TOXICOLOGICAL EFFICACY OF FLOWER EXTRACTS FROM *CHRYSANTHEMUM CINERARIIFOLIUM* AND LEAF EXTRACTS FROM *EUCALYPTUS CAMALDULENSIS* AND *NICOTIANA TABACCUM* ON *ANOPHELES GAMBIAE S.S.* GILES MOSQUITO LARVAE.

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2018

Toxicological Efficacy of Flower Extracts From *Chrysanthemum cinerariifolium* and Leaf Extracts From *Eucalyptus camaldulensis* and *Nicotiana tabaccum* on *Anopheles gambiae s.s.* Giles Mosquito Larvae.

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A Thesis Submitted in Partial Fulfilment for the Degree of Doctor of Philosophy in Public Health in the Jomo Kenyatta University of Agriculture and Technology

2018

DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

This thesis is dedicated to my late parents, Mr. Nahashon Araka and Mrs Teresia Kerubo Araka, whose loving care made me what I am today.

ACKNOWLEDGEMENT

I wish to thank my supervisors Prof. John Ochora of Jomo Kenyatta University of Agriculture and Technology, Prof. Johnstone Wakhisi of Moi University and Dr. Festus Tolo of Kenya Medical Reasearch Institute/Institute of Tropical Medicine and Infectious Deseases (KEMRI) for their willingness to supervise my work right from its initial stage until its completion. I acknowledge induction given to me in mosquito rearing by the Director of KEMRI, Kisumu, and his technical staff which gave me knowledge and skills in mosquito rearing. Iam grateful to Mr Joseph Mahelo and his colleagues namely Oketch, Cheruiyot, Tanui, Barasa, and Ondigi who were with me in the field for the collection of the adult *Anopheles* mosquitoes for laboratory rearing. These workers had knowledge of differentiating mosquito species, and knew about mosquito care. Mr Mahelo's vast experience as a mosquito supervisor for a period of 42 years in the Municipal Council of Eldoret no doubt was of great value to my research work. Prof. B.O. Khwa Otsyula, the Dean of Medicine, Moi University gave perrmission for my use of a room in the Anatomy and Physiology Department, of which I am pleased. I also greatly appreciate the assistance accorded to me by Dr. Robert Too in the statistical analysis. I greatly express my gratitude to Mr George Onyango of Seme Carpentry Workshop, Eldoret, for keenly making the mosquito rearing cages which enabled this study become a success.I specifically remember and wish to thank Messrs Teratisio Ndwiga and Eheyya, Senior and Chief Technicians of Moi University and Eldoret University, respectively, for their input in the preparation of the standard solutions of the extracts that I used. I will not forget the great work done in the Eldoret Secretarial Bureau by Mrs Jemimah Maina who wholeheartedly typed my research work all through from its proposal to thesis level. I heartly appreciate the encouragement given to me by my wife Mrs Marion N. Araka and my children Nyabuto, Tele, Onsongo, Kemunto, Nyaboke and Kerubo especially at the time when I was downhearted as a result of my study workload.

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ABBREVIATIONS AND ACCRONYMS

AMCA American Mosquito Control Association

ATSDR Agency for Toxic Substances and Disease Registry

BCB Baker Cleansers Bitters

CCA Canonical-Correlation Analysis

CDC Centre for Disease Control and Prevention (based in Atlanta, USA)

DCM Dichloromethane

DDT Dichlorodiphenyltrichloroethane

DNA Deoxyribonucleic Acid, is the hereditary material in humans and almost all other organisms for the storage of information in the form of a code made up of four chemical bases: adenine (A), guanine, (G), cytosine (C) and thymine (T).

ED This stands for Effective Dose. It is the dose or amount of a substance that produces a response or desired effect in some fraction of the subjects the substance is administered to.

EIR Entomological Inoculation Rates

ELISA Enzyme Linked Immunosorbent Assay. ELISA is now often used to determine whether mosquito salivary glands are positive for sporozoites.

EPA Environmental Pollution Agency

FAO Food Agricultural Organization

GPIRM Global Plan for Insecticide Resistance Management

IACUC Institutional Animal Care and Use Committee (of Alaska University, USA).

IRR Incidence Rate Ratio

IRS Indoor Residual Spraying

ITNs Insecticide-treated Nets

ITROMID Institute of Tropical Medicine and Infectious Diseases**IUPAC** International Union of Pure and Applied Chemistry

IVC Integrated Vector Control

kdrWest or kdrEast (kdrW or kdrE) : Resistance to pyrethroids in *A. gambiae* commonly associated with a single base-pair mutation in the voltage-gated sodium channel referred to as knockdown resistance (kdr). In Africa there two forms of this mutation, the west African form (kdrW) strongly associated with the S molecular form which results from a leucine to phenylalanine substitution (TTA/TTT) and the east Africa form which results from a leucine to serine

substitution (TTA/TCA). A luicine is a branched chain α-amino acid with the chemical formula $HO_2CCH(NH_2)CH_2CH(CH_3)_2$. An essential amino acid, leucine cannot be synthesized by animals. Consequently, it must be ingested, usually as a component of proteins.

LC_{50} Lethal Concentration 50. This is astandard measure of toxicity of the surrounding medium that will kill half of the sample population of a specific test-animal in a specific period through exposure via inhalation (respiration). Lc_{50} is measured in micrograms (or milligrams) of the material per litre, or parts per million (ppm), of air or water; lower the amount, more toxic the material.

LC_{90} Lethal Concentration 90. This is the concentration of a chemical that can kill 90% of the test population.

LD (Light: Dark)

LD_{50} Is an abbreviation for "Lethal Dose, 50" or median lethal dose. It is the amount of the substance required (usually per body weight) to kill 50% of the test population.

LLINs Long-lasting insecticidal nets

LSM Larval Source Management

MED Minimum Effective Dose. Defined as the lowest dose level of a substance that provides a significant response in average efficacy. This response refers to the average test organism population**.**

MLD Also expressed **LD_{min}** is the least amount of substance that can produce death in a given organism species under controlled conditions.

NDVI Normalized Difference Vegetation Index

PBO Piperonyl Butoxide.Is an organic compound used as a component of pesticide formulation to act as a synergist.

P*f*PR Expresses a function of Parasite Prevalence Ratio

RUP Restricted Use Pesticide

SNP Single-Nucleotide-Polymorphism. This is an array type of DNA microarray which is used to detect polymorphisms within a population.

SPSS Means Statistical Package for the Social Sciences. This is a Window based programme that can be used to perform data entry and analysis and to create tables and graphs. SPSS is capable of handling large amounts of data and can

perform all of the analyses covered in the text and much more.

TD Means Toxic Dose. The calculated dose of a chemical introduced by a routeother than inhalation, to cause a specific toxic effect.

TL (TL_{50}) Statistically derived average time interval during which 50% of a given population may be expected to die following acute administration of a chemical or physical agent (radiation) at a given concentration under a defined set of conditions.

USA United States of America

WHO World Health Organization

ABSTRACT

In this study crude flower extract of *Chryasathemum cinerariifolium* , and crude leaf extracts of *Eucalyptus camaldulensis* and *Nicotiana tabaccum* were tested for their larvicidal activity against the third instar larvae of *Anopheles gambiae s.s.* **Giles** (Diptera: Curicidae), a member of *Anopheles gambiae* complex. Six different solvents were used to extract the oils namely ethanol, methanol, DCM, hexane, ethylacetate and aqueous. Larvae had 24 hour exposure and observed separately in control at 50,100,150,200,250 and 300 ppm concentrations of the extracts. The six different solvent extracts of the plants showed good larvicidal activity. The highest potency was recorded by DCM extract of *C.cinerariifolium* (LC_{50}= 164.68 ppm, LC_{90} = 255.17 ppm) achieving 100% mortality of the larvae. Similarly, ethanol of *C. cinerariifolium*, DCM and methanol of *E. camaldulensis*, and ethanol of *N. tabaccum* exhibited 100% larval mortality at LC_{50} and LC_{90} for which the concentrations were respectively 187.78 ppm, 268.26 ppm; 168.65 ppm, 315.85 ppm; 197.46 ppm, 329.68 ppm; and 189.58.58 ppm, 320.75 ppm. Second to these was methanol of *C. cinerariifolium 98% mortality* (LC_{50}222.45 ppm) and ethanol of *E. camaldulensis* 96% mortality at 210.15 ppm. The rest of the extracts also indicated appreciable results ranging from 80% (ethyl acetate of *E.camaldulensis* at LC_{50} of 260 ppm) as lowest to hexane *C. cinerariifolium* and DCM *N.* tabaccum both at 88% larval mortality and LC_{50}s of 230.66 ppm and 229.72 ppm respectively. There was no mortality observed in controls. A general observation made was that the larvae were susceptible to all treatments. The larvicidal activity of the treatments were dose and time independent i.e. larval mortality increased as the dose and time increased and all of the volatile oils showed significant larvicidal activity against the larvae on 24 hours exposure. The LC_{50} and LC_{90} with their 95 percent confidence limits of the oils were determined using log probit analysis test and was found statistically significant ($p = < 0.05$) . From these results it was observed that the five extracts namely DCM of *C. cinerariifolium*, DCM of *E. camaldulensis*, ethanol of *C. cinerariifolium*, methanol of *E. camaldulensis*, and ethanol of *N.tabaccum* contained toxic compounds to mosquito larvae. However, the DCM flower of *C. cinerariifolium* and DCM leaf extract of *E.camaldulensis* showed the highest activity on the larvae than all extracts. In the determination of synergism and antagonism, seventeen activities were synergistic at the combination ratio of 1:1(100 ppm: 100 ppm). Amongst them were *C. cinerariifolium* methanol + *N. tabaccum* aqueous; *C. cinerariifolium methanol + Nt aqueous; and C. cinerariifolium DCM + E.camaldulensis* hexane. In that order they demonstrated to be the best synergist combinations at their combination levels. The weakest synergist combinations were *C. cinerariifolium methanol + E.camaldulensis* ethyl acetate; and *E.camaldulensis* DCM + Nt hexane. There were ten antagonistic activities. Of these the combination between methanol of *C. cinerariifolium* and hexane of *N. tabaccum* indicated best antagonist followed by methanol of *C. cinerariifolium* in combination with ethyl acetate of *E. camaldulensis in the ratio* 1:1.(100 ppm : 100 ppm). The former showed the best antagonism in their LC_{50} each at 224.45 ppm and 224.35 ppm giving a combination antagonistic activity of 232.66 ppm. This is the only combination that gave a small range between the combination activities and the single crude leaf toxic concentrations i.e. a difference of 7.20 ppm for methanol and 4.20 ppm for hexane. In this case methanol of C. *cinerariifolium* was a synergist to *N. tabaccum* hexane. All extracts were tested on the susceptible and field strain larvae against Resistance Ratio (RR). It was revealed that the laboratory susceptible strain showed complete larval mortality at high concentrations (DCM C. *cinerariifolium*, 0.996; *E. camaldulensis* methanol; 0.998, *E. camaldulensis* DCM; 0.993 and Ethanol *N. tabaccum*, 0.999) while the field strain was susceptible to low concentrations of all extracts (RR varying from 1.003 to 1.891). Results indicated presence of cross-resistance among the field strain in 24 h post-recovery period, especially during the long rain periods. For persistence the results of the three plants indicated that under light regime *C. cinerariifolium* took 5 hours and 30 minutes to completely decompose under light regime and 28 days to decompose under darkness. *E. camaldulensis* decomposition under dark regime was 12 days and that of light regime was 35 days. The dark-light degradation periods for *N. tabaccum* slightly stretched higher than those of other plants and disappeared within 18 days of light and 28 days of darkness. All the extracts showed potential in the control of the malaria vector mosquito and therefore can be developed for use.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

This study was laboratory based. Natural crude oils of flower and leaves of three local plants namely, *Chryasathemum cinerariifolium* (Pyrethrum), *Eucalyptus camaldulensis* (Red Gum) and *Nicotiana tabaccum* (Tobacco) were extracted for testing their efficacy against third instar larvae of *Anopheles gambiae s.s.* **Giles** mosquito. The vegetative products of these plants were collected from the field, dried, ground and their oils extracted using six solvents i.e. ethanol, methanol, Dichloromethane (CDM), hexane, ethyl acetate and aqueous. From the ground powders, 18 (i.e. 3 plants x 6 solvents) stock solutions were prepared and out of these stock solutions the working standards of 50, 100, 150, 200, 250, and 300 ppm of each solvent were prepared for testing the larvae in objectives 1 to 4. Although the larvae were included in the tests of objective 5, efficacy of the oils was not tested against the larvae but rather persistence of the oils was evaluated.

