinene	624674	0.19
11.6862	*p*-Cymene	825199	0.26
11.8032	1,8-Cineole	11985555	3.74
12.3173	γ-Terpinene	1556276	0.49
12.4872	Tricyclene	107291	0.03
12.7862	α-Terpineol	140068	0.04
13.0462	(E)-beta-Ocimene	249577	0.08
13.1487	*cis*-Thujone	176705236	55.10
13.3214	*trans*-Thujone	31150745	9.71
13.4878	Chrysanthenone	1252229	0.39
13.6167	Iso-3-thujanol	13204590	4.12
13.722	*trans*-Sabinol	1671690	0.52
13.8039	Verbenene	691378	0.22
14.1022	Neoiso-3-thujanol	52479210	16.36
14.307	Terpinen-4-ol	10247317	3.20
15.6993	α-Pinene	589416	0.18
16.0854	2-Allyl-4-methylphenol	1831516	0.57
17.2203	α-Cubebene	505672	0.16
17.6883	4-propyl-1,6-heptadien-4-ol	390817	0.12
17.8287	(Z)-Caryophyllene	206419	0.06
18.1855	Ethyl-2-octynoate	462615	0.14
18.2674	α-Humulene	239124	0.07

The total ion chromatogram for compounds present in the EO of *A. afra* is shown in Figure 4.1.

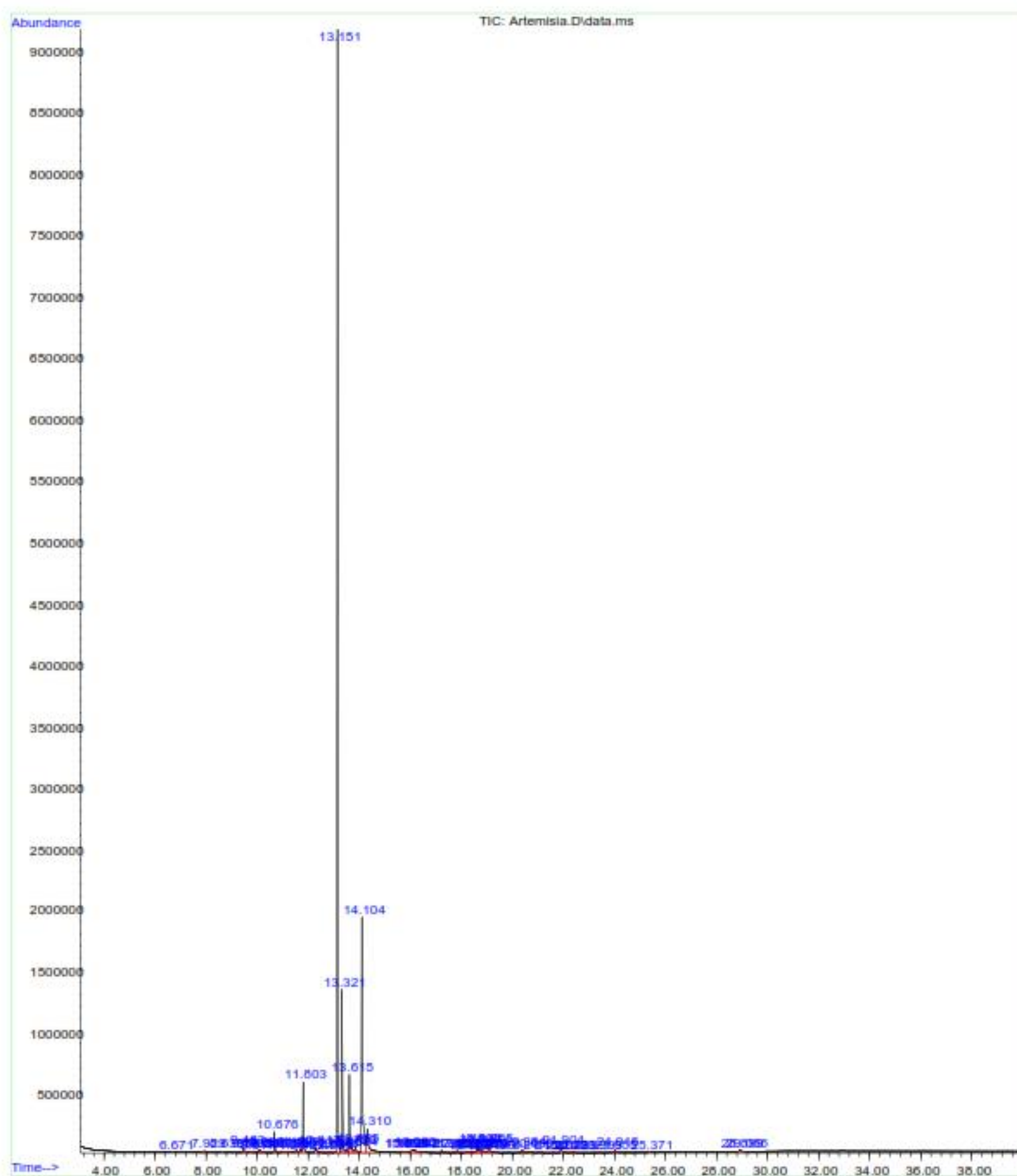


Figure 4. 1: Total ion chromatogram for compounds present in the EO of *A. afra*.

Results from the GC-MS analysis of the EO of *O. kilimandscharicum,* are presented in Table 4.2. A total of 42 compounds, constituting about 99.39% of the compounds present in the EO, were identified. A section of the GC-MS spectrum for compounds present in the EO of *O. kilimandscharicum* is shown in Appendix VIII. The major compounds identified in the EO were 1,8-cineole (1.96%), beta-(*Z*)-ocimene (8.41%), beta-(*E*)-ocimene (5.04%), eugenol (5.25%), methyl eugenol (58.19%), (*E*)-caryophyllene (3.94%), and germacrene D (10.38%).

Table 4. 2: Compounds identified in the EO of *O. kilimandscharicum*

RT	Library/ID	Area	Abundance (%)
11.2913	*α*-Phellandrene	1168438	0.09
11.5285	*δ*-2-Carene	559446	0.04
11.6864	3,5-dimethylene-cyclopentene	422922	0.03
11.7566	Sylvestrene	1645953	0.12
11.7976	1,8-Cineole	26229874	1.96
11.9263	(*Z*)-beta-Ocimene	112723231	8.41
12.1135	(*E*)-beta-Ocimene	67530576	5.04
12.3065	*γ* -Terpinene	2458922	0.18
12.5698	*δ*-3-Carene	278978	0.02
12.5790	*α*-cis Bergamotene	737770	0.06
12.7804	*α*-Terpinene	172428	0.01
13.1197	Linalool	4185033	0.31
13.2991	*β*-thujone	1042475	0.08
13.5085	*Allo*-Ocimene	2076876	0.15
14.3657	Terpinen-4-ol	488496	0.04
14.6407	(*E*)-Anethole	947118	0.07
14.7401	*p*-Mentha-1(7),8-diene	272166	0.02
16.0388	Tridecane	550684	0.04
16.8344	α-Cubebene	537906	0.04
16.9125	Eugenol	70356330	5.25
17.2146	*α*-Copaene	12685278	0.95
17.3492	*β*-Bourbonene	6532677	0.49
17.5481	Methyl eugenol	780201669	58.19
17.8289	(*E*)-*β*-Caryophyllene	52823076	3.94
17.94	Longifolene	3584207	0.27
18.022	*β*-Cubebene	1627376	0.12
18.1565	*cis*-Muurola-3,5-diene	2068565	0.15
18.2618	*α*-Humulene	6101822	0.46
18.3847	*trans*-Muurola-4(14),5-diene	7267906	0.54
18.5133	*γ*-Muurolene	2533640	0.19
18.6128	Germacrene D	139151909	10.38
18.7941	Caryophyllene oxide	4770016	0.36
18.9111	Limonene	1218774	0.09
18.9755	5,8-dimethyl-quinoline	3501996	0.26
19.0808	*δ*-Cadinene	9020130	0.67
19.1861	*α*-Himachala-1,4-diene	835203	0.06
19.2446	Daucene	936914	0.07
19.3616	*α*-Calacorene	598602	0.04
19.8998	Humulene II epoxide	944455	0.07
20.7071	Guaiol	949109	0.07
20.9177	Guaiazulene	361679	0.03
21.1517	Heptadecane	346149	0.03

The total ion chromatogram for compounds present in the EO of *O. kilimandscharicum* is shown in Figure 4.2.

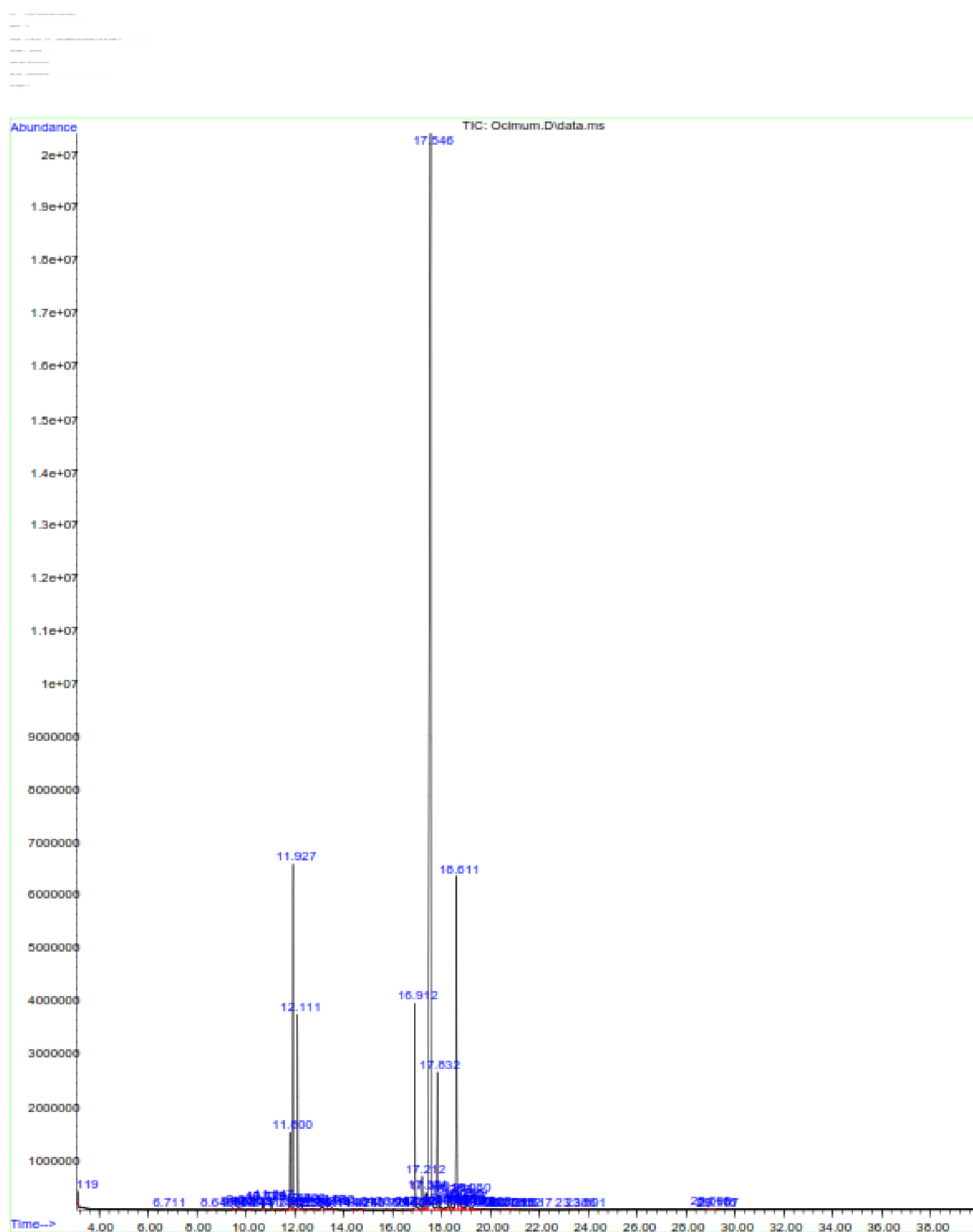


Figure 4. 2: Total ion chromatogram for compounds present in the EO of *O. kilimandscharicum*

Results from the GC-MS analysis of the EO of *T. minuta,* are presented in Table 4.3. A total of 38 compounds, constituting about 99.78% of the compounds present in the EO, were identified. A section of the GC-MS spectrum for compounds present in the EO of *T. minuta* is shown in Appendix IX. Some of the major compounds identified in the EO include: dihydro-tagetone (15.65%), (*Z*)-ocimenone (15.95%), cis-thujone (13.34%), car-3-en-2-one (12.04%), *Z*-tagetone (12.90%), (*Z*)-*β*-ocimene (10.46%).