The adult male and female mosquitoes were collected from the field in the ratio of 3:1 (male:female) and brought to the laboratory for rearing in cages in the combination of 3:1 (male:female) in each cage of the three cages. Larvae were reared to 3rd stage then batches of 25 larvae tested in the order of the concentrations 50, 100, 150, 200, 250 and 300 ppm of each plant WHO,(2018).

All the experiments were conducted under laboratory controlled conditions bearing in mind that the tests were being conducted on living organisms. The results were recorded using tables and line graphs.

In objective 5, the percent recovered oils under degradation was determined by regular short intervals of time sampling ranging from daily, 12 hourly, hourly and every 30 minutes. However, the sampling intervals was determined by the rate of oils

biodegradation of each plant and more regular sampling was conducted in faster biodegrading extract. The samples were analysed using Gas Chromatography-Mass-Spectrometry (GC/MS) technique until zero point of degradation of each extract was reached.

1.1.1 Differentiating features for male and female *Anopheles gambiae*

A number of features were used to differentiate male from female *Anopheles gambiae* mosquito. These include abdominal segments, hind tarsus, legs, wings, pulps, proboscis and body size. The latter four are quite prominent features and often used: In the female, the wings have 2 pale spots on vein 5.1, the pulps have 4 pale bands, the proboscis as a blood sucking organ is longer than the pulps and when the two mosquitoes are close together on a landing surface, the thinner mosquito is always the male. In the male, the wing has one pale spot, three pale bands in the palps and the proboscis is shorter or equal to the pulps.

1.1.2 *Anopheles gambiae s.s.* Giles mosquito.

Anopheles gambiae **Giles** is the most efficient vector of human malaria in the Afrotropical Region. Thus, it is commonly called the African malaria mosquito (CDC 2010).

The *Anopheles gambiae* mosquito consists of six species complex and these are: *Anopheles gambiae s.s.* **Giles**, (1902); *Anopheles merus* **Donitz**, (1902); *Anopheles melas* **Theobald**, (1903); *Anopheles arabiensis* **Patton**, (1905); *Anopheles quadrianulatus* **Theobald**, (1911); *and Anopheles bwambae* **White**, (1985). All belong to *Anopheles* Cellia. *A. Gambiae s.s.* **Giles** and *A. arabiensis* are both highly anthropogenic in their biting. However, *A. gambiae* is exphilic (bitng indoors) and slightly exophilic (biting outdoors) unlike *A. arabiensis* which is wholly zoophilic and exophilic. *Anopheles melas* demonstrate high exophily (Fornadel *et al*. 2010*).* Collectively they are sometimes called *Anopheles gambiae sensu lato*, meaning 'in the wider sense.' None of these species occur

in North America. The intensiy of the invectivity of the malaria vector, *Anopheles gambiae s.s.* **Giles** mainly depends on the distribution of the mosquito and the availability of the host (human). The distribution of the vector is governed by a number of factors namely altitude, humidity, agricultural activities (land use), temperature and rainfall.

Past and present studies cover both the low land and highlands stretching from the coastal region, to the western highlands including the Kano plains, Mwangangi *et al.* (2013); Kipyat *et al.* (2013); Walker *et al.* (2013); O'Loughlin *et al.* (2016); and Charlwood (2017) to the Western highlands, Munga *et al.* (2013); Omukunda *et al.* (2012b); Eliningaya *et al.* (2012); Ndenga (2015); Onchuru *et al.* (2016); Siteti *et al.* (2016) ; Obino *et al.* (2013) and Lili *et al.* (2009) ,Western plains (including the Kano plains in the lake region) Mwangangi *et al.* (2013) and in Central Kenya notably Mwea Irrigation Scheme Mwangangi *et al.* (2010 and 2013).

Further, in contrast to other studies it was observed that there has been a shift from human to animal feeding for both *A.gambiae s.s.* **Giles** (99% to 16%) and *A. funestus* (100% to 30%) Mwangangi (2013). Mwangangi also indicated that there has been on average a significant reduction in the abundance of *A. gambiae s.l.* over the years (IRR=0.94, 95% Cl 0.90-0.98), with density standing at low levels of an average 0.006 mosquito/house in the year 2010. Resulting from this, it was concluded that reduction in the densities of the major malaria vectors and a shift from human to animal feeding have contributed to the decreased burden of malaria along the Kenyan coast. Vector species composition remains heterogeneous but in many areas *An. arabiensis* has replaced *An. gambiae* s.s. **Giles** as the major malaria vector.

1.1.3 *Anopheles gambiae s.s.* **Giles** mosquito larvae.

Anopheles species can be distinguished through larval habitat. *Anopheles* larvae are adapted to a variety of aquatic habitats, but occur predominantly in ground water. Some

species require aerated water, others brackish water and some in habitat cavities such as tree holes (Plumbeus Group, subgenus *Anopheles*) and the axils of epiphylic plants (subgeneus Kerteszia, except for *A. bambusicolus* which inhabits bamboo) (Merchant 2016; Kansas State University 2018). Specific habitats contain stagnant water or water that is slowed down by vegetation or objects in specific niches occupied by the larvae. The larvae of all species feed and breath at the water, where they attach to the surface film by spiracular apparatus, palmate setae and special notched organs on the prothorax. They rotate the head 180^0 so that particles of food at the surface can be swept into the mouth by currents produced by the mouth brushes. The larvae generally rest with the end of the abdomen against objects and are therefore found in greatest numbers in areas with emergent vegetation at the margins of the habitats.

1.1.4 Toxicological efficacy

The toxicological efficacy of an extract is governed by three factors: (i) yield during extraction (ii) the solvent used for extraction (iii) concentration of the compounds in the extract (Mustafayeva and Serkerov, (2015); and (Oeung and Chea (2017).

Fourty four compounds are characterized in *Eucalyptus camaldulensis* essential oil. These lasts are dominated by 1.8-cineole (47.54 to 52.47%), limonene (16.5 to 19 α - pinene (7.3 to 11.2%) and p-cymene (6.0 to 8.8%). These could improve the efficacy of *E. camaldulensis* extracts (Ndiaye *et al*, 2017). In *C.cinerariifolium*, a total of 23 compounds have been identified. The major two compounds which also may influence the efficacy of the oil are Camphor (1) (25.29%) and L. Borneol (21.84%) (Mustafayeva and Serkerov, (2015). *Nicotiana tabaccum* composition consists of alkaloids, steroids, and terpenoids of the order 0.85%, 0.56% and 11.5% respectively. Terpenoids which include nicotine is a major component likely to influence the efficacy of the tobacco oil

(Oeung and Chea (2017). Murnasari and Subiyaleto (2015) attributes the composistion of tobacco leaves to contain Solanone (4.86%), Geranylacetone (0.60%), 3-Decen-1-yne (1.78%), Cis-11-Tetradecenylacetate (73.28%) an 5-ethyl-2-heptanone (1.05%).

1.1.5 All flower and leaf extracts

Pure essential oils do not go ransid. Over a time , however, essential oils can oxidise, deteriorate and gradually lose their therapeutic value and aromatic quality. The life span vary tremendously from one botanical to the next. Key factors that can affect the shelf life of an essential oil include the following (Turek and Stintzing (2013) and (Life of Essential Oils and How to Make Them Last Longer) In: https://www.usingeossafely.com/shelf-life-essential-oils-and-how-to-make-them-last-longer/ Assessed 10.6.2018.

(i)Composistion of natural chemical constituents present in the essential oil.

(ii) The method of distillation.

(iii) The conditions and care used during the distillation.

(iv) The quality of the botanical used.

(v) The care in bottling, storage and handling,

(vi) The storage conditions of the oil once you have received it.

If the essential oil has deteriorated, the aroma will change, the oil will thicken and the essential oil will become clowdy. It is important to store essential oils under cold temperatures preferably at 4^0 C.

1.1.6 Laboratory

The laboratory also known as the insectary was of the size 4.88 metres (16 feet) by 3.66 metres (12 feet). The room was adequate in size. It was well ventilated and lighted and in good repairs. The concrete slabs 1m high from the floor made the performance of the experiments easier since the slabs were used as stands for the mosquito rearing cages.

Other facilities that were provided in the laboratory to ease experimental procedures included rearing cages, a dissecting microscope, personal protective equipment (overcoats, gloves, boots, goggles, masks), trays, basins, jars, volumetric cylinders, bowls, pippets, towels, curtains, electric bulbs, microscope, cloth filters, Whatman's filters, magnifying glasses, curtains, dehumidifying towels and mosquito food.

1.2 STATEMENT OF THE PROBLEM AND JUSTIFICATION

1.2.1 Statement of the Problem

Malaria is life-threatening and devastating parasitic disease transmitted by mosquitoes and in sub-Saharan Africa, *Anopheles gambiae* complex is the malaria vector. The disease is endemic throughout most of the tropics. Of the approximately 3 billion people worldwide who are exposed annually, more than 240 million develop symptomatic malaria causing nearly 1 million deaths of which 86% of deaths occur in sub-Saharan Africa. Those who are most at risk are young children and pregnant women. Children are at risk because they lack developed immune systems to protect against the disease (WHO, 2013; WHO, 2015). Pregnant women and their unborn children are 2.3 times more likely to suffer from malaria during this time, due to a lower immune system during pregnancy. Malaria is a major cause of maternal mortality and low birth weight (WHO, 2015).

The WHO African Region continues to carry a disproportionately high share of the global malaria burden. In 2016, the region was home to 90% of malaria cases and 91% of malaria deaths. Some 15 countries – all in sub-Saharan Africa, except India – accounted for 80% of the global malaria burden. In areas with high transmission of malaria, children under 5 are particularly susceptible to infection, illness and death; more than two thirds (70%) of all malaria deaths occur in this age group. The number of under-5 malaria deaths has declined from 440 000 in 2010 to 285 000 in 2016. However, malaria remains

a major killer of children under five years old, taking the life of a child every two minutes (WHO, 2015).

According to the latest *World Malaria Report*, released in November 2017, there were 216 million cases of malaria globally reported in 91 countries in the year 2016, up from 211 million cases in 2015. The estimated number of malaria deaths stood at 445 000 in 2016, mostly among children under the age of five in Africa who represent 77% of all malaria deaths (WHO, 2018). A similar number of deaths (446 000) occurred the previous year. The WHO African Region continues to carry a disproportionately high share of the global malaria burden. In 2016, the region was home to 90% of malaria cases and 91% of malaria deaths. Some 15 countries – all in sub-Saharan Africa, except India – accounted for 80% of the global malaria burden.

Because malaria causes so much illness and death, the disease is a great drain on many national economies. Since many countries with malaria are already among the poorer nations, the disease maintains a vicious cycle of disease and poverty (CDC, 2012).

WHO (2016) gives Regional aspect of malaria cases (c) and deaths (d):- African (194 million (c), 407,000 (d); Americas (875,000 (c), 650 (d); Eastern Mediteranean (4.3 million (c), 8,200 (d) South-East Asia (14.6 million (c), 27,000 (d); Western Pacific (1.6 million (c), 3,300 (d); World total cases (216 million (c), 445,000 (d).

1.2.2 JUSTIFICATION

In Kenya malaria is the most important infection. Endemicity of the disease ranges from holo-endemic at the coast and Lake Victoria regions to hypo-endemic in the plains (those lying approximately between latitudes 60^0 N and 40^0 S). Thus in the first instance, there is reason why malaria epidemics in Kenya and other sub-Saharan countries should be controlled through eradication of the vectors for which the natural herbicides under efficacy trials are intended. Secondly, if the essential oils under toxicological test are

proved efficacious in the organism, then their use in the control of mosquitoes would be more preferred than the use of chemical insecticides. This is so because the natural oils extracts from botanical sources will be environment friendly as a result of their fast biodegration, less persistence and hence eluded toxicity within a limited period of time. The chemical insecticides such as organochlorines have the characteristics of persistence in the environment, bioaccumulation in fat tissues of organisms and long period extended toxicity. Thirdly, the potency of these oil extracts has not been attempted in Kenya in respect of *Anopheles* mosquitoes, the malaria vector. This trial therefore will serve a good reference for the future researchers who will wish to undertake studies along the same line.