Table 4. 3: Compounds identified in the EO of *T. minuta*

RT	Library/ID	Area	Abundance (%)
9.7012	Isoamylisobutyrate	81544	0.03
10.6743	3-methyl-2-hexanol	9054767	2.78
11.0545	Myrcene	134002	0.04
11.5252	α-Terpinene	404579	0.12
11.6766	*p*-Cymene	439535	0.14
11.7507	Limonene	1524574	0.47
11.8037	1,8-Cineole	2628154	0.81
11.9145	(*Z*)- *β*-Ocimene	33998481	10.46
12.1053	(*E*)-*β*-Ocimene beta	425287	0.13
12.1953	Dihydrotagetone	50875884	15.65
12.6034	(*E*)-Sesquilavandulol	187367	0.06
13.0463	Lavandulyl isobutanoate	873541	0.27
13.1371	*cis*-Thujone	43382372	13.34
13.3243	*trans*-Thujone	4612803	1.42
13.4942	*allo*-Ocimene	2199467	0.66
13.5759	dihydro-Eugenol acetate	363116	0.11
13.7537	(*Z*)-Tagetone	41937202	12.90
13.9034	(*E*)-Tagetone	23819308	7.33
14.1619	3-Thujanol	6795636	2.09
14.3071	Terpinen-4-ol	1438233	0.44
15.0969	(*Z*)-Ocimenone	51870115	15.95
15.2256	Car-3-en-2-one	39140330	12.04
15.4361	Thymol	281176	0.09
16.0495	Tridecane	626490	0.19
17.2145	*α*-Copaene	158313	0.05
17.6825	Premnaspirodiene	133044	0.04
17.8229	(*E*)-Caryophyllene	146716	0.05
18.2617	Linalool propanoate	835381	0.26
18.3845	*β*-Copaene	431804	0.13
18.5074	Valencene	180545	0.06
18.6017	Germacrene D	132756	0.04
18.6774	*β*-Selinene	1610361	0.50
18.7943	Bicyclogermacrene	390344	0.12
19.0807	*trans*-Cadina-1(6),4-diene	947965	0.29
19.8119	Spathulenol	588786	0.18
20.3677	*δ*-Cadinene	258637	0.08
24.0064	Sandaracopimara-8(14),15-diene	138904	0.04
25.7789	(*E*)-*γ*-Atlantone	1367010	0.42

The total ion chromatogram for compounds present in the EO of *T. minuta* is shown in Figure 4.3.

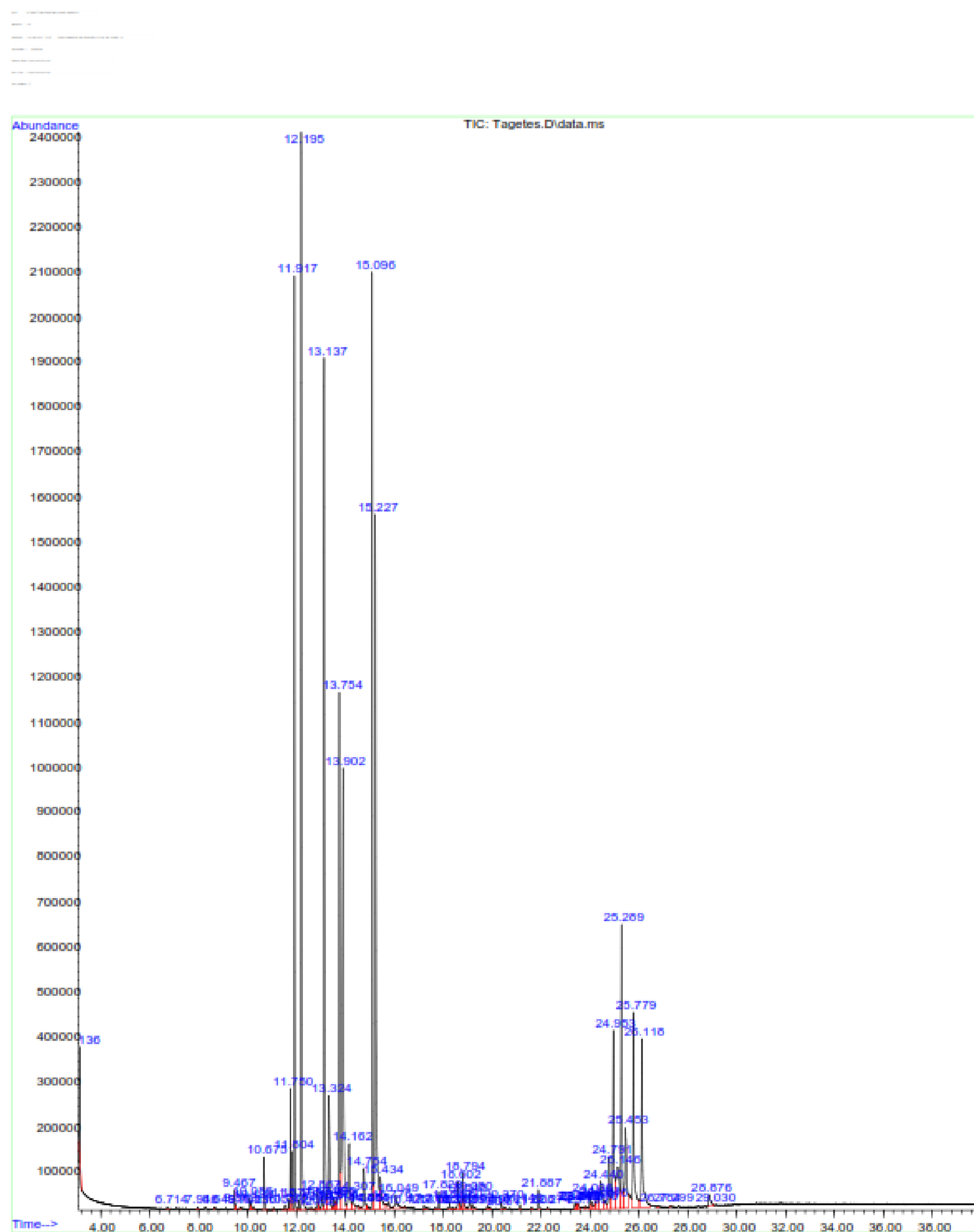


Figure 4. 3: Total ion chromatogram for compounds present in the EO of *T. minuta*

4.3 Larvicidal activity of the Crude Essential Oils against *Anopheles gambiae* s.s.

The larvicidal activities of the crude EOs of *A. afra, O. kilimandscharicum* and *T. minuta* are presented in Figure 4.4. It was observed that the larvae became restless and hyperactive upon exposure to the treatment for the first thirty minutes after which the hyperactivity slowed down and the larvae began to fall towards the bottom of the tray. Larvae in higher dose treatment solutions showed more curling up, restlessness and vigorous body movements. From the range of doses used, larval mortalities after 24- and 48- hours exposure were found to be dose dependent, with the lowest dosage having the lowest mortality while the highest dosage had the highest mortality as shown in Figure 4.4. The EO of *O. kilimandscharicum* was the most effective against the larvae followed by EO of *A. afra* while the EO of *T. minuta* was the least efficacious.

The dosage of crude EOs required to kill 50% (LC_{50}) and 90% (LC_{90}) of the larvae varied with concentration and duration of exposure as shown in Table 4.4. In this study, there were significant ($P < 0.001$) differences in the LC_{50} and LC_{90} of the mosquito larvae subjected to the crude EOs which varied with concentration and duration of exposure. Among the treatments, *O. kilimandscharicum* had the lowest concentration of LC_{50} and LC_{90} values of 48.50 ppm and 91.40 ppm while the highest LC_{50} of 58.80 ppm was recorded in *T. minuta* which was unable to cause 90% larval mortality up to a concentration of 200 ppm after 24 hours of exposure, respectively. However, the positive control had a significantly lower ($P < 0.001$) concentration than the crude EOs tested.

Larvae that survived exhibited body surface deformities including loss of hair and integumental abrasion, and prolonged developmental periods of 2 to 3 days beyond

those in negative controls, with larval-pupal intermediates. The general trend in larvicidal activity observed when the crude EOs were tested against larvae of *An. gambiae* s.s., alongside positive (C(+)) and negative (C(-)) controls, was: C(+) > OK > AA > TM > C(-).

Table 4. 4: LC_{50} and LC_{90} values of mortalities of *An. gambiae* s.s. larvae caused by the crude EOs after 24- and 48- hours exposure periods

Exposure time	Lethal concentration	Positive Control	*O. kilimandscharicum*	*A. afra*	*T. minuta*	F-value	P-value
24 h	LC_{50}	44.10^{a}	48.50^{b}	54.40^{c}	58.80^{d}	2707.1068	<0.001
	LC_{90}	86.70^{a}	91.40^{b}	Na	Na	645.4000	<0.001
48 h	LC_{50}	26.50^{a}	30.90^{b}	36.80^{c}	49.20^{d}	3175.5902	<0.001
	LC_{90}	60.30^{a}	72.20^{b}	92.60^{c}	Na	4738.6286	<0.001

Values with different superscripts across the row are significantly different ($P \leq 0.05$). Na denotes not attained.

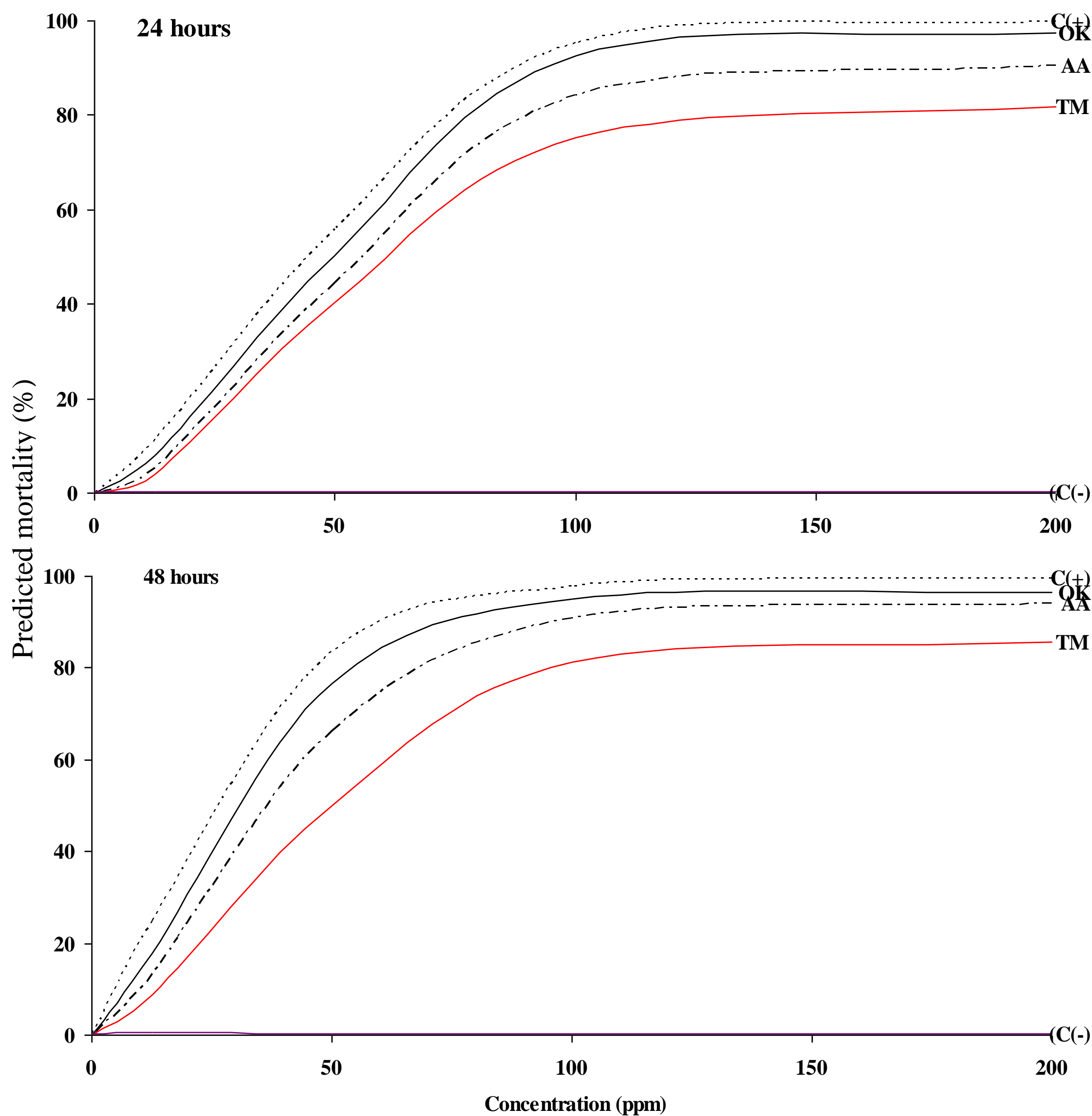


Figure 4. 4: Mortalities of *An. gambiae* s.s. larvae caused by crude EOs of *A. afra, O. kilimandscharicum*, and *T. minuta* after 24- hours and 48- hours of exposure.

Key:

C (+) – Positive Control

C (-) – Negative control

AA- Essential oil of *Artemisia afra*

OK- Essential oil of *Ocimum kilimandscharicum*

TM- Essential oil of *Tagetes minuta*

4.4 Larvicidal activity of Blends AA-3, OK-4 and TM-4 against *Anopheles gambiae* s.s. under Laboratory Conditions

The larvicidal mortality caused by blends AA-3, OK-4 and TM-4 against *An. gambiae* s.s. are presented in Figure 4.5. Based on the laboratory bioassays using selected blends, it was observed that larval mortalities followed a dose-response pattern, with lower mortality recorded at lower exposure doses and higher mortality at higher exposure doses. Among the blends tested, larvicidal efficacy was highest in treatment solutions containing blend OK-4, LC_{50} 59.50 ppm and 37.70 ppm while the least efficacy was observed in blend TM-4 with LC_{50} 69.60 ppm and 53.70 ppm after 24- and 48- hours of exposure, respectively.

The LC_{50} and LC_{90} values obtained when blends AA-3, OK-4 and TM-4 were tested against *An. gambiae* s.s. larvae after 24- and 48-hours of exposure, are presented in Table 4.5. During the experiment it was established that the differences in the LC_{50} and LC_{90} values of the mosquito larvae subjected to blends AA-3, OK-4 and TM-4 were significant ($P < 0.001$). Blend OK-4 had the lowest concentration required to kill 50% and 90% of the *An. gambiae* s.s. larvae, followed by blends AA-3 and TM-4, respectively.

In comparison with the positive control, there were significant differences ($P < 0.001$) between each of the selected blends and the positive control. The overall trend in larvicidal activity observed when the selected blends of constituents in EOs of *A. afra*, *O. kilimandscharicum*, and *T. minuta* were tested against larvae of *An. gambiae* s.s. alongside positive and negative controls was: C (+) > OK-4 > AA-3 > TM-4 > C (-).

Table 4. 5: The LC_{50} and LC_{90} values of larval mortalities of third larval instar of *An. gambiae* s.s. caused by Blends AA-3, OK-4 and TM-4 after 24- and 48- hours exposure periods

	Exposure time			
	24 h		48 h	
Treatments	LC_{50}	LC_{90}	LC_{50}	LC_{90}
Blend AA-3	65.30^{c}	Na	45.00^{c}	98.60^{c}
Blend OK-4	59.50^{b}	93.80^{b}	37.70^{b}	79.80^{b}
Blend TM-4	69.60^{d}	Na	53.70^{d}	Na
Positive control	44.10^{a}	86.70^{a}	26.50^{a}	60.30^{a}
F-value	134.9730	886.0000	973.0800	5124.0000
P-value	<0.001	<0.001	0.0005	<0.001

Values with different superscripts along the column are significantly different ($P \leq 0.05$). Na denotes not attained.

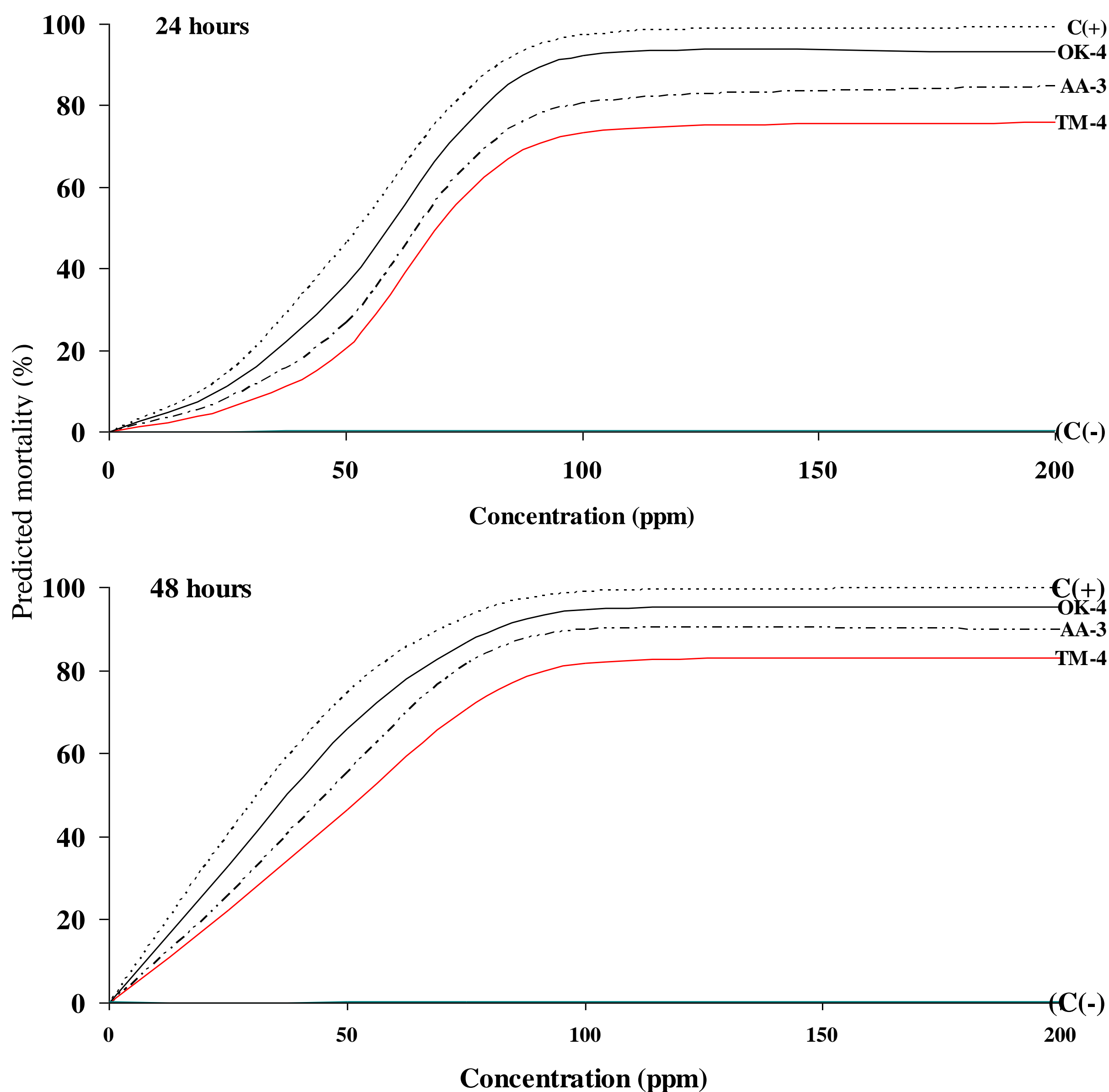


Figure 4. 5: Mortalities of third instar larvae of *An. gambiae* s.s. caused by Blends AA-3, OK-4 and TM-4 after 24- hours and 48- hours of exposure.

Key:

C (+) – Positive Control

C (-) – Negative control

AA-3 - Blend of major constituents in the EO of *A. afra*

OK-4 - Blend of major constituents in the EO of *O. kilimandscharicum*

TM-4 – Blend of major constituents in the EO of *T. minuta*

4.5 Comparison between Larvicidal activity of Crude EOs and their Blends

When a comparison was made between the larvicidal activity of each crude EO and its derived blend, the bioactivity of the crude EO was significantly higher ($P < 0.001$) than their respective selected blends. The LC_{50} values obtained when crude EOs and their respective blends were tested against *An. gambiae* s.s. after 24 and 48 hours are shown in Table 4.6.

Table 4. 6: LC_{50} values of EOs and their blends after 24 and 48 hours

LC_{50} value after:	Essential oil	Blend	F-value	P- value
	A. afra	AA-3		
24 hours	54.40^{a}	65.30^{b}	1526.0500	< 0.001
48 hours	36.80^{a}	45.00^{b}	952.2000	< 0.001
	O. kilimandscharicum	OK-4		
24 hours	48.50^{a}	59.50^{b}	1243.5400	< 0.001
48 hours	30.90^{a}	37.70^{b}	989.1100	< 0.001
	T. minuta	TM-4		
24 hours	58.80^{a}	69.60^{b}	918.0400	< 0.001
48 hours	49.20^{a}	53.70^{b}	764.2800	< 0.001

4.6 Larvicidal Activity of Blends AA-3, OK-4 and TM-4 against third instar larvae of *An. gambiae* s.s. under Simulated Field Conditions

The larvicidal activities of blends AA-3, OK-4 and TM-4 against third instar larvae of *An. gambiae* s.s. under the SFCs are shown in Figure 4.6. The mortality trends observed under SFC experiments were similar to those observed under laboratory conditions and exhibited dose- and time-dependent patterns. However, the mortality rates were significantly higher ($P<0.001$) under SFCs compared to those observed under laboratory conditions. Blend OK-4 had the highest larvicidal activity against *An. gambiae* s.s. larvae under SFCs with LC_{50} values of 40.00 ppm and 29.20 ppm after 24 and 48 hours of exposure, respectively. Blend TM-4 had the lowest larvicidal

activity under SFCs with LC_{50} values of 56.80 ppm and 44.70 ppm after 24 and 48 hours, respectively.