1.2.3 Significance of the expected results

It is expected that the results of this research will be useful to mankind. Once the LC_{50} is ascertained it will be applied in the field to control mosquito breeding as a major step in the control of malaria in Kenya using botanical essential oils. It is hoped that technical officers in the relevant governments i.e. Central and county governments will be trained on larvicides application for the control of mosquito breeding and consequently the prevention of malaria outbreaks. Importantly, these crude leaf extracts could be constituted intro powder, tablet or liquid forms for use in mosquito control. This move no doubt will encourage the commercial growing of the three plants with a view of selling their needed vegetative parts for the exploitation of larvicide development.

1.3 RESEARCH QUESTIONS

1.3.1 What concentration of the extracts from the plants *Chrysanthemum cinerariifolium* (Pyrethrum (P), *Eucalyptus camaldulensis* (E) *and Nicotiana tabaccum* (Tobacco (T) that can cause mortality of 50 and 90% of the mosquito larvae?

1.3.2 What are the synergistic extracts when the extracts of the three plants are alternately applied as P+E; P+T; and E+T and administered on the 3rd instar lavae of *A. gambiae s.s* **Giles** mosquito?

1.3.3 What are the antagonistic extracts when the extracts of the three plants are alternately applied as P+E; P+T; and E+T and administered on the 3rd instar lavae of *A. gambiae s.s.* **Giles** mosquito?

1.3.4 What will be the resistance ratio (RR) of the tested *Anopheles gambiae* larvae against the extracts applied independently?

1.3.5 What level of persistence is expected of the crude oils when applied in the mosquito habitat?

1.4 HYPOTHESIS

The crude oil extracts from the natural plants *Chrysanthemum cinerariifolium, Eucalyptus camaldulensis* and *Nicotiana tabaccum* will not efficaciously be toxic to 3rd instar larvae of *Anopheles gambiae s.s.***Giles** mosquito and may neither kill 50% and 90% of the larvae population nor likely to be synergistic, antagonistic, offer any resistance to the larvae and be persistent in the environment when applied.

1.5 OBJECTIVES

1.5.1 Overall Objective

To determine the lethal concentration (LC_{50} and LC_{90} ppm) values of crude oil flower and leaf extracts from *Chrysanthemum cinerariifolium, Eucalyptus camaldulensis* and *Nicotiana tabaccum* that will kill 50% and 90% of 3rd instar larvae of *Anopheles gambiae s.s.***Giles** mosquito, and be synergistic, antagonistic, offer resistance to the larvae and persistence to the mosquito habitat.

1.5.2 Specific objectives

1.5.2.1 To determine the median lethal concentrations (LC_{50} and LC_{90}) to kill 50 and 90 percent of the treated 3rd instar larvae of *Anopheles gambiae s.s.* **Giles** mosquito when each of the essential oils from *Chrysanthemum cinerariifolium, Eucalyptus camaldulensis* and *Nicotiana tabaccum* is applied independently.

1.5.2.2 To determine the **Synergistic** effects of the flower/ leaf extracts to kill 50 and 90 percent of the 3rd instar larvae of *Anopheles gambiae s.s* **Giles** mosquito when the flower and leaf extracts are alternately combined as of P+E, P+T and E+T.

1.5.2.3 To determine the antagonistic effects of the flower/ leaf extracts to kill 50 and 90 percent of the 3rd instar larvae of *Anopheles gambiae s.s* **Giles** mosquito when the flower and leaf extracts are alternately combined as of P+E, P+T and E+T.

1.5.2.4 To determine the **Resistance Ratio (RR)** of the mosquito larvae against each crude oil extract of the three plants.

1.5.2.5 To determine the **persistence** (**Residuals)** of each of the three essential oil extracts as a function of time when applied under light-dark photoperiods.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 INTRODUCTION

The three essential oils under investigation are derivatives from the natural plants, *Chryasathemum cinerariifolium* (Asteraceae), *Eucalyptus camaldulensis* **Dehnh** (Myrtaceae) and *Nicotiana tabaccum* (Tobacco L.) all commonly grown in Kenya and have been exploited commercially. These plants are grown in other countries of the world for economical reasons. In some countries tobacco smoke is used ceremonially to facilitate peaceful communications between those in ceremony, in others as Shamanic tool (throughout the Amazon), as entheotic powers, sometimes smoked during Ayahuasca ceremonies and added to the brew (http://forums.mycotobia.net/director), 2014 Php?ur/=http3A%2F%.2Fwww.bouncingbearbotanicals.com%,2Fsacred-tobacco-p-157.html, 2014).

Chrysanthemum cinerariifolium and *Nicotiana tabaccum* are grown in Kenya under controllable management system i.e. the crop harvest is delivered to the managing industry for farmers to be paid. *Eucalyptus* does not enjoy this facility but rather its marketing is by individual's initiative and through received orders for energy use, timber, fencing posts and house building.

In Kenya *Eucalyptus* and *Nicotiana tabaccum* essential oils have not been fully exploited while *Chrysanthemum cinerariifolium* has been used as an insecticide for many years, despite lack of documentation in its use as an insecticide.

2.2 *Chrysanthemum cinerariifolium*

Chrysanthemum cinerariifolium, which is perhaps the most widely used botanical insecticide, is derived from the flowers of *Chrysanthemun* which belongs to the family Asteraceae. This genus contains many species of which only a few e.g. *Chrysanthemum*

reseum and *Chrysanthemum cinerariifolium*) produce insecticidal substances which have been exploited at one time or the other.

The pattern of the world production of pyrethrum has been greatly influenced by the effects of world wars. Dalmatia remained the main source of pyrethrum up to World War 1, following which Japan took over as the principal producer. The late 1920s saw the introduction of pyrethrum into the highlands of Eastern Africa, including Kenya, Tanzania, Rwanda, and Zaire. Due to the higher flower yields per unit area and superior pyrethrins content, production increased faster in Africa than in any other part of the world. By 1941, Kenya overtook Japan as the main world producer and, following the outbreak of World War II, Japan ceased to be a significant producer (Burnett *et al.* (2002)

In Eastern Africa Kenya remains the main source of supply. The estimated total production of pyrethrum from all sources (Kenya, Tanzania, Rwanda, Tasmania, and Papua New Guinea) in 1992 was 18,100 metric tons to which Kenya contributed about 69% (MacDonald, 1994) and later 70% (Africa Business Start (2014). However, latest production data is not available. It is worthwhile to note that in Kenya, pyrethrum is cultivated almost entirely by small-scale farmers currently numbering between 50,000 and 60,000, who depend on the crop as their source of income (Gachie 2018)**.** Most of the world's supply of pyrethrin and *Chrysanthemum cinerariifolium* comes from Kenya, which produces the most potent flowers.

Figure 2.1 (Top): *Chrysanthemum cinerariifolium* flowers picking in a farm at Kiambereria, Molo.

Fig.2.2 (Bottom): Mature Pyrethrum flower showing head and petals. Courtesy of Molo Photographers.

Climatic conditions suitable for growing pyrethrum are found in four major regions in Kenya- Lake Victoria (Southern Nyanza Province), North Rift Valley, South Rift Valley and Mount Kenya region. One hectare accommodates 52,000 plants producing about 1,000 kg of dried pyrethrum flowers annually. This quantity yields about 25 kg of highly refined extract. Ready flowers are picked in intervals of two weeks with picking continuing for nearly a year from July to April. Although pyrethrum is a perennial crop, a typical plantation lasts for three to four years (www.kenya-pyrethrum.com/growing.html/Softkenya.com/farming/pyrethrum-farming/ 2014.

Marketing of pyrethrum in Kenya is under the direct control of the Government through the Pyrethrum Board of Kenya (PBK), whose mandate is to register and licence growers and to control production, extraction and marketing of pyrethrum in Kenya. *Chrysanthemum cinerariifolium* is also marketed through licenced marketing agencies based in Europe. Pyrethrum products from Kenya are of high quality and in great demand in the world market. Most of the pyrethrum produced is for the export market with the local market consuming less than 2% and the rest exported mainly to U.S.A (51%), Europe (30%), Asia/Pacific (14%) and Africa (5%) (Statistical Abstract 2003, Kenya). PBK stopped regular payments to farmers in 2008. Between 2001 and 2005 *Chrysanthemum cinerariifolium* products worth Kshs. 1.8 billion (US$18 million) could not be accounted for. Those who depended on pyrethrum farming for their livelihood in the past are counting their loses and hence the solution is liberalization (Mulagoli, 2011).

Pyrethrin as an insecticide in Kenya has been used in its solid (mosquito coils), powder and liquid forms to control insect vectors, however, scientific information on its application on mosquito control is not documented. There are forty four compounds of both pyrethrin and pyrethrods. The later are referred to as synthetic pyrethroids and are relatively more stable in air and light than the pyrethrins (natural derivatives). While all

the six natural pyrethrins (pyrethrin 1; cinerin, jasmolin 1, pyrethrin II, cinerin and jasmolin II) decompose in air and light with loss of insecticidal activity and only little stabilization is feasible {(United States Environmental Pollution Agency (USEPA) (2017).

Pyrethroids: There are two types **o**f pyrethroids. Type 1 have a negative temperature coefficient, similar to that of DDT, whereas type 11 have a positive temperature coefficient, showing increased kill with increase in ambient temperature. The stimulating effect of pyrethroids is much more pronounced than that of DDT. Pyrethroids dominated world insecticide use from the 1980s to the start of the current century representing an example of synthetic pesticide chemistry based on botanical model. As modern pyrethroids bear little structural resemblance to the natural pyrethrins, their molecular mechanism of action differs as well (Soderland , 2012; Khater, 2012).

2.3 *Eucalyptus*

Eucalyptus is a diverse genus of flowering trees (and a few shrubs) in the myrtle family, Myrtaceae. Members of the genus dominate the tree flora of Australia. There are more than 700 species of *Eucalyptus* mostly native to Australia and a very small number are found in adjacent areas of New Guinea and Indonesia and one as far as far north as the Philippine archipelago and Taiwan (Blakely, 1965; Benett, 2014). Only 15 species occur outside Australia, and only 9 do not occur in Australia. The generic name is derived from the Greek word Ev (eu), meaning “well”, and κ∝λυ™πτος (kaluptos/kalyptos), meaning cover, “well-covered”, which refers to the operculum on the calynx that initially conceals the flower {(The International Plant Names Index (IPI), (2015)}

Figure 2.3: *Eucalyptus camaldulensis* mature tree at Kiambereria, Molo.
(Source: Molo Photographers**).**

Figure 2.4: *Eucalyptus camaldulensis* mature leaves for sampling at Kiambereria, Molo.
(Source: Molo Photographers**).**

Eucalyptus was first introduced from Australia to the rest of the world by Sir Joseph Banks, a botanist on the cook expedition in 1770. It was subsequently introduced to many parts of the world, notably Califonia, Portugal, South Africa, Uganda, Israel, Galicia and Chile. Several species have become invasive and are causing major problems for local ecosystems, mainly due to the absence of wildlife corridors and rotations management.

2.3.1 Cultivation, uses, environmental effects and ecological problems

2.3.2 Cultivation

Species of *Eucalyptus* are cultivated throughout the tropics and subtropics including the Americas, Europe, Africa, the Mediterranean Basin, the Middle East, China and the Indian Subcontinent (International Plant Names Index (IPI) (2015)**.** Many *Eucalyptus* trees are known as *gum trees* because many species exude copious sap from any break in the bark (e.g. Scribbly Gum). Several *Eucalyptus* are among the tallest trees in the world. *Eucalyptus regnans*, the Australian Mountain Ash, is the tallest of all. As of today, the tallest measured specimen named *Centurion* is 99.6m (327 feet) tall (Tasmania's Ten Tallest Giants). Only Coast Redwood is taller and Coast Douglas-for about the same; they are conifers (Gymnosperm). Six other *Eucalypt* species exceed 80 meters in height *Eucalyptus obliqua, Eucalyptus delegatensis, Eucalyptus deversicolor,Eucalyptus nitens, Eucalyptus globulus* and *Eucalyptus viminalis*, (Everett, 2013).

2.3.3 Uses

Eucalyptus have many uses which have made them economically important trees, and have become a cash crop in poor areas such as Timbuktu (a town in the west African nation of Mali situated 15 km. north of River Niger) Africa, (World Watch Institute, 2007) and the Persian Andes (Luzar, 2007) despite concerns that the trees are invasive in some countries like South Africa (Benett 2014; Heuler, 2013). Due to their fast growth, the foremost benefit of these trees (Karri and Yellow box varieties) is their wood, use as

ornament, timber, firewood and pulpwood. *Eucalyptus* is also used in a number of industries, from fence posts and charcoal to cellulose extraction for biofuels. Fast growth also makes *Eucalyptus* suitable as windbreaks (as in the case of blue gum, *E.globulus* in Califonia for highways) and to reduce erosion, while they are also admired as shades and ornamental trees in many cities and gardens. They can also be used in other areas which incude: lowering the water table and reduce soil salination, used as a way of reducing malaria by draining the soil e.g. as it happened in Algeria, Lebanon, Sicily (Bahadaret *et al.* 2016), elsewhere in Europe, and Califonia (Wolf and DiTomaso (2015) habitat for mosquito larvae, its oil from leaves used in food supplements, especially sweets, cough drops and decongestants, has insect repellent properties, its nectar as high-quality monofloral honey dyesmaking, (Moore *et al.* 2018). *Eucalypt* wood is also commonly used to make digeridoos, a traditional Australian Aboriginal wind instrument.

2.3.4 Environmental effects and ecological problems

Eucalyptus trees can also be an ecological disaster by draining land of its water. Similarly *Eucalyptus* forest tend to promote fire because of the volatile and highly combustible oils produced by the leaves, as well as the plantation of large amounts of litter which is high in phenolics, preventing its breakdown by fungi and thus accumulates as large amount of dry, com bustible fuel (Fensham and Fairfax (2009**).** Consequently, dense eucalyptus plantings may be subject to catastrophic firestorms. For example, mature Tasmanian blue gum trees creat a safety hazard in public places because they tend to drop limbs. Leaves and branches decompose very slowly. Due to flammable plant compounds, dense growth of fine branches and leaf and branch litter, groves are highly combustible and increase the risk of fire under drought conditions. The flowers are attractive to native humming birds, but the nectar has been implicated in clogging their beaks, causing the birds to starve. Frost dieback can exacerbate accumulation of dry, flammable leaves and branches

making fire danger extremely high. *Eucalyptus* obtain their long-term fire survivability from their ability to regenerate from epicormic shoots and lignotubers (Fensham and Fairfax (2009)**,** or by producing serotinous fruits. The 1991 Oakland Hills firestorm which destroyed almost 3,000 homes and killed 25 people was partly fuelled by large numbers of *Eucalyptus* close to the houses (Wheat/Corn Flour Mill (2017)**.** What the blue gum, *Eucalyptus* globules have in common rather than being allelopathic (inhibiting germination and growth of native plant species) is that there is a layer of leaf litter under them that suppresses germination and growth of other plants because it forms asphysical barrier to the soil (Million trees Permalink, 2013).

2.4 *Nicotiana tabaccum*

The word tobacco may refer either to the various species of broad-leafed plants comprising the genus *Nicotina* of the nightshade family or to the dried leaves of these plants. There are more than 70 species of tobacco, of which 45 are native to the Americas (http://www.lycos.com/info/tobacco-plants, 2010). By some accounts in America tobacco has been cultivated for a very long time, 10,000 years and the plant began to be cultivated about 8,000 years ago in South America.

Figure 2.5 *Nicotiana tabaccum* plant in a Marakisi farm

Fig. 2.6 *N. tabaccum* leaves under drying

The tobacco producing countries worldwide in 2016 {(in 1,000 metric tons-(mts)} were China (2805.62 mts), India (761.32 mts), Brazil (675.55 mts), U.S.A. (285.18 mts), Indonesia (196.15 mts), Zimbabwe (172.27 mts), Zambia (124.64 mts), Pakistan (116.16 mts), Tanzania (102.47 mts) and Argentina (93.67 mts) (Statista, the Statistics Portal, 2016).

Nicotiana tobacum is not found wild and may be a hybrid of other species. Its use and cultivation spread throughout most of South and North America. Some authorities have it that tobacco was used by native cultures of America by around 3000 BC and has a long history of ceremonial use in native America cultures. It has played an important role in the political, economic and cultural history of the United States and is the largest non-food crop by monetary value in the world today (http://en.wikipedia.org/wild/Nicotiana, 2010).

Although cotton is grown on more surface area, tobacco is the most widely grown nonfood crop in the world; it is produced in approximately 124 countries and on every continent. In consumption it most commonly appears in the forms of smoking (cigarettes or pipe), cigars, chewing, snuffing, or dipping tobacco, or snus/snuff, (http://en.wikipedia.org/wiki/Tobacco #cite-ref-1, 2010).

However, over 80% of the world production is consumed as cigarettes, currently estimated nearly 5.6 trillion annually. China, the United States, Brazil and India produced over 60% of total world production in 1995, which was estimated at 6.8 million tones (Hu and Lee (2015); while Eriksen *et al.* (2012) states that in the year 2007, four countries (China, Brazil, India and the United States) produced two- thirds of the world's tobacco.

Tobaccos, grown in 124 countries, occupy 3.8 million hactares of agricultural land. Worryingly, 20,000 hactares of forests across the globe annually are being cleared to cure

tobacco. The World Health Organization (WHO) and UNCTAD (2015) Food and Agricultural Organization (FAO, FAQ, UNICEF, WFP, and WHO 2018), estimated that nearly 3.9 million hactares of land throughout the world were under tobacco cultivation during the year 2009 with a global production of the crop exceeded 7.1 million tonnes at the same time (The Tobacco Atlas, Growing Tobacco (Statistics and facts about the tobacco industry,www.tobaccoatlas.org/industry/growing-tobacco/text/, Accessed 2013). Tobacco production of the world is largely dominated by China, Brazil, India and the United States. The global tobacco industry produced approximately 7.6 million metric tones in 2011. The leading producer is China, which harvested some 3.2 million metric tonnes of tobacco in the same year. Other major producers are India and Brazil, (www.statista.com/topics/1593/tobacco/, Accessed 2013). According to FAO figures Pakistan had varieties of tobacco cultivated on nearly 50,000 hectares in the year 2009 with an aggregate tobacco production of nearly 105,000 tonnes (Arshad, 2011). The total value of global tobacco production stands somewhere between 600 and 700 billion US dollars. The leading tobacco company worldwide is Philip Morris International, generating some 31 billion US dollars of revenue in 2012 (Tobacco – Statista Dossier, 2013).

In 2009, six of the 10 tobacco producing countries, including India, had undernourishment rates between 50% and 27% resulting from cleared land for tobacco instead of food production. For example about half of the tobacco leaves produced in developing countries in Africa and Asia are cured (dried out for cigarette production) with wood. An average of 7.8 kg of wood is needed to cure 1 kg of tobacco. The Indian Institute of Forest Management, Bhopal estimated that the historical use of fuel wood between 1962 and 2002, for tobacco curing and manufacture of cigarettes, has destroyed

and degraded 680 sq km of scrub forests, or nearly 868 million tonnes of wood through successive extraction (Kounteya, 2012).

China has more tobacco consumers than any other country, with an estimated 301 million tobacco users, India comes in second with 275 million users. Sixty four percent (64%) of tobacco users smoked manufactured cigarettes, although smokeless tobacco use, such as loose-leaf chewing tobacco in India (206 million users) and Bangladesh (www.medicalnewstoday.com/articles/249233.php, 2013). The share of tobacco produsedin the developing world increasing from 57% in 1961 to 86% in 2006, the share of land under tobacco worldwide increasing from 70% in 1961 to 90% in 2006 (Jew *et al*. (2017). Tobacco cultivation was introduced in Kenya in 1907 and it is currently grown by about 30,000 small scale farmers on about 15,000 hectares of land. Tobacco growing is mainly through contract farming and involves three main players: Alliance One Kenya (AOK), Mastermind Tobacco Kenya (MTK) and British American Tobacco Kenya (BATK). BATK controls 70% of the market share for finished tobacco products, MTK 29% while the remaining 1% is shared between other manufacturers and importers (University of Bath (2018).

As the use of tobacco grew, some people became concerned about its users: King James 1 of England in 1604; John Hill (an English doctor in 1761); American Samuel Thomas Von-Soemmering (in the year 1797); in 1912, American Dr. Isaac Adler; in 1929, Fritz Lickint of Dresden, Germany; and in 1964, Luther L.Terry, UD, Surgeon General of the United States Public Health Service, released the report of the Surgeon General's Advisory Committee on Smoking and health. All these warned against smoking and cancers of the lungs, nose, lip , and lung respectively. The latter's warning was based on over seven thousand scientific articles that linked tobacco use with cancer and other diseases. This report led to laws requiring warning labels on tobacco products and to

restrictions on tobacco advertisements. From then tobacco smoking in the United States begun to decline. By 2004, nearly half of all Americans who had over smoked had quit (CDC, 2017).

The usage of tobacco is an activity that is practiced by some 1.1 billion people, and up to 1/3 of the adult population. The world Health Organization reports it is the leading preventable cause of death worldwide and estimates that it currently causes 5.4 million deaths per year (WHO, 2018; US Surgion General's Report 2004). Rates of smoking have levelled off or declined in developed countries, however, they continue to rise in developing countries. The poisonous principle in tobacco is an alkaloid nicotine, which in the pure state is a colourless fluid; slightly heavier than water. "Black leaf" tobacco contains only 2.7% *nicotine*. "Black leaf 40" is a concentrated tobacco extract containing 40% *nicotine* sulphate and is used at strengths varying from one part in 800 parts of water to one part in 1,600 parts. *Nicotiana rustica* (wild tobacco) contains about 10 times the nicotine of *N.tabacum*. (http:en.wikipedia.org/wiki /tobacco#cite-ref-I, 2010)

2.5 Malaria

Malaria occurs in nearly 100 countries worldwide. According to the 2018 world malaria report, there were more than 216 million malaria cases in 2016. An estimated 445,000 people died from malaria in 2016, 90% of them in sub-Saharan Africa. Most of those who die are women and children under the age of 5.

The principal vectors of malaria are *Anopheles gambiae*s.s. **Giles** and *Anopheles funestus.* Oringanje *et al*. (2011) and Sanden *et al*. (2012) identified *Anopheles gambiae s.l*., *Anopheles gambiae s.s.* **Giles** and *Anopheles funestus* as the malaria vectors. *Anopheles gambiae s.l.* is split into six sibling species defined by their reproductive barriers and cytotaxanomic distribution.

There are four species of human malaria, all caused by species of genus *Plasmodium* (P). The species are *P. falciparum, P. vivax, P. ovale and P. malariae* (WHO,2014)**,** describes these *Plasmodium* and their incubation periods: *P. falciparum* (malignant tertian malaria, subtertian), *P. vivax* (benign tertian malaria), *P. ovale* (ovale tertian malaria) and *P. malariae* (quartan malaria). The length of sporogony which determines plasmodia transmission depends on temperature. At 28^0C, spology takes from 9 to 10 days for *P. fulciparum*, 8 to 10 days for *P. vivax*, 12 to 14 days for *P. ovale* and 14 to 16 days for *P. malariae*. At 20^0C,it takes 3 weeks for *P. falciparum*. When the temperature is below 18^0C, *P. falciparum* is generally no longer transmitted. This is also true for *P. vivax* and *P. malariae* at temperatures below15^{0C}. Summer isotherms corresponding to minimal temperatures and allowing transmission delineate the regions where each *Plasmodium* can be endemic. LabSpace OpenLearn (2014)**,** describes malaria incubation period briefly: the infected person may feel normal from 7 to 21 days when infected with *Plasmodium* parasites, *P. falciparum*has a shorter incubation period (7 to 14 days) than *P. vivax* (12 to 18 days),*P. ovale* (12 to 18 days) *Plasmodium malariae* tends to have a much longer incubation period, (18 to 40 days).

There exists a large variation in the capacity of different *Anopheles* species to transmit different kinds of plasmodial species. For given *Anopheles* and *Plasmodium* species, vectorial capacity may vary according to geographical origin. The capacity is largely genetically determined and corresponds to a co-adaptation between *Plasmodium* and vector. Of more than 480 species of *Anopheles* only about 50 species transmit malaria, with every continent having its own species of these mosquitoes. *Anopheles gambiae* complex in Africa; *Anopheles freeborni* in North America; *Anopheles culicifacies, A.fluriatilis, A. munimus, A. philippinensis, A. stephensis*, and *A. sundaicus* in the Indian subcontinent*; Anopheles leucosphyrus, A. lateens, A. cracens, A. hacker, A.dirus*, have

been identified as the vectors for the transmission of *Plasmodium* knowlesi (United Nations, 2011).