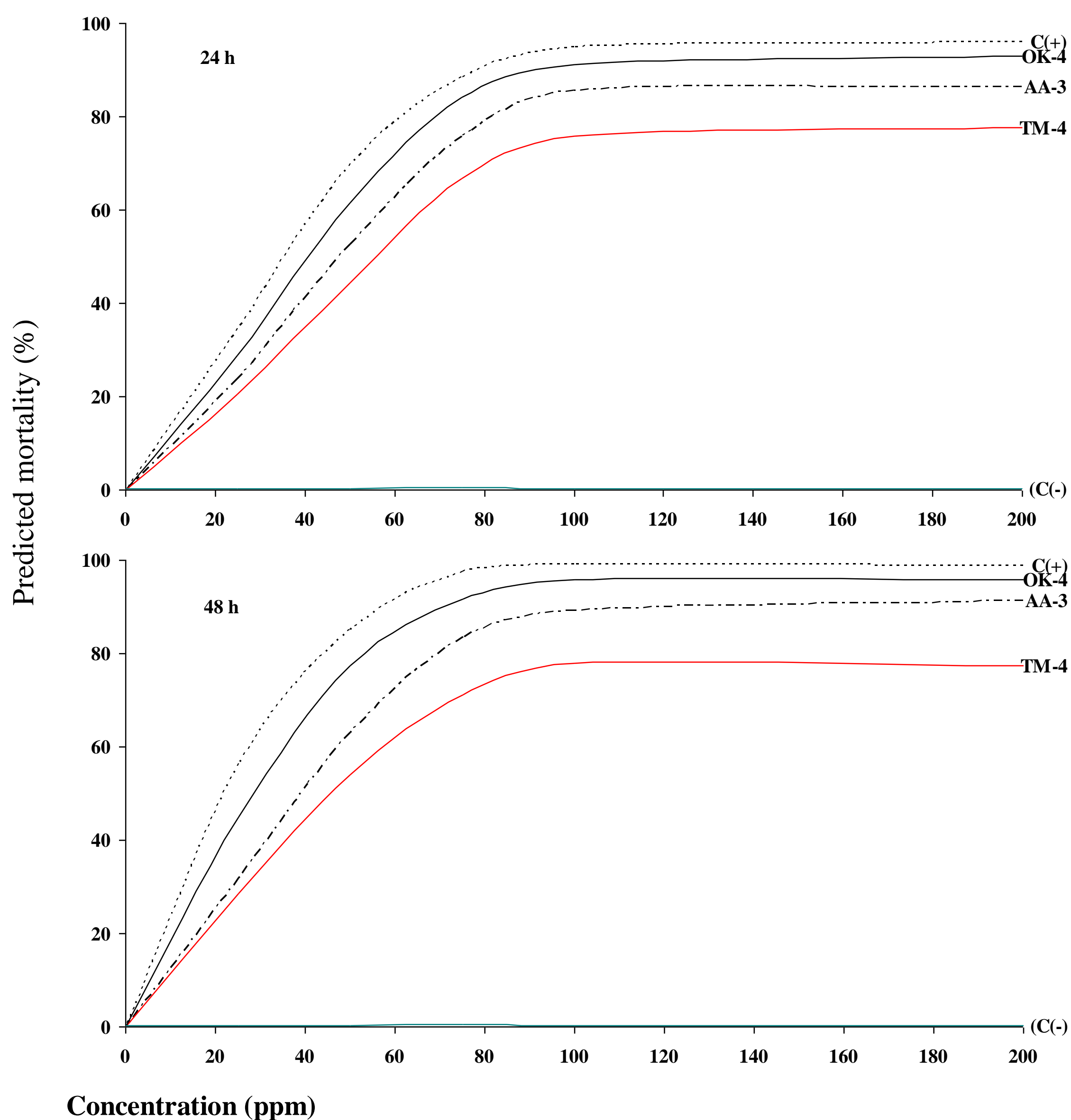
The LC_{50} and LC_{90} values obtained when blends AA-3, OK-4 and TM-4 were tested against *An. gambiae* s.s. larvae after 24- and 48- hours exposure to treatments under SFCs are shown in Table 4.6. There were significant differences ($P < 0.001$) in the LC_{50} and LC_{90} values obtained when *An. gambiae* s.s. larvae were subjected to the selected blends from EOs of *A. afra, O. kilimandscharicum* and *T. minuta*, alongside positive and negative controls, under SFCs. Of the blends tested, blend OK-4 was the most efficacious against *An. gambiae* s.s. larvae, with the least LC_{50} and LC_{90} values, respectively. Only two blends, OK-4 and AA-3, were able to cause up to 90% larval mortalities after 48- hours of exposure while blend TM-4 was unable to cause up to 90% mortality in concentrations of up to 200 ppm in the same exposure duration.

Each of the blends had, however, comparatively lower larvicidal activity than the positive control. The overall trend in larvicidal activity observed when blends AA-3, OK-4 and TM-4 were tested against larvae of *An. gambiae* s.s. alongside the positive and negative controls was: C (+) > blend OK-4 > blend AA-3 > blend TM-4 > C (-).

Table 4. 7: The LC_{50} and LC_{90} values of larval mortalities of third larval instar of *An. gambiae* s.s. caused by Blends AA-3, OK-4 and TM-4 after 24- and 48- hours of exposure under SFCs.

	Exposure time			
	24 h		48 h	
Treatment	LC_{50}	LC_{90}	LC_{50}	LC_{90}
Blend AA-3	48.20^{c}	Na	37.70^{c}	93.80^{c}
Blend OK-4	40.00^{b}	84.90^{b}	28.80^{b}	72.00^{b}
Blend TM-4	56.80^{d}	Na	44.70^{d}	Na
Positive control	35.40^{a}	75.30^{a}	21.50^{a}	58.40^{a}
F-value	686.9039	269.6925	168.7421	400.5494
P-value	<0.001	<0.001	<0.001	<0.001

Values with different superscripts along the column are significantly different ($P \leq 0.05$). Na denotes not attained.



Concentration (ppm)

Figure 4. 6: Mortalities of third instar larvae of *An. gambiae* s.s. caused by Blends AA-3, OK-4 and TM-4 after 24- and 48- hours of under SFCs

Key:

C (+) – Positive Control

C (-) – Negative control

AA-3 - Blend of major constituents in the EO of *A. afra*

OK-4 - Blend of major constituents in the EO of *O. kilimandscharicum*

TM-4 – Blend of major constituents in the EO of *T. minuta*

4.7 Ovicidal activity of Blends AA-3, OK-4 and TM-4 against Ova of *An. gambiae* s.s.

The hatchability of *An. gambiae* s.s. ova after 72 hours exposure to blends AA-3, OK-4 and TM-4 are presented in Figure 4.7. The hatchability of the ova reduced in a dose-response pattern with the ova in blend OK-4 showing the highest reduction in hatching at higher doses with only up to 2% eggs hatching at 200 ppm. This was followed by blend AA-3 in which only up to 6% ova hatched at 200 ppm. Blend TM-4 recorded the highest hatching rate of *An. gambiae* s.s. ova of up to 25% at 200 ppm.

The LC_{50} and LC_{90} values obtained when *An. gambiae* s.s. ova were treated with selected blends from EOs of *A. afra, O. kilimandscharicum* and *T. minuta* after 72 hours of exposure are presented in Table 4.7. The lowest concentration that hindered 50% of ova of *An. gambiae* s.s. from hatching was the test solution treated with blend OK-4 (LC_{50} 17.50 ppm) followed by blend AA-3 (LC_{50} 25.80 ppm) then blend TM-4 (LC_{50} 35.90 ppm). The concentrations that hindered 50% and 90% of ova of *An. gambiae* s.s. from hatching were significantly different ($P < 0.001$) among the selected blends of EOs, and the positive control. The trend in ovicidal activity was: C (+) > Blend OK-4 > Blend AA-3 > Blend TM-4 > C (-).

Table 4. 8: LC_{50} and LC_{90} values for hatchability of *An. gambiae* s.s. ova exposed to blends AA-3, OK-4 and TM-4 for 72 hours

	Positive control	Blend AA-3	Blend OK-4	Blend TM-4	F-value	P-value
LC_{50}	16.40^{a}	25.80^{c}	17.50^{b}	35.90^{d}	299.5032	<0.001
LC_{90}	46.70^{a}	84.20^{c}	63.70^{b}	Na	892.736	<0.001

Values with different superscripts across the row are significantly different ($P \leq 0.05$). Na denotes not attained.

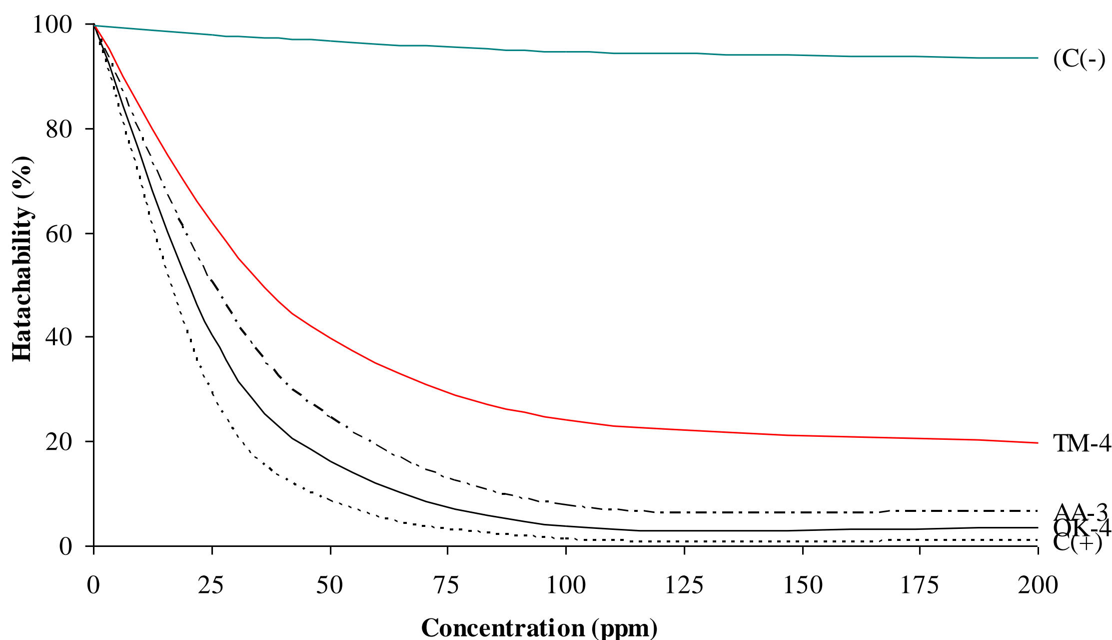


Figure 4. 7: Hatchability of the *An. gambiae* s.s. ova after 72 hours exposure to blends AA-3, OK-4 and TM-4

Key:

C (+) – Positive Control

C (-) – Negative control

AA-3 - Blend of major constituents in the EO of *A. afra*

OK-4 - Blend of major constituents in the EO of *O. kilimandscharicum*

TM-4 – Blend from the EO of *T. minuta*

4.8 Pupicidal activity of Blends AA-3, OK-4 and TM-4 against Pupae of *An. gambiae* s.s.

The pupicidal activities of blends AA-3, OK-4 and TM-4 against pupae of *An. gambiae* s.s. after 24 hours of exposure are presented in Figure 4.8. In each case, the pupicidal activity increased in a dose-response pattern with treatment solution containing Blend OK-4 showing the highest pupal deaths (LC_{50} 69.20 ppm) as well as highest impediment to adult emergence at higher doses, while the least pupicidal

activity was observed in treatment solution containing Blend TM-4 (LC_{50} 123.30 ppm).

The LC_{50} and LC_{90} values obtained when *An. gambiae* s.s. pupae were subjected to treatment solutions containing blends AA-3, OK-4, and TM-4 after 24 hours exposure are presented in Table 4.8. The concentrations that elicited 50% and 90% mortality of *An. gambiae* s.s. pupae were significantly different among the blends ($P < 0.001$). In comparison with the positive and negative controls, there were significant differences ($P < 0.001$) between them. The trend in pupicidal activity observed when the blends AA-3, OK-4 and TM-4 were tested against pupae of *An. gambiae* s.s., alongside the positive and negative controls was: C (+) > OK-4 > AA-3 > TM-4 > C (-).

Table 4. 9: LC_{50} and LC_{90} values of mortalities of *An. gambiae* s.s. pupae caused by Blends AA-3, OK-4 and TM-4 after 24 hours exposure.

	Positive control	Blend OK-4	Blend AA-3	Blend TM-4	F-value	P-value
LC_{50}	60.80^{a}	69.20^{b}	79.20^{c}	123.30^{d}	175.2139	<0.001
LC_{90}	119.20^{a}	132.50^{b}	153.40^{c}	Na	144.2062	0.0002

Values with different superscripts across the row are significantly different ($P \leq 0.05$).
Na denotes not attained.