2.5.1 *Anopheles* mosquitoes taxonomy

Throughout the world, 528 species of *Anopheles* mosquitoes have been discovered, and approximately 50 of them pay an important role as vectors of malaria, filarial nematodes and ancephalitis virus. Among these, at least 20 taxa represent species complexes, which comprise about 115 sibling species members. The existence of species complexes in *Anopheles* vectors leads to difficulty in precisely identifying sibling species (isomorphic species) and / or subspecies (morphologic/cytologic/ polymorphic races). In addition, these members may differ in biological characteristics (e.g. microhabitats, resting and biting behaviour, sensitivity or resistance to insecticides, susceptible or refractory to malaria parasites), which can be used to determine their potential for transmitting disease agents. Incorrect identification of individual members in *Anopheles* species complexes may result in failure to distinguish between a vector and non-vector, and lead to complications and/ or unsuccessful vector control (Choochote and Saeung, 2013).

Giles, (1902) defines *A. gambiae* complex: This is a complex of at least six (6) morphologically indistinguishable species of mosquitoes in the genus *Anopheles.* This complex includes the most important vectors of malaria in sub-Saharan Africa particularly of the most dangerous malaria parasite, *Plasmodium falciparum* (Walter Reed Army Institute of Research, 2013). It is one of the most efficient malaria vectors known (Otarigho and Falade (2013). This species complex consists of six species (Otarigho and Falade (2013): *Anopheles arabiensis, Anopheles bwambie, Anopheles merus,Anopheles melas, Anopheles quadriannulatus, and Anopheles gambiae s.s.* **Giles.**

2.6 External morphological differentiation

2.6.1 Morphological differentiation

Morphological differentiation is of great importance particularly will be useful in identifying species when collecting adult mosquitoes from the field. The following characters have been investigated and documented (Buhura et al. (2016); Stanuszek, 2013); Northern Territory Government, 2013; (profwaqarhussain.blogspot.com/1212/10/ differentiate-culexAnopheles-and-aedes.html;www.majordifferences.com/2013/10/ difference-between-Anopheles-and-vs.html#U8VL90BvIX 2013) and will be used to identify male and female *Anopheles* mosquitoes:

Table 2.1 Differentiating features of vector mosquitoes

Feature	Mosquito Species		
	Anopheles	*Culex*	*Aedes*
Colour			
Palps	Same as proboscis in length	Female: shorter than proboscis Male: longer than proboscis	Fem: shorter than proboscis Male: same as proboscis - clubb at the tip
Wings	With white and black spots	-Wings are unspotted -Wing scales narrow	Without white and black spots
Legs	-With or without white rings -Hind legs held outstretched	-Without white rings -Hind legs curled up over the back	-With white rings(legs/abdomen) back - Hind legs curled upwards
Body	Body is slender	Body is stout	__
Proboscis	Is line with body Male: Straight line with body Female: Proboscis and body at an angle	Proboscis at an angle to surface	Proboscis and body to an angle
Head	__	___	Slightly bent downwards
Antennae Abdomen	Male: Hairy Female: Plume-shaped	Blunt-tipped abdomen Abdomen sterna covered with scales	Pointed abdomen with pale band basally
Thorax	___	No spiracular/ postspiracular bristles	___
Eyes	__	__	Well separated eyes

The same writers have given morphological differentiation of the immature stages of *Anopheles* and *Culex* species of mosquitoes:

Table 2.2 Immature stages of *Anopheles* and *Culex* species of mosquitoes

Stage	*Anopheles*	*Culex*
Eggs	Laid singly and have air floats	Laid in cluster called raft with no air floats
Larvae	-Head is longer than broad - Feeds on water surface No siphon tube	-Head is rounded -Feeds below water surface (called wriggler)
Pupa	Respiratory trumpets short and broad	Respiratory trumpets are long and narrow

2.6.2 Leg banding

The Africa populations of the vector species *gambiae* and *arabiensis* can be differentiated from *quadriannulatus* and *merus*. The differences are that the hind tarsal segment in the *gambiae* and *arabiensis* are pale bandings three in number and two are short and similar in length while the last and third segment is small approximately half of each of the two. In *merus* and *quadriannulatus* there are likewise three pale bandings of which the first two anterior bandings are equal in length and longer than those of *gambiae/arabiensis* and are twined. The posterior third banding is twice the length of the *gambiae/arabiensis* (Kabbale et al. 2016) Sharp *et al*. 1989).

2.6.3 Bionomics

Anopheles species can be distinguished through larval habitat. *Anopheles* larvae are adapted to a variety of aquatic habitats, but occur predominantly in ground water. Some species require aerated water, others brackish water and some in habitat cavities such as tree holes (Plumbeus Group, subgenus *Anopheles*) and the axils of epiphylic plants

(subgeneus Kerteszia, except for *A. bambusicolus* which inhabits bamboo). Specific habitats contain stagnant water or water that is slowed down by vegetation or objects in specific niches occupied by the larvae. The larvae of all species feed at the water, where they attach to the surface film by spiracular apparatus, palmate setae and special notched organs on the prothorax. They rotate the head 180^0 so that particles of food at the surface can be swept into the mouth by currents produced by the mouth brushes. The larvae generally rest with the end of the abdomen against objects and are therefore found in greatest numbers in areas with emergent vegetation at the margins of the habitats.

Distinguishing features of vector mosquitoes are as indicated in the Figure2.5 . Among the mosquitoes there are two groups that suck human blood and may transmit disease.

The anophelines; the genus *Anopheles* is best known for its role in transmitting malaria, but in some areas it can also transmit filariasis.

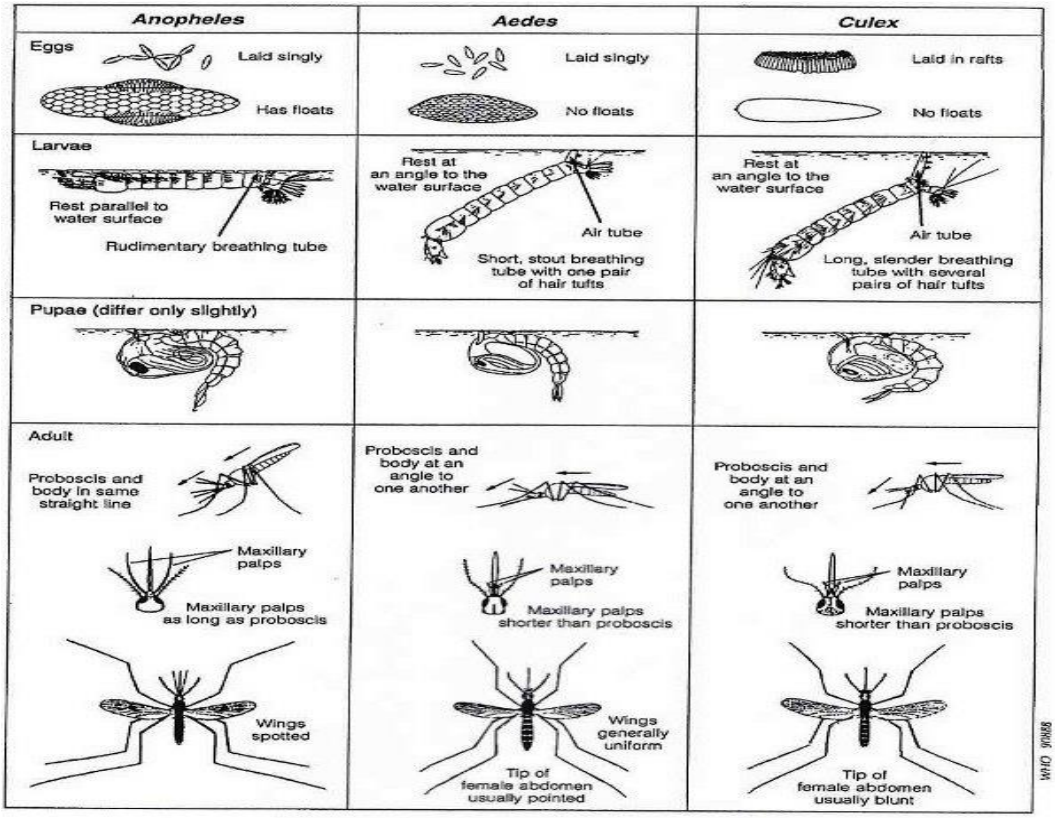


Fig 2.7 Some of the main characteristics for differentiating *Anopheles*, *Aedes* and *Culex* mosquitoes (Source: ISGlobal Barcelona Institute of Global Health (2017).

The difference between*A.gambiae s.s.***Giles** and A. *arabiensis* are quite marked. Both species are highly anthropophilic but, when alternativee mammalian hosts are available, *arabiensis* shows a much greater tendency to feed on animals. Since domestic animals in many areas are kept outside or in flimsy shelters at night, it is not surprising that *arabiensis* shows a greater tendency to feed and rest outdoors. *A. arabiensis* also appears to be relatively exophagic and have been caught biting outdoors immediately after sunset and before sunlight, potentially circumventing some of the protective effects of ITNs (Dambach *et al.* (2018); and also described as zoophilic, exophagic, and exphilic species (Map malaria atlas project. In:www.map.ox.ac.uk/explore/mosquitoe-malaria-vectors/ bionomics/Anopheles-arabiensis/ 2013). Studies in Taveta county, Kenya coast indicate that malaria transmission is occurring both indoors and outdoors. The main vectors are *A. arabiensis, A. funestus* and *A. coustani* indoors while *A.coustani* is playing a major role in outdoor transmission (Mwangangi *et al*. (2013). A study which was carried out in the villages of Kangichiri, Mathangauta, Kiuria, Karima and Kangai in Mwea Central Kenya indicated that *A. arabiensis* is highest in irrigated rice agroecosystems, intermediate in irrigated French beans agroecosystems, and lowest in the nonirrigated rice agroecosystems. In the use of insecticide sumithion (irrigated rice ecosystems) and dimethoate and alpha cypermethrin (irrigated French beans agroecosystems), *A. arabiensis* adult survivorship lower in irrigated rice ecosystems than in irrigated French beans agroecosystems. These findings indicate that agricultural practices may influence the ecology and behaviour of malaria vectors and ultimately the risk of malaria transmission.

In Ghana Akogbeto *et al.* (2018) observes that A. *melas* demonstrate high exophily while *A. gambiae*s.s. **Giles**bit people more frequently indoors and did so more often during the dry season than in the rainy season. In the experimental huts in two regions of north-east

Tanzania Kitau *et al*. (2012) found out that LLINs and ITNs treated with pyrethroids were more effective at killing A. *gambiae* and *A. funestus* than *A. arabiensis*. This could be a major contributing factor to the species shifts observed in East Africa following scale up of LLINs.

2.7 *Anopheles* mosquitoes distribution in Kenya

2.7.1 Kenyan Coast

Past and recent studies cover both low land and highlands stretching from the coastal region to Western highlands including the Kano plains in the lake region. Mwangangi *et al.* (2013) in their study on the role of *Anopheles arabiensis* and *A. coustani* in indoor and outdoor malaria transmission in Taveta district, Kenya found in their mosquito collection that that malaria transmission was majorly attributed to *A. gambiae s.l.* and complimentary by *A. funestus* as opposed to *A. arabiensis* and *A. coustani*. The results produced by Kipyab *et al.* (2013) by a study carried out in Malindi through entomological sampling carried out in Garithe village located 27 km north of Malindi town, Kenya showed different results to those of Mwangangi *et al.* (2013) in Taveta district both lying in the same region. The results were that A. *merus* comprised 77.8% of the 387 *A. gambiae s.l.* adults that were collected. Other sibling species of *A. gambiae s.l.* identified in the study included *A.arabiensis* (3.6%) and *A. gambiae s.s.* **Giles** (8%). The researchers observed that the coastal part of Garithe consists of mangrove trees and the area experiences high tides every month leaving pools of water during the low tides. These pools of salty water provide suitable habitats for *A. merus* breeding. The area also has numerous pockets of man-made ponds; *melas* **Theobald** and *merus* **Donitz** occurring respectively on the West coast and on the East coast. Conclusively these findings suggested that *A. merus* can play a major role in malaria transmission along the Kenyan coast.

Using data collected from 1990 to 2010 a period of 20 years, Mwangangi *et al*. (2013) employing various techniques examined vector density, species composition, blood-feeding patterns, and malaria *A. funestus* transmission intensity along the Kenyan coast. The results showed that in some areas along the Kenyan coast, *Anopheles arabiensis* and *Anopheles merus* have replaced *A. gambiae s.s.* **Giles** and as the major mosquito species. Further, in contrast to other studies it was observed that there has been a shift from human to animal feeding for both *A.gambiae s.s.* **Giles**(99% to 16%) and *A. funestus* (100% to 30%). Mwangangi also indicated that there has been on average a significant reduction in the abundance of *A. gambiae s.l.* over the years (IRR=0.94, 95% Cl 0.90-0.98), with density standing at low levels of an average 0.006 mosquito/house in the year 2010. Resulting from this it was concluded that reduction in the densities of the major malaria vectors and a shift from human to animal feeding have contributed to the decreased burden of malaria along the Kenyan coast. Vector species composition remains heterogeneous but in many areas *An. arabiensis* has replaced *An. gambiae*s.s. **Giles**as the major malaria vector.

In another study Mwangangi *et al*. (2013) collected indoors and outdoors in 4 ecologically different villages and examined for infection with *P. falciparum* sporozoites and blood feeding preferences using enzyme-linked immunosorbent assay (ELISA). A total of 4,004 mosquitoes were collected. The sporozoite transmission was found to be occurring both indoors and outdoors. The overall sporozoite infectivity was 0.68% (n=2,486) indoors and 1.29% (n=1,243) outdoors. In the four villages, Mwarusa had the highest EIRs with *An. arabiensis, A. funestus* and *A. coustani* contributing to 23.91, 11.96 and 23.91 infectious bites per person per year (ib/p/year) respectively. In Kiwalwa and Njoro outdoor EIR was significantly higher than indoors. In conclusion this study showed that malaria transmission was occurring both indoors and outdoors (Mwangangi *et al*.

2013). The main vectors are *An. arabiensis*, *A. funestus* and *A. coustani* indoors while *A.coustani* is playing a major role in outdoor transmission.

Walker *et al.* (2013) carried out a study in three villages at the Kenya Coastal region to determine *Anopheles* mosquito distribution. The villages were Jaribuni, Majajani and Mtepeni in Kilifi County. The density of *Anopheles* mosquitoes was highest in Jaribuni (*A. gambiae s.s.* ***Giles*** and *An. funestus* per household was 3.1 and 6.6 respectively). *A.funestus* was thirteen-fold and fifteen-fold larger in Jaribuni compared with Majajani and Mtepeni respectively. Rainfall (seemed to maintain larval stage of mosquitoes) and a permanent river to which Jaribuni lies was the favourable conditions.

In another study Kipyab *et al.* (2013) made an indoor collection of mosquitoes in Malindi County, Kenya and collected 387 *A. gambiae s.l.* adults. They found that *A.merus* comprised 77.8% of 387 *A. gambiae s.l.* collected. Other species identified included *A. arabiensis* (3.6%) and *A. gambiae s.s.* **Giles** (8%). However, since the human blood index for *An. merus* was 0.12, while sporozoite rate was 0.3% the findings suggest that *A. merus* can play a minor role in malaria transmission along the Kenyan coast.

O'Loughlin *et al.* (2016) in their study of population structure of *Anopheles gambiae*s.s. **Giles** along the Kenyan coast observed that in the tropics *Anopheles* mosquito abundance is greatest during wet season and decline significantly during the dry season as larval habitat shrink. Their findings suggest that along the Kenyan coast, seasonality and site specific ecological factors can alter the genetic structure of *A. gambiae s.s.* **Giles** population. Further O'Loughlin *et al.* (2016) suggested that population size fluctuations between wet and dry seasons may lead to variations in distribution of specific alleles within natural *Anopheles* populations and a possible effect on the population genetic structure.

Charlwood (2017) studied mosquito distribution in thirty villages in South-eastern Kenya-Malindi, Kilifi and Kwale. Environmental data were derived from remote-sensed satellite sources of precipitation, temperature, specific humidity, Normalized Difference Vegetation Index (NDVI), and elevation. Spatial analysis indicated positive autocorrelation of *A. arabiensis* and *A. funestus* transmission, but not of *A. gambiae s.s.* **Giles**, which was found to be widespread across the study region. The spatial clustering of high entomological inoculation rates (EIRs) value for *A.arabiensis* was confined to the lowland areas of Malindi, and for *A. funestus* to the southern counties of Kilifi and Kwale. Overall, *A. gambiae s.s.* **Giles** and *A. arabiensis* had similar spatial and environmental trends, with higher transmission associated with higher precipitation , but lower temperature, humidity and NDVI measures than those locations with lower transmission by these species and/or in locations where transmission by *A. funestus* was high.

2.7.2 The western plains.

Studies of mosquito distribution in the western plains of Kenya, namely the Kano plains which includes Kisumu City holds the earliest mosquito study area in Kenya. This dates as far back as 1929 when Graham suggested that a relationship existed between the onset of the long rainy season and increase in populations of *Anopheles gambiae* s.s. **Giles**

At Kombewa where malaria is holoendemic located on 34^0 30′ East and 0^0 17′South in Kisumu county; about 30km from Kisumu city mosquito distribution study was carried out by Omukunda *et al.*(2012a). A total of 1973 larvae were collected of which *A.gambiae s.l.* was abundant (56.9%). It was observed that farmland had higher proportions of malaria vectors (27.2% and 24.6% *A. gambiae*s.s. **Giles** and *A. funestus* respectively); followed by pastures during the rainy season.

In Asembo and Seme, Omukunda *et al*. (2012) carried out a study on the association of land cover with larval *Anopheles* habitats both in wet and dry seasons at an area of

3.216m by 3.216m ($10m^2$) located at 34^0 23′E , 0^0 11′S in Nyanza Province Western Kenya, approximately 50 km west of the city of Kisumu. In the wet season they collected 19,776 larvae which comprised 86% *Anopheles* larvae and on morphological identification *Anopheles gambiae s.l.* (a species complex of *A. gambiaes.s.* **Giles** and *A. arabiensis*) represented 50.69% of the total followed by *A. coustani* (21.7%). In the dry season 586 larvae were collected. Analysis of 1,078 of the larvae identified morphologically as *A. gambiae s.l*. by the PCR method revealed that 796 (73.8%) were *A. arabiensis*, 19.8% were *A. gambiae s.s* . **Giles** and 69 (6.4%) did not react in the test used. Obala *et al*. (2012) used pyrethrum spray catch for adult mosquitos' collection in Kopere village located in a sugar plantation region at an elevation ranging between 1,260 m and 1,440 m above sea level. The majority of the vectors captured indoors were *Anopheles gambiae s.s.* **Giles** only a small proportion consisted of *A. arabiensis.* A historical population decline of *A. gambiae s.s.* **Giles** associated with an increase of ITN coverage in Nyanza and Western provinces, Kenya is reported by Ochomo *et al.* (2017), also reported the disproportion of the 2 species in Western Kenya and found that the frequency of *A. gambiae s.s.***Giles** varied by site, with frequencies of < 15% at sites west of Kisumu and along the lakeshore (Asembo and Kisiani) but > 80% at sites further from the lakeshore (Busia, Bungoma, Kakamega and Malaba). They attributed the decline in *A. gambiae s.s.***Giles** at the two sites along the lakeshore (Asembo and Kisiani) to the rollout of LLITNs. Similar findings in the south of the region on the opposite side of Lake Victoria (Mbita County) were recently reported by Hancock *et al.* (2018). The present results might indicate that the same decline in the proportion of *A.gambiae s.s.***Giles** relative to *A. arabiensis* described above has occurred broadly in southern and western Kenya in the 10 years since the rollout of LLITNs.

2.7.3 Western Kenya highlands

A number of studies on the *Anopheles* mosquito abundance have been conducted in western Kenya highlands. Munga *et al.* (2013) on the response of *Anopheles gambiaes.l.* (Diptera: Culicidae) to larval habitat age in western Kenya highlands confirmed that *Anopheles gambiae s.l.* larvae were significantly more abundant (P= 0.0002) in habitats that were cleared every 10 days compared to the other habitats. In particular, there were 1.7 times more larvae in this habitat age compared to the ones that were cleared every 30 days. Hence a confirmation that age of the habitat significantly influences the productivity of malaria vectors in western Kenya.

Omukunda *et al*. (2012b) conducted a study on the effect of swamp cultivation on distribution of *anopheline* larval habitats in western Kenya. The study was conducted in Marani (a highland area located 34^0 48′east and 0^0 35′ south in Kisii county) and Kombewa (a low lying area located at 34^0 30′ East and 0^0 07′ South in Kisumu county, about 30km from Kisumu town western Kenya. In their study a total of 5023 anopheline were larvae counted for the months of February, May, August and November at both the sites – Kombewa (1973 larvae) and Marani (3050 larvae). In Kombewa lowland, *A. gambiae s.l.* was the most abundant (49.1%) in *anopheline* larvae collected while in the highland of Marani, it was *A. marshallii*(57.3%). Of the four species found in November, only two were present in August (*A. coustani* and *A. marshallii*). Two major malaria vector species were found, *A. gambiae* and *A.funestus*. Of the vector larvae collected, *A. gambiae s.s.* **Giles** from Marani, and *A. gambiaes.l.* from Kombewa were the most abundant (94.6% and 56.9% respectively. Their results also showed a higher percent of *A. gambiae s.s.* **Giles** (70.9%) than *A. funestus* (29.1%). They found that the distribution of larval breeding is confined to the valley bottom and that land use type influenced the occurrence of positive breeding habitats in the highland.

Igulu village, Ikolomani constituency, a malaria epidemic-prone area in western Kenya highlands has also been investigated in line with *anopheline* larval habitatas seasonality and species distribution. Eliningaya *et al.* (2012) carried out a study on this subject. A total of 51 aquatic habitats positive for *Anopheles* were surveyed. A total of 46,846 immature stages of mosquitoes were sampled from all the habitats during the 85 weeks of study and sampling being done weekly. Out of these *A. gambiae s.l.* accounted for 48.21% (n= 22,583). *A. funestus* larvae and pupae accounted for 11.59% (n=5,4428), other anopheline including *A. coustani, A. squamous, A. ziemanni* and *A. implexus* accounted for 9.46%% (n=4,433) and culicine larvae accounted for 30.74% (n=14,402). As a matter of time the researchers identified 616 *A. gambiae s.l.* larvae using PCR from random samples amongst which77.60% (n=478) were identified 154) as *A. gambiae s.s.* **Giles** while 18.34% (n=113) were *A.arabiensis* and specimens with no PCR product amplifications were constituted of 4.06% (n=25).

In their attempt Kweka *et al.* (2015), carried out a study on the productivity of malaria vectors from different habitat types in western Kenya highlands. The total samples made were 918 and of these 659 (71.8%) had water and 259 (28.2%) were dry. In all the 659 samples, 213 (32.3%) did not have any mosquito larvae whereas all the 147 (22.3%) samples that had *culicine* larvae also had *anophelines*. *Anopheline* larvae were found in 58.6% (n=386) of all samples, *culicines* in 31.4% (n=207) and mosquito pupae in 34.9% (n=230). Further to this study Ndenga *et al.* (2011) made another contribution by a study in three highland sites in western Kenya highlands, which were classified as natural, swamp, cultivated swamp, river fringe, puddle, open drain or borrow pit. All surveyed habitat types were found to produce adult malaria vectors. Mean adult productivity of *Anopheles gambiae sensu lato* in puddles ($1.8/m^2$) was 11-900 times higher than in the

other habitat types. Puddles were most unstable habitats accounting for 43% having water whilst open drains accounted for 72%.

In another study Onchuru *et al*. (2016) looked into the factors influencing differential larval habitat productivity of *Anopheles gambiae* complex mosquitoes in a western Kenya village. The study revealed that out of 500 adults subjected to PCR analysis 358 (71.6%) were *Anopheles gambiae* **Giles**, 127 (25.4%) *A. arabiensis*.

Siteti *et al.* (2016) contribution is also of great importance. They made a study on *Anopheles* species diversity and breeding habitat distribution and the prospect for focused malaria control in the western highlands of Kenya. Their results showed that *Anopheles gambiae sensu lato* was the most prevalent known vector contributing 95.4%, *A. funestus* and *A. arabiensis* each 2.3%.