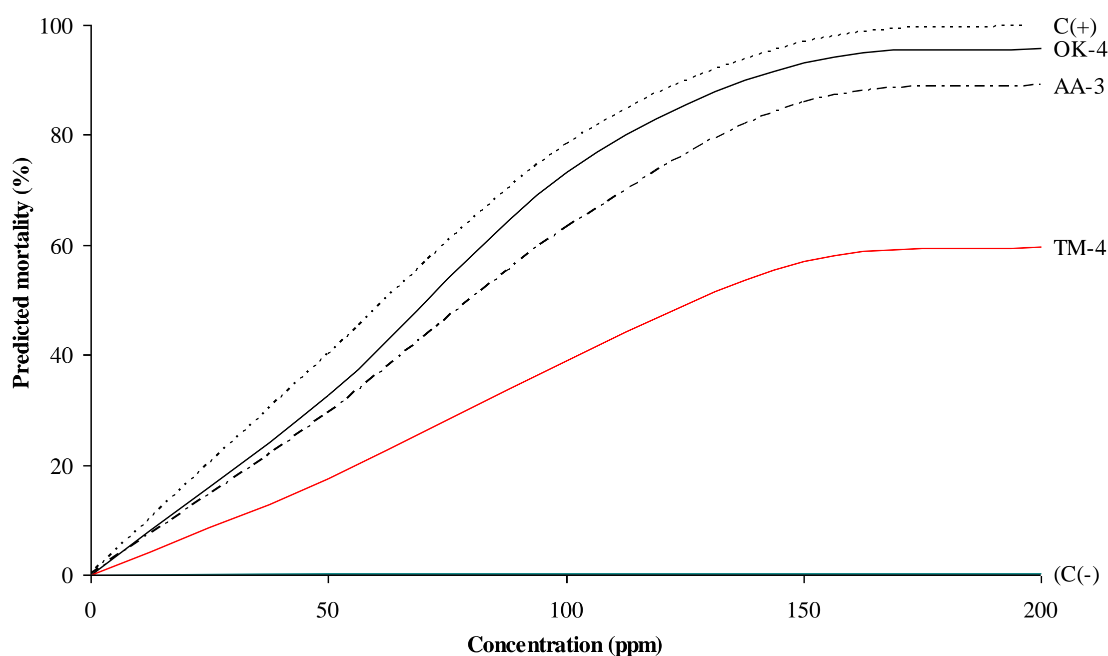


Figure 4. 8: Mortalities of *An. gambiae* s.s. pupae caused by blends AA-3, OK-4 and TM-4 after 24 hours of exposure

Key:

C (+) – Positive Control

C (-) – Negative control

AA-3 - Blend of major constituents in the EO of *A. afra*

OK-4 - Blend of major constituents in the EO of *O. kilimandscharicum*

TM-4 – Blend from the EO of *T. minuta*

4.9 Effects of Blends AA-3, OK-4 and TM-4 on Oviposition Site Selection by Gravid Females of *An. gambiae* s.s.

The effects of blends AA-3, OK-4 and TM-4 on oviposition site selection by gravid females of *An. gambiae* s.s. under laboratory conditions are shown in Figure 4.9. The number of eggs laid after 24- hours by gravid females of *An. gambiae* s.s. reduced with increased doses of treatments.

In the five-choice oviposition assay, the overall oviposition preference hierarchy for the blends from EOs of *A. afra*, *O. kilimandscharicum,* and *T. minuta* was found to be Blend OK-4 < Blend AA-3 < Blend TM-4 < C (-). All the selected blends had negative OAI values of -0.9375, -0.8765 and -0.4305 for blends OK-4, AA-3 and TM-4 at 200 ppm, respectively.

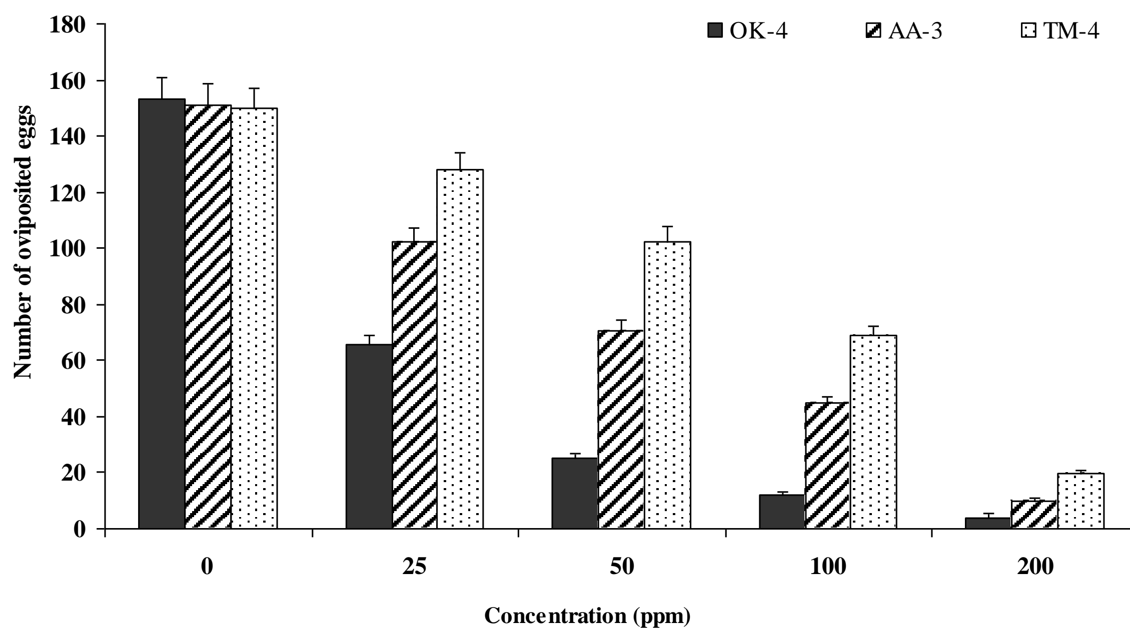


Figure 4. 9: Effects of blends AA-3, OK-4 and TM-4 on oviposition by gravid females of *An. gambiae* s.s

Key:

AA-3 - Blend of major constituents in the EO of *A. afra*

OK-4 - Blend of major constituents in the EO of *O. kilimandscharicum*

TM-4 – Blend from the EO of *T. minuta*

4.10 Repellent activity of Blends AA-3, OK-4 and TM-4 against Blood-starved Females of *An. gambiae* s.s.

The landing success/repellency of blood-starved females of *An. gambiae* s.s. on human hands treated with blends AA-3, OK-4 and TM-4 are shown in Figure 4.10. The landing and probing by female *An. gambiae* s.s. mosquitoes reduced in a dose-dependent pattern with the landing success on hands treated with Blend OK-4 showing the least probing and landings at higher doses (up to 3.2% at 200 ppm) followed by hands treated with blends AA-3 and TM-04 (up to 8.2% and 33% at 200 ppm, respectively), conferring a protection efficiency (PE) of up to 96.8%, 91.8%, and 67%, respectively. The complete protection time (CPT) was found to be 40, 70 and 90 minutes for blends TM-4, AA-3 and OK-4, respectively.

The LC_{50} and LC_{90} values of the landing success of blood-starved females of *An. gambiae* s.s. on human hands treated with blends AA-3, OK-4 and TM-4 are presented in Table 4.9. The concentrations that prevented up to 50% and 90% probing and landings of blood-starved females of *An. gambiae* s.s. were significantly ($P < 0.001$) different among the blends tested.

The treatment solution with the lowest concentration that hindered up to 50% landings of *An. gambiae* s.s. females contained Blend OK-4 (LC_{50} 61.60 ppm), while the least deterrence was observed in treatment solution containing Blend TM-4 (LC_{50} 84.70 ppm). Only one blend, OK-4, deterred at least 90% of blood-starved females of *An. gambiae* s.s. from landing on arms treated with up to 200 ppm, with an LC_{90} values of 127. 30 ppm.

There were significant differences ($P < 0.001$) between the selected blends and the controls. The overall trend in repellent activity observed when the selected blends

were tested against blood-starved females of *An. gambiae* s.s., alongside the positive and negative controls was: C (+) > Blend OK-4 > Blend AA-3 >Blend TM-4 > C (-).

Table 4. 10: LC_{50} and LC_{90} values of repellent activities of blends AA-3, OK-4 and TM-4 against blood-starved females of *An. gambiae* s.s. after 30 seconds exposure

	C (+)	Blend OK-4	Blend AA-3	Blend TM-4	F-value	P-value
LC_{50}	53.90[a]	61.60[b]	69.30[c]	84.70	312.5137	<0.001
LC_{90}	114.20[a]	127.30[b]	Na	Na	201.7425	<0.001

Values with different superscripts across the row are significantly different ($P \leq 0.05$). Na denotes not attained.

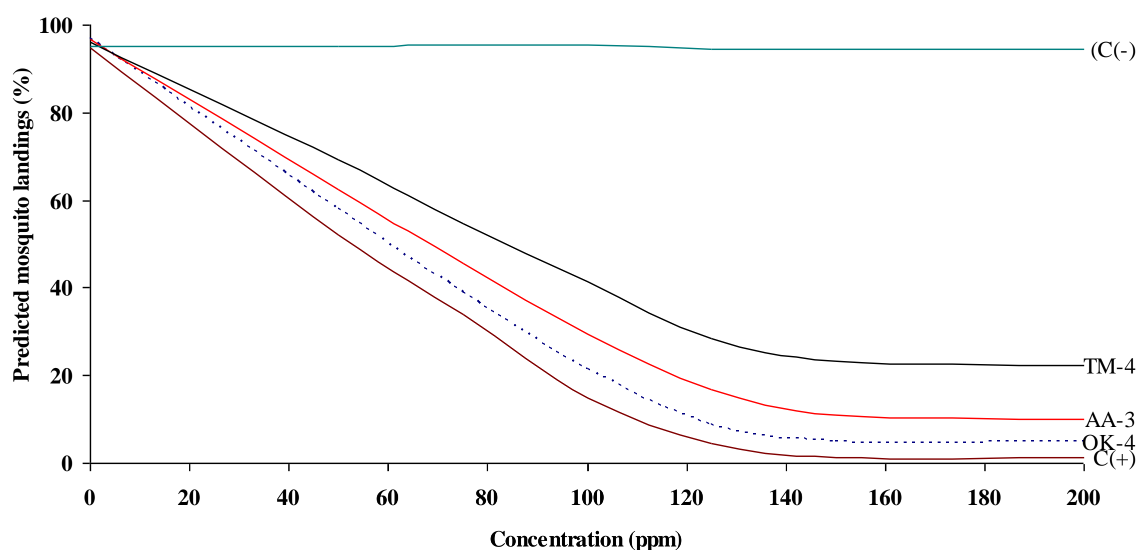


Figure 4. 10: Repellent activities of blends AA-3, OK-4 and TM-4 against blood-starved females of *An. gambiae* s.s. after 30 seconds exposure

Key:

C (+) – Positive Control

C (-) – Negative control

AA-3 - Blend of major constituents in the EO of *A. afra*

OK-4 - Blend of major constituents in the EO of *O. kilimandscharicum*

TM-4 – Blend from the EO of *T. minuta*

CHAPTER FIVE

DISCUSSIONS

The essential oils (EOs) of *A. afra*, *O. kilimandscharicum* and *T. minuta* used in this study were found to possess larvicidal activity against *An. gambiae* s.s. The EO of *O. kilimandscharicum* was more efficacious than EOs of *A. afra* and *T. minuta* against third instar larvae of *An. gambiae* s.s. This difference led to the rejection of the first hypothesis of this study, and was in agreement with the findings of Runyoro *et al.* (2010) in which 323.6 ppm of *O. kilimandscharicum* and 457.1 ppm of *A. afra* EOs caused 50% mortality of *Cx. quinquefasciatus* larvae. The higher biocidal activity of *O. kilimandscharicum* than *T. minuta* observed in this study also agrees with Runyoro *et al.*, who found that 1000 µg of *O. kilimandscharicum* and *T. minuta* exhibited antimicrobial inhibition zones of 21.0 mm and 12.5 mm, respectively.

The concentration of *O. kilimandscharicum* used in this study was much lower with 91.40 ppm killing 90% of *An. gambiae* s.s. larvae within 24 hours. This could be attributed to various agroecological factors that affect the essential oil composition, and to various handling procedures of the plant extract. According to Arena *et al.* (2018) and Pineda-Cortel *et al.* (2019), variations in insecticidal activities of plant extracts are due to factors such as plant species, part of the plant used, age of the plant and the targeted vector species.