Two estates within Kisii town namely Mwembe, and Maili mbili and 3 villages in Keumbu location in outskirts of Kisii town were investigated for mosquito distribution by Obino *et al*. (2013). They confirmed that *A. gambiae*s.s. **Giles**and *An. funestus* forms about 80% of mosquito density and is the main malaria vector in the highlands. They observed that environmental changes have an effect on the vector population. Lower vector productivity by mosquitoes was observed during the mid-rain season (April), when temperatures were at mean of 20^0C.

Lili *et al.* (2009) in their study on *Anopheles* species diversity and breeding habitat distribution and the prospect for focused malaria control in the western highlands of Kenya found that the spatial patterns of larval habitats had significant temporal variability both seasonally and inter-annually.

A study was conducted in three highland villages (Fort Ternan in Kericho County located on the slopes of Nandi Hills, and Lunyerere in Vihiga County) and one village (Nyalenda, peri-urban area Lake Victoria basin) by Imbahale *et al.* (2012). The results were that

Anopheles gambiae sensulato larvae were found in all study sites. *A.arabiensis* was more abundant (93%) in Nyalenda (Lake Victoria basin) and Fort Ternan (highland area; 71%). In Lunyerere (highland area) *A. gambiae* **Giles** comprised 93% of the total *A. gambiae s.l.* larvae. Larvae of *A. gambiae s.l.* mosquitoes were present in both temporary and permanent habitats with monthly variations depending on rainfall intensity and location. *Anopheline* larvae were found in man-made as opposed to natural habitats. Grassy habitats were preferred and were, therefore more productive of *anopheline* larvae compared to other habitat types. Weekly rainfall intensity led to an increase or decrease in mosquito larval abundance depending on the location.

2.7.4 Mosquito distribution in Rift Valley

A survey carried out for the period 1990 to 2009 by published and unpublished sources using reference source materials and the researchers' initiative for larval searches, indoor house catches, baited traps and finally performing species identification based only on morphological examinations at 194 sites (39%) using PCR methods at 298 (60%) sites and DNA probes at only six sites various species of *anopheline* were identified: *A. gambiae* 16, *A. arabiensis* 11, *A .merus* 0 (zero), *A. .funestus* 28, *A. pharoensis* 0 (zero), *A. nili* 0 (zero) Ministry of Health, Kenya (2016).

In the highlands of Kenya (North Nandi County in the highland areas of Kipsamoite and Kipsisiywa each with 7 and 10 villages, respectively, located at 1500m above sea level, Siteti *et al.* (2016) carried out a study to determine the abundance of malaria vectors. From the performed indoor adult mosquito collection they found that the known human malaria vectors were *A.gambiae s.s. Giles, A. funestus* and *A. arabiensis* comprising 11% in 3 species. *A. gambiaes.l.* was the most predominant known malaria vector species while the other two species were rare.

Mosquitoes sampled between 2007 and 2010 at thirteen sites across seven administrative provinces and ecological zones using CDC light traps. Over 524,000 mosquitoes were collected and identified into 101 species. The highest collection of anopheline mosquitoes was made in Tana Delta (31.8%), followed by *A. coustani* (20.4%) mostly in Kisumu (41.7%). *A. gambiae* were predominant in Baringo (45.3%) and *A. cellia* (squamosus in Naivasha (83.3%).

2.7.5 Mosquito distribution in Central Kenya.

Anopheles mosquito studies in the Central Province of Kenya have been extensively undertaken at Mwea Irrigation Scheme located in Kirinyaga County by Mwangangi *et al.* (2013). The study was carried out in three rice agro-village complexes namely, Mbui Njeru (planned rice cultivation village, 1000m above sea level), Kiamachiri (unplanned rice cultivation, 1200m above sea level) and Murinduko village (non-irrigated, 1350m above sea plane They found that *A. arabiensis* is the predominant vector of malaria, and the only sibling species of the *A. gambiae* species complex recorded in the area. Seven anopheline species were morphologically identified: *A.arabiensis* (82.1%), *A. pharoensis* (7.8%), *An. rufipes* (2.6%), *A. funestus* (2.5%), *and A. ruvorulum* (2.1%) *A. maculipalpis* 2, and *A. coustani* (1%). Larval density was highly variable in different habitat types and during the seasons. In Murinduko, water reservoirs had high larval densities. Other habitats were tree holes and rock pools.

In Mbui Njeru, favourable habitats were rice fields, canals and temporary pools. In Kiamachari larvae were found in four habitat types including rice fields, canal, marsh and temporary pools. In all the three villages rice fields and canals had highest densities of anopheline larvae than the other habitat types. Peridomestic habitats (pools) in the three villages had higher densities of *Anopheles* larvae.

2.7.6 Mosquito distribution in Eastern and North-eastern regions of Kenya

So far there is no data available on mosquito distribution in these two provinces.

2.8 Prevention and control of mosquitoes

2.8.1 Prevention

2.8.1.1 Mosquito education.

An educational effort that relates understanding of factors contributing to breeding involves the public need to be considered as an essential component of integrated mosquito control. The aim of this strategy is to create and encourage public awareness about mosquitoes, in order to foster and survival of these insects, whose activities adversely affect human health in a variety of ways. In this case advocacy of the use of a wide range of activities including media, the use of printed materials (pamphlets and posters which can illustrate mosquito life cycle, gender characteristics etc), talks and lectures supported by demonstrations could be given to school children, students or the general public and messages delivered through the use of radio and television will provide an effective communication system to promote mosquito education.

2.8.1.2 ITBNs and EDT

World Health Organization advocates the combined approach of ITBNs and EDT in its Roll Back Malaria initiative, but there has been little study of the combined efficiency of these approaches Dambach *et al.* (2014).

Scale-up of malaria control programmes has helped to greatly reduce malaria cases and deaths (WHO, 2013b; WHO (2018). Since 2000, eight African countries have experienced at least a 75% reduction in newly reported malaria cases; 37 countries outside of Africa have experienced at least a 75% reduction in newly reported malaria cases (WHO, 2013). Between 2000 and 2010, the number of reported annual malaria cases in 34 malaria-eliminating countries decreased by 85% from 1.5 million in 2000 to

232,000 in 2010 (WHO (2018) WHO,2011)**.** Most of these are attributable to *P. falciparum,* but *P. vivax* and *P. knowlesi* can also cause severe disease. Malaria deaths peaked at 1.82 million in 2004 and fell to 1.24m in 2010 (714,000 children < 5 years and 524,000 individuals ≥ 5 years); Over 80% of the deaths occur in sub-Saharan Africa WHO (2018); Murray *et al.* 2012)**.**

Visibly the standing evidence now at hand is that the three species in *Anopheles gambiae* complex occur symmetrically in Kenya including *A. gambiae sensu stricto* (hereafter referred to as*gambiae,A. Arabiensis,* and *A. Funestus* (Diptera: Culicidae) and are the ones with vectorial capacity. Again there is no doubt that their habitats are well known for example in sub-Saharan Africa the abundance and distribution of *Anopheles* mosquito species is dependent on environmental factors and ecological zones as well as on human population changes and anthropological effects, which may lead to land-use changes ultimately affecting vector distribution and abundance (Mattah *et al.* (2017). An accurate and predictive understanding of the geographic distributions of these species would permit efficient planning of strategies for targeted interactions i.e. prevention and control measures.

A potentially important target of malaria vector control is *anopheline* a larva, (Philips, 2014) gives an account of the three WHO elements of malaria control. First, it is the selective application of vector control by, for example, reduction of the numbers of vector mosquitoes either by eliminating, where feasible, or reducing mosquito breeding sites; destroying larval, pupal, and adult mosquitoes; and reducing human mosquito contact. Second, are early diagnosis and effective and prompt treatment of malarial disease (which also reduces a source of parasites for infection of mosquitoes as well as reducing morbidity and mortality, in all areas where people are at risk, whatever the economic and social circumstances. The third element is early detection of forecasting of epidemics and

rapid application of control measures. It is also stated that chemicals continue as the mainstay of mosquito control and broadly fall into five groups: petroleum oils (forming a film, prevent larvae and pupae from breathing through the surface of the water); pyrethrins and later synthetic derivatives, the pyrethroids; organochlorines which include dichlorodiphenyltrichloroethane (DDT) and dieldrin; the organophosphates such as malathion and temephos; and carbamates, such as propoxur. The last two groups are relatively dangerous in handling, requiring specialist equipment for their use. The rationale for use of these chemicals is indoor spraying with a persistent insecticide, i.e. , it remains active on the sprayed surface for weeks or even months to kill or at least repel the adult female mosquito.

In addition to the above elements the World Health Organization (WHO, 2013) further recommends that Larval Source Management (LSM) as the only method of malaria vector control. LSM is the management of aquatic habitats (water bodies) that are potential larval habitats for mosquitoes, in order to prevent the completion of immature stages of mosquito development, the egg, larvae, and pupae. WHO suggests four categories of LSM:

2.8.1.3 Integrated vector control (IVC)

As applied to mosquito control, the IVC approach draws on several strategies including prevention of mosquito–man contact, elimination of active and potential breeding sites and reduction or destruction of all developmental stages including adults.

The strategy is increasingly making use of biological control and environmental management intervention, including personal protection. Methods available for mosquito control, which could be incorporated into an integrated programme, include (1) Personal protection measures (WHO, 2006) referring to: (a) location of human settlements away from breeding sources; (b) screened housing, (c) use of insecticidal spray; (d) application

of repellents; (e) protective clothing; and (f) use of bed nets; (2) Mechanical control: involves the use of attractants especially light traps, for catching and killing mosquitoes with special interest in blood fed and gravid samples; (3) Behaviour manipulation: involves manipulating chemicals communication systems of mosquitoes in order to regulate their behavior. This may be achieved through the use of semiochemicals (chemical produced by animals and used as a means of communicating). Semiochemicals can be used as attractants, repellents and edcoys (traps), to have vector and pest mosquitoes away from their host (4) Host attractants: Mosquitoes make use of some specific chemicals attractants given off by the host in order to identify their specific hosts. For example, it has been demonstrated that *A. gambiae s.s.* **Giles**is specifically attracted by the odour of human feet, whereas *A. atroparvus* is lured by breath chemicals. Such attractants could be amplified for the removal of vector populations from the environment, (5) Natural repellents: Examples of such as the Neem (*Azadirachtaindica*) and several species of *Ocimum.* These are plants used traditionally to keep mosquitoes and other arthropods away from homes because of their repellent potency. Exophilic mosquitoes avoid the environment where such repellent vegetation occurs, (6) Zooprophylaxis: Several mosquito vectors are equally attracted to human and animals.

It has been demonstrated that placing domestic animals such as cattle, horses, pigs and sheep between human habitations and mosquito breeding sites diverts mosquitoes away from human populations. The role of domestic animals in protecting human population from mosquito bites and malaria has been observed in Italy as early as 1903 and 1933 where the biting and disease transmission mosquitoes turn to feed on domestic animals instead of humans.The use of DDT: WHO (2018) position statement on the use of DDT in malaria vector control and about the safety of DDT WHO (2018).

This position statement highlights WHO's commitment to achieve sustainable malaria control in the context of Stockholm Convention. The convention has given an exemption for the production and public health use of DDT for indoor application to vector-borne diseases, mainly because of the absence of equally effective and efficient alternatives. It was originally published in 2007 and subsequently revised in 2011. The decision adapted by the conference of the parties to the Stockholm Convention at the Expert Group fourth meeting from 3rd-5th December 2012 and in January 2013 the Dichlorodipheny ltrichloroethane (DDT) Expert Group assessed and recognized its continued need (WHO, 2013).

2.9 Mosquito control

2.9.1 Habitat modification: A permanent alteration to the environment, aimed at eliminating larval habitats, including landscaping, surface water drainage, filling and land reclamation.

2.9.2 Habitat manipulation: Temporary environmental changes to disrupt vector breeding, including water level manipulation, e.g. flushing, drain clearance to eliminate pooling. Also shading or exposing habitats to the sun depending on the ecology of the vector.