The larvicidal activity of each of the selected blends were significantly lower ($P < 0.001$) than the respective crude EOs. This implies that some of the compounds present in the EOs in trace concentrations contribute to the overall bioactivity of each crude EO. This observation is in agreement with the findings of Govindarajan (2009) and Elango *et al.* (2012), that a mixture of active compounds acts synergistically by

showing greater overall bioactivity compared to individual compounds. However, the contribution of different compounds in different EOs varies according to composition. For instance, the no observable effects concentrations (NOEC) for linalool and thymol, according to Faraone *et al*. (2015), have been found to be approximately 1% v/v and 2% v/v, respectively. These two compounds were separately found in the crude EOs of *O. kilimandscharicum* and *T. minuta,* albeit in trace concentration, in which they could have contributed to the overall performance of the individual EOs.

The trends in larval mortality observed in experiments done under semi-field conditions correlated with those observed in bioassays done under laboratory bioassays in being dosage- and exposure time-dependent. However, actual mortality rates were comparatively higher under semi-field conditions than those recorded under laboratory conditions in similar treatment concentrations, which led to the rejection of the second hypothesis of this study. This finding conformed to earlier findings by Kweka *et al*. (2011a) and Nyamoita *et al*. (2013), with both groups attributing the higher larval mortalities under SFCs to contributions of other variables in the surroundings, a normal adaptive biodiversity output.

According to Kweka *et al*. (2011a), the exposure of plant extracts to sunlight triggers degradation of phytochemicals to their secondary metabolites, to which higher larval mortalities under SFCs than laboratory conditions had been attributed. Besides, since the larvae used in this study were laboratory bred as opposed to wild forms, their mere introduction into a new set of habitat conditions could in its own make them more susceptible to the treatments to which they were subjected compared to their usual laboratory conditions.

The results of the current study, however, differed from those reported by Karunamoorthi *et al*. (2014), who while testing the larvicidal efficacy of EOs from *Juniperus procera* against *An. arabiensis*, found larval mortality under laboratory conditions to have been significantly higher with LC_{50} and LC_{90} values of 14.42 and 24.65 mg/L, which were significantly 10 mg/L lower than concentrations recorded under SFCs (LC_{50} and LC_{90} values 24.518 and 34.212 mg/L), respectively. However, while laboratory reared larvae were used under both laboratory and SFCs in this study, Karunamoorthi *et al*. (2014) had used laboratory reared and wild larvae for laboratory and semi-field experiments, respectively. This could be the reason for the difference in the findings between their study and the current one.

Nevertheless, Karunamoorthi *et al*. (2014) still found laboratory reared anopheline larvae to have been more susceptible than wild collected ones. On the other hand, the findings of this study were in line with those of Nyamoita *et al*. (2013), who while testing the activity of *Vitex payos* against laboratory reared *An. gambiae* s.s larvae, found those under SFCs to have been more susceptible than the ones under laboratory conditions.

The hyperactivity exhibited by mosquito larvae upon exposure to treatment solutions containing crude EOs and their blends, suggests that the treatments had an effect on the nervous system leading to paralysis and consequently, death. The restlessness observed in exposed larvae, especially at higher doses could be as a result of treatment seepage into the larval body, causing damage therein. This view is in line with previous reports by Rattan (2010), AhbiRami *et al*. (2014), Uragaya *et al*. (2015), Hakil *et al*. (2017), and Bekele (2018) in which the apparent hyperactivity is

linked to inhibiting acetylcholinesterase (Ache), ultimately blocking nerve functions by the treatments.

The observation that larval mortalities were directly proportional to the concentration of treatments used and to the duration of exposure was consistent with earlier reports by Kweka *et al*. (2011a), Murugan *et al*. (2012), Kyarimpa *et al*. (2014), Bekele, (2018), and Mwanjulu *et al*. (2018). This could be attributed to the fact that at lower doses the treatments took longer to accumulate to lethal concentrations in systemic poisoning. On the other hand, at higher doses, the lethal concentrations were attained after a short duration.

The progressive darkening of dead mosquito larvae from the head region towards the abdomen, observed a few hours after their death in this study, concurred with the findings of AhbiRami *et al*. (2014) and Chaaban *et al*. (2019). The former reported darkened and twisted abdomen of dead larvae of *Ae. albopictus* and *Ae. aegypti* exposed to solvent extracts of *Ipomoea cairica,* while the latter reported darkening and dryness of the cuticle of *Lucilia cuprina* larvae exposed to the EO of *T. minuta.* This indicated systemic poisoning through the alimentary canal, an implication that the treatments were ingested alongside larval food by the larvae. Similar sentiments have been put forth by Uragaya *et al*. (2015), Mwanjulu *et al*. (2018) and Chaaban *et al*. (2019).

The loss of dermal bristles and the disintegration of the cuticle of larvae exposed to the treatment solutions, observed in this study, has also been documented. According to Ghosh (2012) and Lee *et al.* (2017), dermal abrasion could be the mode of action through which the EOs and conventional insecticides penetrate the larval body surfaces, causing harm and eventual death of the exposed larvae. The loss of dermal

bristles, whose role is to aid in larval movement in water, according to Benelli (2015), restricts larval movement to the surface to replenish air. Consequently, the inability to replenish air hastens larval death by suffocation because larvae are known to remain under water for a finite period (Lee *et al*., 2017).

Morphological deformities and prolonged developmental periods (2 to 3 days) observed in this study among exposed larvae that survived in treatment solutions compared to those in negative controls have also been documented. Yu *et al.* (2015), reported that besides causing death of mosquito larvae, the intoxication effect of sea weed (*Laurencia dendroidea*) extracts on *Ae. Aegypti* also manifested through aberration of structures such as the anal papillae. The authors linked the deleterious effects of anal papillae to interruption of ion regulation and homeostatic imbalance of affected larvae. Similarly, the disrupted growth of larvae that survived after being exposed to treatment has been reported by Chaaban *et al*. (2019).

Upon testing the hatchability of *An. gambiae* s.s. ova exposed to various treatments in this study, it was found that the hatching rate reduced in a dose-response pattern with blend OK-4 showing the highest reduction in hatching at higher doses with only up to 2% hatching at 200 ppm. Govindarajan (2009) and Kweka *et al*. (2011a), found the relationship between hatchability percentage and treatment concentration to be inversely proportional. The low hatchability recorded in solutions containing higher doses of blends used in this study could, therefore, be attributed to the detrimental effects of biocidal compounds in the blends since there was almost 100% hatchability in the negative control used during the study. First instar larvae that hatched from eggs in treatment solutions were observed to die almost immediately. This could be as a result of larval poisoning through ingestion.

The concentrations of blends that induced 50% and 90% mortalities of *An. gambiae* s.s. larvae were also found to hinder a higher percentage of ova of the same species from hatching. Pineda-Cortel *et al*. (2019), documented similar findings while using crude ethanol extract of *Artocapus blancoi* against ova and larvae of *Ae. Aegypti*, although their concentrations were much higher than those recorded in this study. This could be as a result of different compositions of EOs used, and differential susceptibility of the different target species used in the two studies.

Among the juvenile stages of *An. gambiae* s.s exposed to treatment solutions containing the blends, pupae were found to be the most tolerant. For instance, the LC_{50} values obtained for pupae were 60.80 ppm, 69.20 ppm, and 79.20 ppm for BiLarv^{TM} (positive control), and blends OK-4 and AA-3, while the corresponding values obtained for third instar larvae were 44.10 ppm, 59.50 ppm, and 65.30 ppm, after 24 hours of exposure, respectively. Similar findings have been reported Elango *et al*. (2012), Kovendan *et al*. (2012), Murugan *et al*. (2012) while testing extracts from different plants against different mosquito species. The remarkable ovicidal and pupicidal activities of the blends of compounds present in EOs of *A. afra*, *O. kilimandscharicum* and *T. minuta* against *An. gambiae* s.s. led to the rejection of the third hypothesis of this study.

While testing the effect of treatments on oviposition site selection by gravid females of *An. gambiae* s.s., it was observed that the number of eggs oviposited reduced with increased doses of treatments. The highest reduction in the number of eggs laid was observed in the treatment solution containing 200 ppm of blend OK-4, which had the lowest OAI value of -0.9375. The negative OAI values indicated that fewer eggs were laid in the treated solutions than in rearing water (negative control). The OAI value

obtained for blend OK-4, a derivative of *O. kilimandscharicum*, fell within the range documented by Kweka *et al.*, (2010) for *O. kilimandscharicum* of between -0.238 to -1 when they used crude EOs of *O. kilimandscharicum* and *O. suave* against *An. gambiae* s.s.

During this study, some blends were found to possess remarkable repellent activity against blood-starved female *An. gambiae* s.s. mosquitoes. The probing and landing of blood-starved female *An. gambiae* s.s. mosquitoes on human hands treated with solutions of selected blends reduced in a dose-response pattern and varied among different treatment solutions used in this study. Blend OK-4 exhibited the highest reduction in landing success at higher doses, permitting up to 3.2% at 200 ppm while blends AA-3 and TM-4 permitted up to 8.2% and 33% at 200 ppm, conferring a protection efficiency (PE) of up to 96.8%, 91.8%, and 67%, respectively. The low OAI and high PE exhibited by the selected blends against adult female mosquitoes during oviposition and blood feeding, respectively, led to the rejection of the fourth hypothesis of this study.

These findings corroborate with those of Kweka *et al*. (2008b) who found that the EO of *O. kilimandscharicum* conferred 90.5% and 89.75% PE against *Cx. quinquefacitus* and *An. arabiensis.* The findings of this study also agree with those of Amerasan *et al*. (2012) who reported that solvent extracts of *Artemisia* species, extracted using methanol and benzene, possessed 90 to 100%, and about 70% PE against malaria vectors and *Cx quinquefasciatus,* respectively.

The CPT recorded in this study varied among the blends tested, with the longest duration observed in arms treated with blend OK-4 (90 minutes), followed by blend AA-3 (70 minutes), then TM-4 (40 minutes). The results were in conformity with

those of Afolabi *et al*. (2018) who reported the CPT conferred by EOs against *An. gambiae* as *Chromolaena odorata* (30 minutes), *ocimum caninum* (90 minutes), *O. gratissium* and *Datura stramonium* (120 minutes). The composition and proportion of constituents in the blends used could be responsible for the disparity.

The composition of the crude EO of *A. afra* used in this study is generally similar to those reported in previous studies except for slight variations in proportions. The high concentration of thujone in the EO of *A. afra* shown in this study is consistent with the findings of Srivastava *et al*. (2012) and Srinivasan *et al*. (2015), who in either case found the compound to comprise the highest percentage composition in the plant's EO. Thujone could have played a major role in insecticidal activities of the crude EO and blend AA-3 based on previous reports. Srinivasan *et al*. (2015) and Hakil *et al*. (2017) attribute the remarkably high insecticidal activity of *A. afra* EO to thujone. Besides thujone, other compounds such as 1,8-cineole, thujanol and terpinen-4-ol could also have contributed to the overall insecticidal activity of the EO in this study.

Most of the major compounds present in the EO of *O. kilimandscharicum* have been reported to possess insecticidal activity. In this study, major compounds present in the EO and which have been reported to possess insecticidal activity include 1,8-cineole, beta-(*Z*)-ocimene, beta-(*E*)-ocimene, eugenol, methyl eugenol, and (*E*)-caryophyllene. These compounds could have contributed immensely to the insecticidal activity of the EO and the derived blend because according to Zoubiri and Baaliouamer (2014) and Lee (2018), the biological effects of EOs are synergistic. However, Essoung *et al*. (2020) and Lambert *et al*. (2020) reported the role of eugenol and methyl eugenol in insecticidal activities of some EOs against various target insects to be overwhelming.

The major compounds present in the EO of *T. minuta* are in the range reported by Arena *et al*., (2018). Most of the compounds present in the EOs of *T. minuta* were also found either in the EO of *A. afra* or that of *O. kilimandscharicum* albeit in different proportions. Most of these compounds, as stated earlier, have been reported to possess insecticidal activities and each could have contributed to the overall effect of the EO on the various stages of *An. gambiae* s.s. tested.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

1. The crude essential oils of *A. afra, O. kilimandscharicum* and *T. minuta* have larvicidal activity against *An. gambiae* s.s., with *O. kilimandscharicum* being more efficacious than *A. afra* and *T. minuta*, respectively.
2. Blends AA-3, OK-4 and TM-4 exhibited significantly lower ($P < 0.001$) larvicidal activity than the crude EOs of *A. afra, O. kilimandscharicum* and *T. minuta* against third instar larvae of *An. gambiae* s.s., respectively. In the same breath, the blends caused higher larval mortalities under simulated field conditions (SFCs) than under laboratory conditions.
3. Blends AA-3, OK-4 and TM-4 had remarkable ovicidal and pupicidal properties against ova and pupae of *An. gambiae* s.s. That notwithstanding, ova were found to be the most susceptible among the juvenile stages while pupae were the most tolerant to treatments.
4. The phytochemicals in all the blends negatively influenced oviposition by gravid female mosquitoes as well as effectively repelled blood starved females.

6.2 Recommendations

1. The development of suitable formulations of biocides from EOs of *A. afra*, *O. kilimascharicum*, and *T. minuta* is feasible with blends from *O. kilimadscharicum* and *A. afra* being more promising.
2. The stability of the EOs and their isolates in natural environments should be established to determine the viability of these products in field applications.
3. The insecticidal mechanisms and toxicity towards non-target cohabiting organisms should be scientifically evaluated and validated to meet the demands of the pest products board of Kenya.

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APPENDICES

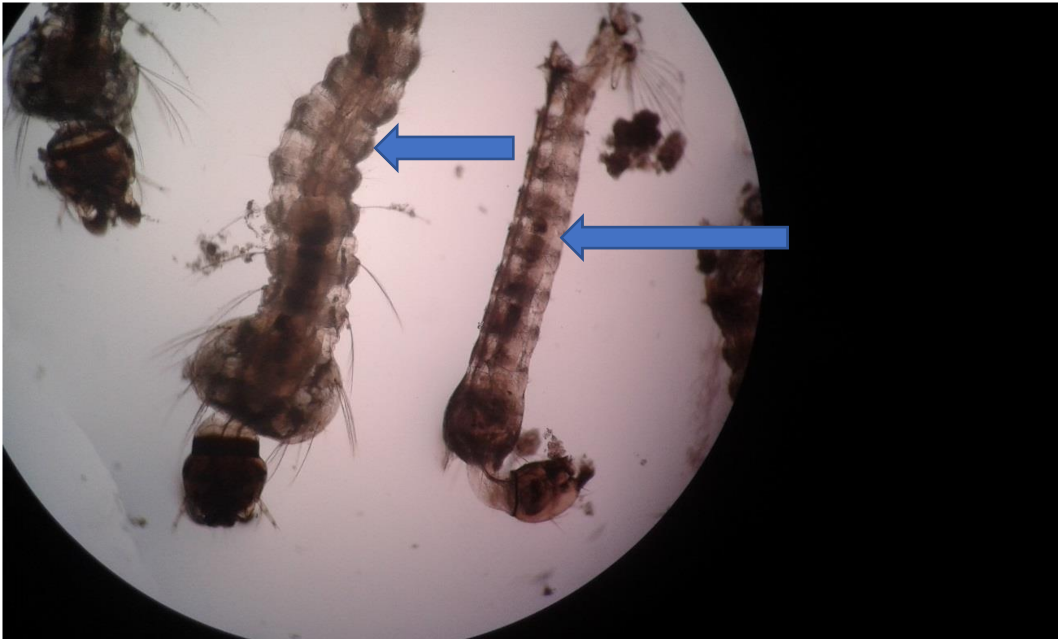
APPENDIX I: STEAM DISTILLATION OF ESSENTIAL OILS

a) Picture showing steam distillation setup

b) Plate showing extracted essential oil of *O. kilimandscharicum* (pointed by arrow)

(Source: Author, 2018)

APPENDIX II: EFFECTS OF ESSENTIAL OILS ON DERMAL SURFACES



a. **Plate showing larval surfaces of larvae from treatment solutions. Note the loss of hair at regions pointed by arrow**. (**Source: Author, 2018**)

b. Plate showing larva from negative control. Note the hairy surface and uniformly coloured body.

(**Source: Author, 2018**)

APPENDIX III: INTERNAL DAMAGE CAUSED BY ESSENTIAL OILS ON MOSQUITO LARVAE

a) **Plate showing darkened anterior region (pointed) of a dead larva 3 hours post-mortality**. (**Source: Author, 2018**)

b) **Plate showing darkened anterior region and yet to abdomen (pointed) of a dead larva 1-hour post-mortality**
(Source: Author, 2018)

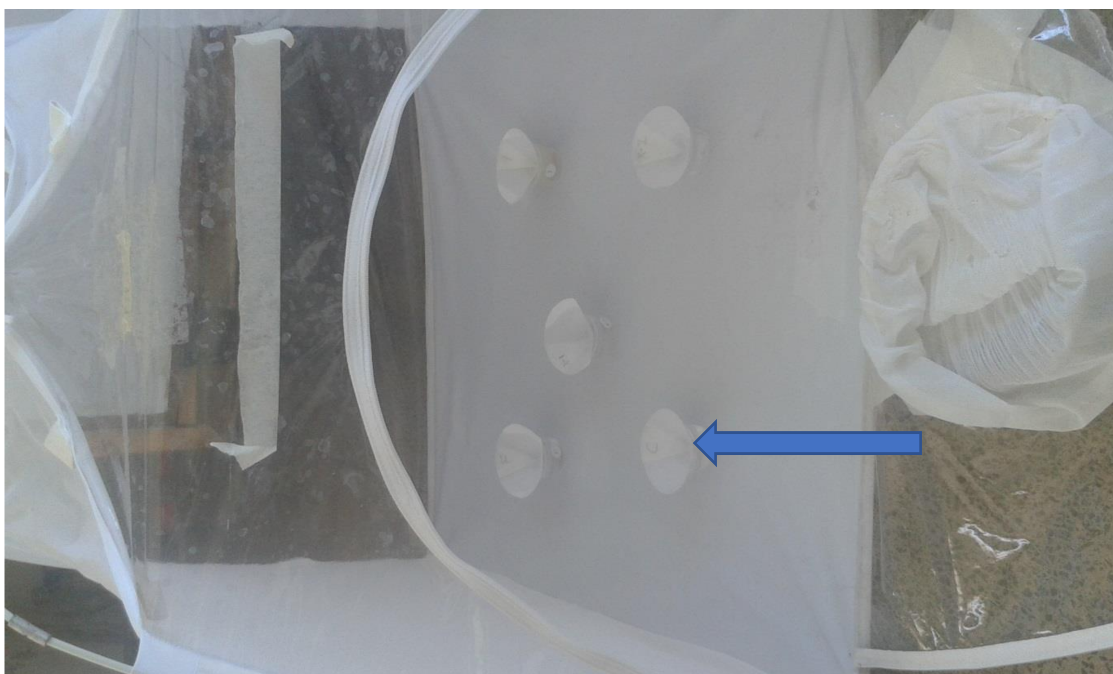
APPENDIX IV: SETUP FOR SIMULATED FIELD TRIALS

Plate showing one of the setups during simulated field trials

(**Source: Author, 2018**)

APPENDIX V: SETUP FOR OVIPOSITION SITE SELECTION

a. Setups for oviposition site preference



b. Setup showing arrangement of treatments for oviposition site preference

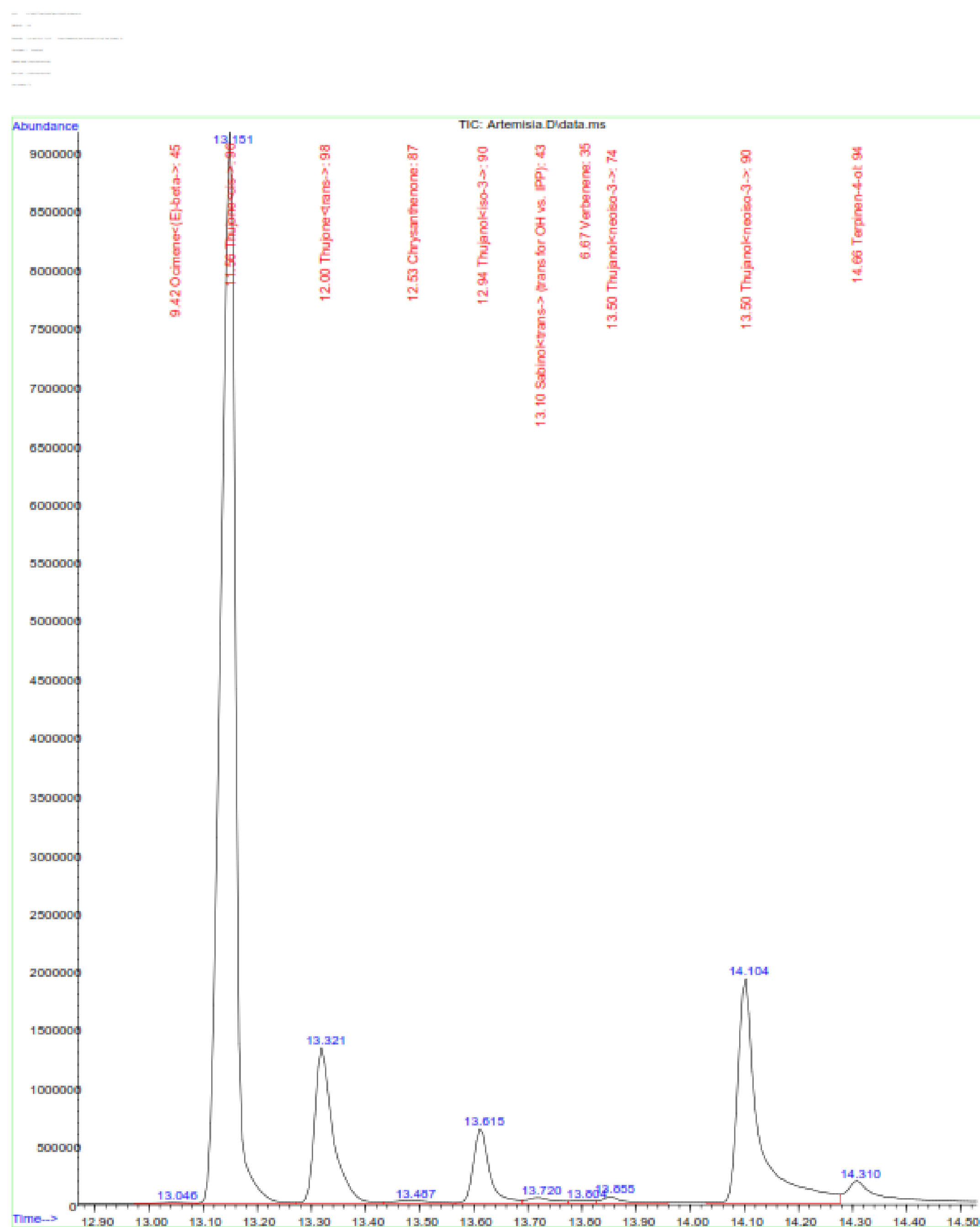
(Source: Author, 2018)

APPENDIX VI: SETUP FOR REPELLENCY BIOASSAY

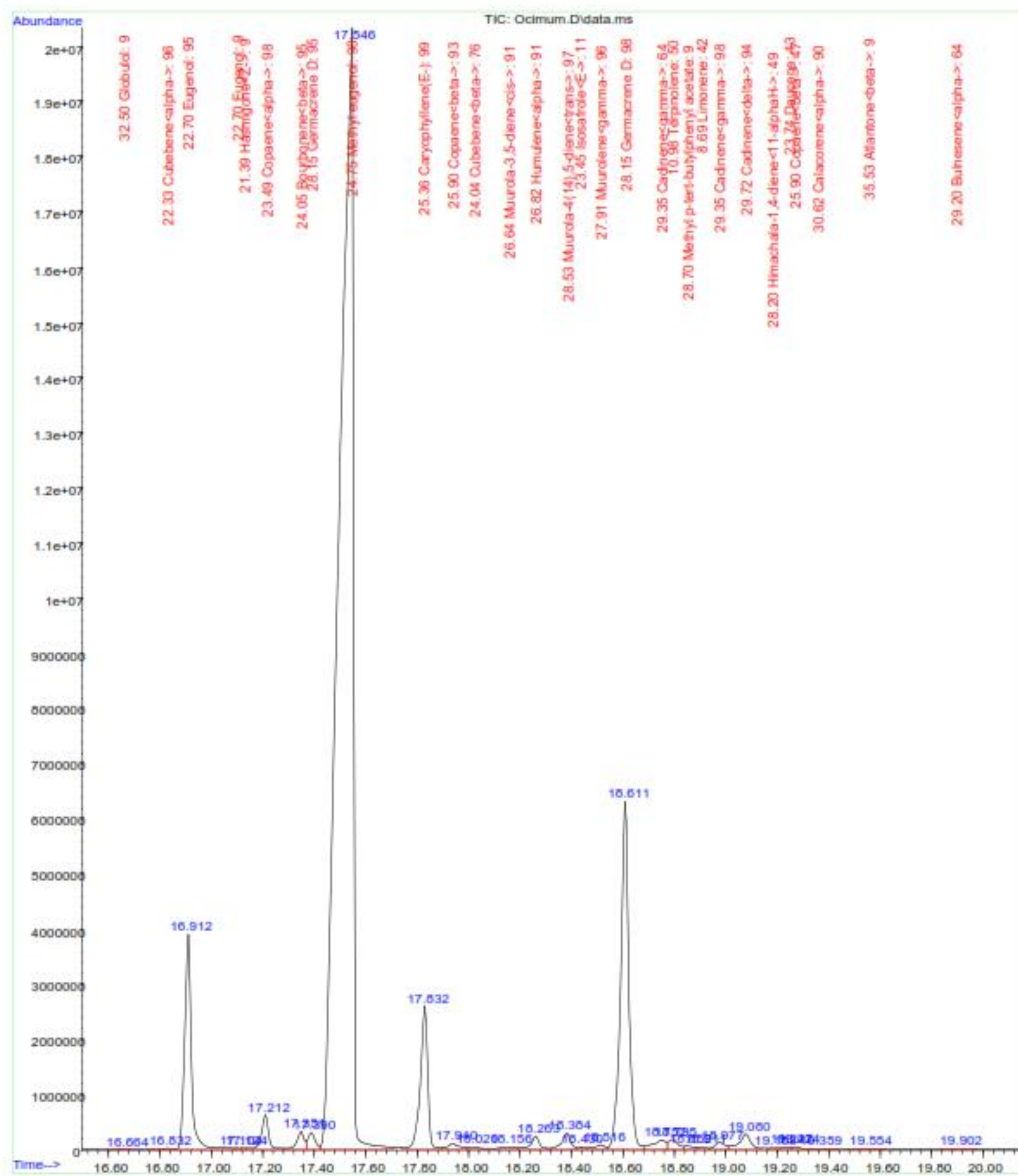
Plate showing one of the tests for repellency

(**Source: Author, 2018**)

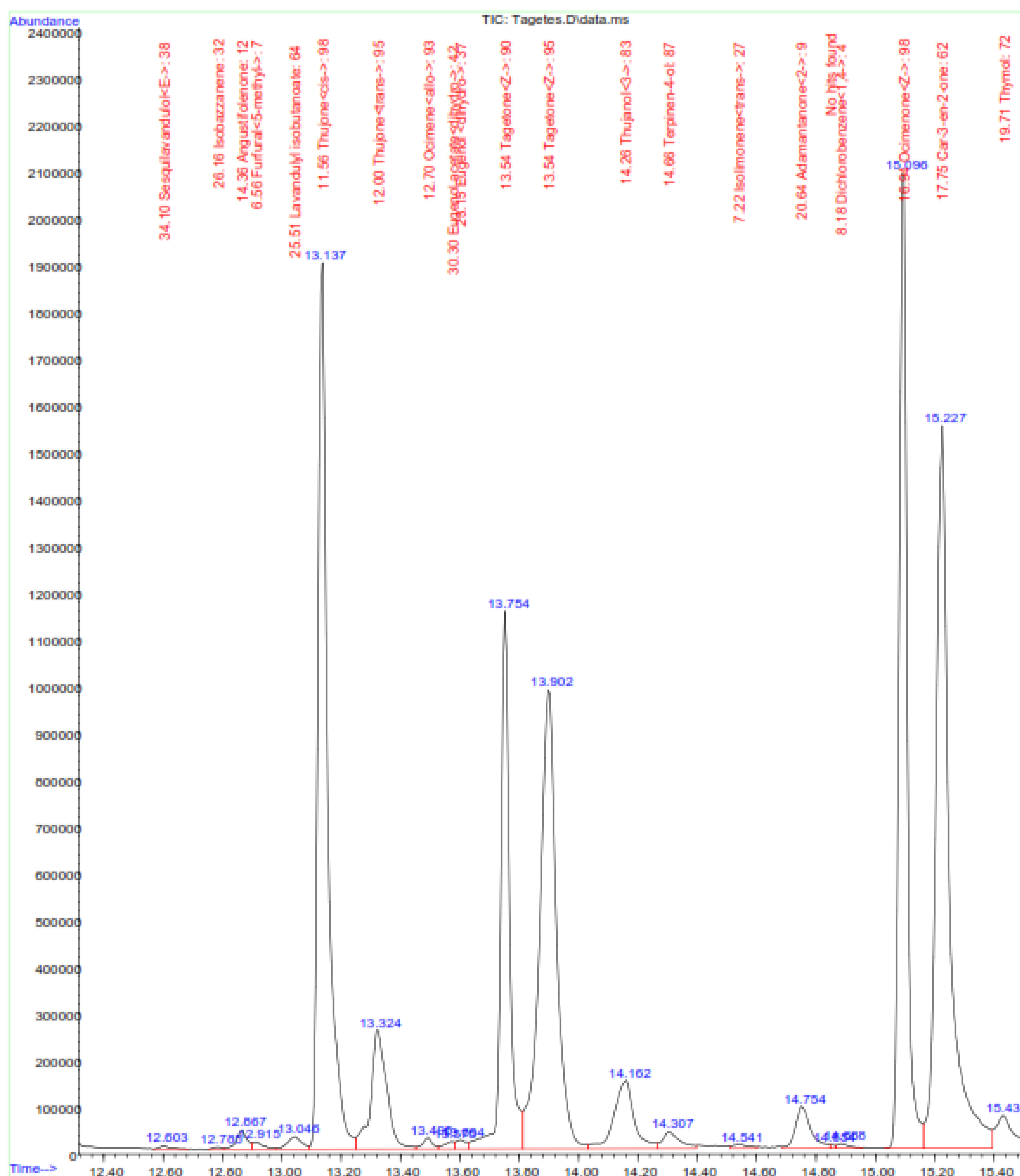
APPENDIX VII: GC-MS SPECTRUM FOR *Artemisia afra* EO



APPENDIX VIII: GC-MS SPECTRUM FOR *Ocimum kilimandscharicum* EO



APPENDIX IX: GC-MS SPECTRUM FOR *Tagetes minuta* EO



APPENDIX X: ETHICAL CLEARANCE

OFFICE OF THE DIRECTOR OF GRADUATE STUDIES AND RESEARCH

UNIVERSITY OF EASTERN AFRICA, BARATON

P. O. Box 2500-30100, Eldoret, Kenya, East Africa

June 25, 2018

Misire Christopher Ong'au (SC/D.PHIL/Z/005/11)
University of Eldoret

Dear Misire,

Re: **ETHICS CLEARANCE FOR RESEARCH PROPOSAL (REC: UEAB/01/6/2018)**

Your PhD thesis proposal entitled "**Bioactivity of Essential Oils of *Tagetes minuta, Ocimum kilimandscharicum* and *Artemisia afra* against *Anopheles gambiae* s.s.**" was discussed by the Research Ethics Committee (REC) of the University and your request for ethics clearance was granted approval, provided that you make the changes indicated on the scanned pages.

This approval is for one year effective June 25, 2018 until June 24, 2019. For any extension beyond this time period, you will need to apply to this committee one month prior to expiry date.

Note that you will need a research permit from the National Commission for Science, Technology, and Innovation (NACOSTI) and clearance from the study site before you start gathering your data.

We wish you success in your research.

Sincerely yours,

Prof. Jackie K. Obey, PhD
Chairperson, Research Ethics Committee

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APPENDIX XI: INFORMED CONSENT FORM

Title of the Study

Evaluation of Repellent activity of Compounds Isolated from Essential Oils of *Tagetes minuta*, *Ocimum kilimandscharicum*, and *Artemisia afra* against *Anopheles gambiae* s.s.

Principal Investigator

Misire Christopher Ong'au,
Department of Biological Sciences,
University of Eldoret,
P.O. Box 1125- 30100,
Eldoret.
+254720890847
misirechris2@yahoo.com

Purpose of the Study

You are being asked to take part in a research study. Before you decide to participate in this study, it is important that you understand why the research is being done and what it will involve. Please read the following information carefully. Please ask the researcher if there is anything that is not clear or if you need more information.

The purpose of this study is to evaluate the repellent activity of compounds isolated from essential oils of three plants, *Tagetes minuta*, *Ocimum kilimandscharicum*, and *Artemisia afra* so as to determine their application in the control of human malaria through repelling the mosquito vector. Since the target is the human malaria vector, you are the most ideal subject for this study.

Study Procedure

During the study, you will be required to apply 1 mL solutions of different compounds isolated from essential oils of the above-mentioned plants following the World health Organization (WHO) standard procedure. To begin with, you will apply the 1 mL ethanol, as recommended by WHO, on a non-scented, clean and dry forearm skin of your left (test) hand and expose it to a batch of laboratory-reared disease-free mosquitoes in a mosquito netting cage for 30 seconds. You will withdraw the arm then separately apply five different concentrations of treatment solutions of the same compound in increasing order and the arm exposed for 30 seconds after each application except at the highest concentration where you will be required to exposes the arm for a little longer until one or two mosquitoes land on it.

You will also be required to separately apply the recommended concentrations of ethanol and a conventional insect repellent (DEET) on your right (control) forearm and expose it to the mosquitoes for 30 seconds in each instance, in order for the researcher to evaluate the effectiveness of the treatment solutions. In all the exposures, you will be required to count the number of mosquitoes that probe and/or land on the treated skin. The only part of your arm that will be exposed to the mosquitoes is the part applied with the solution as the rest of the hand will be protected. You will be provided with protective clothing. Since the study will be done on five different days, you will be required to avail yourself on the agreed days to

perform repeat procedures using a fresh batch of uninfected mosquitoes on each day, with each experimental session estimated to last about two hours.

Risks
Though highly unlikely, your skin may be irritated by the treatment solution and/or the control solutions, and by the mosquito bites. In such cases, you will be provided with calamine lotion to apply on the affected area and if need be, further remedies may be provided. You may decline to participate partially of fully and you may terminate your involvement at any time if you choose to.

Benefits
There will be no direct benefit to you for your participation in this study. However, your participation in this study is significant because the information obtained may be useful in developing alternative cheap and ecofriendly repellents affordable and acceptable to all.

Confidentiality
Your participation in this study will be anonymous. Participant data will be kept confidential except in cases where the researcher is legally obligated to report specific incidents.

Compensation
You will be provided with lunch and non-scented body cream.

Contact Information
If you have questions at any time about this study, or you experience adverse effects as the result of participating in this study, you may contact the researcher whose contact information is provided on the first page.

Voluntary Participation
Your participation in this study is voluntary. It is up to you to decide whether or not to take part in this study. If you decide to take part in this study, you will be asked to sign a consent form. After you sign the consent form, you are still free to withdraw at any time and without giving a reason. Withdrawing from this study will not affect the relationship you have, if any, with the researcher. If you withdraw from the study before data collection is completed, your data will be returned to you or destroyed.

Consent

I have read and understood the provided information and have had the opportunity to ask questions. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving a reason and without cost. I understand that I will be given a copy of this consent form. I voluntarily agree to take part in this study.

Participant's signature ________________________________ Date __________

Investigator's signature _______________________________ Date __________

APPENDIX XIII: SIMILARITY REPORT

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SC/D.PHIL/Z/005/11 By Ong'au Misire Christopher

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