In addition to the above control methods WHO recommends other several methods:

2.9.3 Biological control: The introduction of natural enemies into rval habitats, including: **(a) (a) Predatory or larvivorous fish:** (*Gambusa spp.: Gambusia affinis, Gambusiaaffinis holbrooki; Tilappia spp.: T. zilli, T. macrochir, T. mossambica, T. nilotica, T.nigra, Oreochromis: Oreochromis spilurus spilurus and Carp*; **(b) Nematodes:***Romanomermis culicivorax*; has highest susceptibility to *Anopheles* mosquitoes. Also effective against species in the genera *Aedes*, *Culex* and *Psorophora*; **(c) Bacteria:***Bacillus thuringiensis (Bti)* and *Bacillus sphaericus;***(d) Fungi:** These are of

the genus *Coelomyces, Langenidium giganteum* and *Culicinomyces*. (e) **Mosquito predators –** *Taxorhynchites*: **(f) Plants** *Citronella* grass (Ceylon grass) **(g) Plant alkaloids**: extracted from the plant called *Annona squamosa* (Family: *Annonaceae*).

The role of fish as predators of mosquitoes is well investigated including the researchers Louca *et al.* (2009) who examined the potential of using native fish species in regulating mosquitoes in the floodplain of the Gambia River, West Africa. A semi field trial was used to test the predatory capacity of fish on mosquito larvae and was established that *Tilapia guineensis* and *Epiplatys spilargyreius* were effective predators, removing all late-stage culicine and anopheline larvae within one day.

2.9.4 Chemical use: WHO (2012) recommended the application of chemicals in mosquito control and of priority was DDT.

2.9.5 Larviciding: Further more WHO (2018) recommends use of larviciding (use of essential oil leaf, root or bark extracts from trees) for malaria control in sub-Saharan Africa.

Based on natural plants, the present study was to investigate whether the essential oil extracts from the flowers of*Chrysanthemumcinerariifolium*, *Eucalyptuscamaldulensis* and *Nicotiana tabaccum*plants can suppress mosquito larvae.

2.9.6 Genetic control. Although genetic control strategies have not been widely exploited for successful mosquito control a number of these techniques are available and can be applied for mosquito control and include:

Sterile-male or hybrid sterility*:* This involves artificially sterilizing large numbers of male mosquitoes and then releasing them into a wild population habitat. The intention is to encourage competition between the sterile males and the natural fertile males for female mating partners. This is expected to result in a substantial decline in progeny production for the next generation. By repeating this process for successive generations

the wild population should eventually decrease to extermination. In the fight against malaria, researchers have devised a way to create spermless male mosquitoes also to convince females to reproduce with them, according to a study in the proceedings of the National Academy of Sciences. Researchers created 100 male mosquitoes without sperm. In the experiment researchers "silenced" a gene called zpg needed for males to produce healthy sperm cells. When they encountered females, they were just as competitive as other males and could mate, but their rendezvous did not result in offspring (English, 2011). In a similar trial Chambers (2011) modified male *Aedes polynesiensis* mosquitoes and released into field cages holding field-collected, virgin females and field- collected wild type males.

The outcome indicated that mass release of modified *Aedes plynesiensis* mosquitoes could result in the suppression of *Aedes polynesiensis* populations. But, Stone *et al.* (2013) on his attempt to provide insight on which vector control methods are likely to be most synergistic, describes a form of sexual coercion, male harassment, whereby the repeated attempts of males to copulate are costly to females because the operational sex ratio becomes heavily skewed over a short timeframe and females potentially encounter males at far greater rates than normal. That male harassment occurs in mosquitoes has been demonstrated in *Anophelesgambiae s.s.* **Giles**: females that were subject to males for only 3 days had 2-d shorter median lifespan than females that were not.

Cytoplasmic incompatibility: This technique has been suggested for a number of mosquito species where incompatible strains have been observed, such as *Culex* pipiens and *A. scutellaris*. In this method certain strains of the same species of mosquitoes when crossed produce a progeny consisting of different reproductive categories. It is possible to use one or two modifications: production and release of the hybrid, which will complete with the target vector for mating partners.

Conditional lethals: In these methods it expected that some genes controlling adaptability to temperature for example, produce normal effects under certain temperatures ranges; at higher or lower temperatures they produce maladjusted or lethal effects. This phenomenon has been observed in some mosquitoes including *Aedes aegypti*. A strain of this species has been formed which when reared at 27-28°c produces normal adults in a 1:1 sex ratio, however, when reaming occurs at 30-34°c half the progeny were fameless and the rest inter-sexes. The latter category resembles normal females and will mate and receive sperm, but will produce no eggs (Ageep *et al.* (2014).

Plasmodium-refractory mosquitoes: Mosquitoes react differently to *Plasmodium* infections. Whereas some individuals are susceptible to the disease others are resistant to the pathogen. It is anticipated that the gene responsible for the resistant behavior can be identified and incorporated into microbial symbiont of the mosquito midgut. Once established in the mosquito midgut, the resistant characteristic will be passed on from the mother to the offspring, with consequent propagation among the wild population.

Zoophilic mosquitoes: A large number of *Anopheles* species are incapable of transmitting human malaria because they are zoophilic and feed on mainly on animals. This biting behavior has been demonstrated in *A. quadriannulatus,* one of the six sibling species of the *An.gambiae* complex. On the other hand *A. gambiae s.s.* **Giles** and to a lesser extent, *A. arabiensis*, are anthropophilic. Anthropophilic habits coupled with the longevity of these two species is undoubtedly responsible for the predominance of the greater proportion (not less than 80%) of the world's malaria in tropical Africa, the exclusive zone of the siblings. If the genes causing zoophilic behavior in *A.quadriannulatus* could be manipulated to fixation in the wild population of *A.gambiae s.s.* **Giles** and *A. arabiensis,* the uncontrollable African malaria problems could be solved.

2.9.7 Toxicology as applied to organisms

2.9.7.1 Toxicology

It was Rowe (2004) who succinctly defined the toxicity of a product as its capacity to cause injury while the hazard attributable to a product represents the probability that it will do so. Barne's definition is considered to be fundamentally correct, and is the definition accepted by the National Academy of Science in the U.S.A.

Paracelsus (1493-1541) phrased this well when he noted, "All substances are poisons; there is none which is not a poison. The right dose differentiates a poison and a remedy". In the Mosby's Medical dictionary Toxicology is defined as the study of poisons, their detection, their effects, and methods of treatment for conditions they produce Mosby's Medical Dictionary (2012). While the Medical dictionary for Health Professions and Nursing defines toxicology as the science of poisons, including their source, chemical composition, action, tests and antidotes (Medical dictionary for Health Professions and Nursing, 2012). The relationship between dose and its effects on the exposed organism is of significance in toxicology. The chief criterion regarding the toxicity of a chemical is the dose, i.e. the amount of exposure to the substance. All substances are toxic under the right conditions.

2.9.7.2 Toxicity, target, route, and duration of exposure

Toxicity*:** Toxicity is expressed generally as a dose response relationship involving the quantity of substance to which the organism is exposed and the route of exposure skin (absorption), mouth (ingestion), or respiratory tract (inhalation). Toxicity is classified usually as (a) Acute: harmful effects produced through a single or short-term exposure. ***Chronic: harmful effects produced through repeated or continuous exposure over an extended period. (c)Subchronic: harmful effects produced through repeated or continuous exposure over twelve months or more but less than the normal lifespan of the organism

(www.bussinessdictionary.com/defination/toxicity.html, May 2012). (d) Subacute: adverse effects occurring as a result of repeated daily dosing of a chemical, or exposure to the chemical, for part of an organism lifespan usually not exceeding 10%. With experimental animals, the period of exposure may range from a few days to 6 months (www.hyperdictionary.com/dictionary/subacute+toxicity, 2012).

The major factors that influence toxicity as it relates to the exposure situation for a specific chemical are the route of administration and the duration and frequency of exposure (EPA, 2014).

Target: Toxicologists talk about the target of a toxicant. They are referring to the particular macromolecule, cell, organ, or biochemical process that the toxicant disrupts. The way the toxicant is able to disrupt that process is called the mechanism of action of the toxicant. Not all toxicants are lethal. They may also cause disease, tissue damage, genetic alterations, cancer, etc. Next you might think about how you came into contact with those xenobiotics. The answer to this question determines the route of exposure. For example, you breath in air pollutants, thus the first contact points in your body would be your nasal passages, airways, lungs, etc. (This would be route of carbon monoxide poisoning). One question you may have imagined is something regarding the length of exposure, or duration. Toxicologists define two types of exposure based on its duration. Acute exposure is of brief duration. Chronic exposure is a persistent exposure, over a long period of time. Duration is not the only significant aspect of exposure. One of the most important questions that toxicologists ask about exposure is- “how much? This is called dose. This is important because for each chemical, a certain dose produces certain biological effects in the individual organism. Any biological effect caused by the exposure is called the response. Most of the time, the greater the dose, the greater the response, but this is not always true.

For example Iwuanyanwa *et al*. (2012) conducted an evaluation of acute and sub-acute oral toxicity study of Baker Cleansers Bitters (BCB)- a polyherbal drug on experimental rats. The result was that single dose of oral administration of BCB to Winstar albino rats at 2000 mg/kg body weight had no effect on mortality and clinical signs e.g. changes in the skin and fur, eyes and mucous membrane (nasal), respiratory rate, circulatory signs (heart rate and blood pressure), autonomic effects (salivation, perspiration, piloerection, urinary incontinence and defecation) and central nervous system (ptosis, drowsiness, gait, tremor and convulsion).

2.9.8 Specialized areas of toxicology

Three areas of toxicology are Forensic (primarily ivoked to aid in establishing the cause of death and elucidating its circumstances in a postmortem investigation), Clinical (an area of professional emphasis within the realm of medical science concerned with disease caused by, or uniquely associated with, toxic substance) and Environmental toxicology (focuses on the exposure of the chemical varieties of toxicants found in the general living environment and its implications on organisms, mostly on the nonhuman population).

Different areas of toxicology have also been described: Descriptive toxicology (focuses on toxicity testing of chemicals or agents of toxicity, usually on animals and then correlated to human conditions; Mechanical toxicology (focuses on the in depth study of how the agent initiates its biochemical or physiologic effect on the organism whether it would be genotoxic, teratogenic, mutagenic, or carcinogenic. Works as toxicokinetics and toxicodynamics) and Regulatory toxicology (focuses on risk assessment and exposure guideline development from gathered information obtained from toxicity testing. Establishes standards for safe exposure in occupational health guideline or governmental regulations to limit the use) (tonicpotato.wordpress.com/tag/descriptive-toxicology/, 2012).

2.9.9 Exposure, environmental exposure, aquatic toxicity, tolerance and survival time

2.9.9.1 Exposure

In this context exposure is defined as the contact between an agent and a target. Contact takes place at an exposure surface over an exposure period. Mathematically, exposure is defined as :$\mathbf{E = \int_{t1}^{t2} C(t)dt}$

Where E is exposure, C(t) is a concentration that varies with time between the beginning and end of exposure. It has dimensions of mass x time divided by volume. This quantity is related to the potential dose of contaminant by multiplying it by the relevant contact rate, such as breathing rate, food intake rate etc. The constant rate itself may be a function of time (http://cfpub.gov/ncea/cfm/ recordisplay.cfm? deid=15263. US EPA Guidelines for Exposure Assessment and disease, 2014.

2.9.9.2 Environmental Exposure

This type of exposure is generally limited to inhalation toxicity or the toxicity of chemical to aquatic animals. Two distinct situations can be attained.

Static: There is an initial concentration of toxicant in the environment and this concentration diminishes because of uptake by the animals' adsorption on to surface degradation etc and there is no source of replenishment of the toxicant in the environment.

Dynamic: The concentration of toxicant in the environment undergoes a process of replenishment off setting any physical removal of the toxicant that may be accruing. Environmental exposures are not generally expressed in terms of dosage but rather the term concentration to produce the defined toxic response is used, time of exposure being an essential variable. Royston and Parman (2013) found that survival time data for mixtures of toxicant can be modeled on the equation:

$$C = C0 + \beta \left[\frac{(MST - T)}{MST\text{-}T_o} \right]$$

When,

C = concentration of A in mixture of A and B

C_O = estimate of the asymptote of concentration of A as survival time increases towards infinity.

β = slope of the curve of concentration versus the reciprocal of the survival time.

MST = means survival time for a group of experimental animals in which all died during the exposure.

To = an estimate of the asymptote of the means survival time approached as concentration of component A increases towards infinity.

2.9.9.3 Aquatic toxicity

Aquatic toxicity testing submerges key indicator species of fish or crustacean or algae which are tested as surrogate species covering a range of trophic levels and taxa and the test methods are highly standardized. Certain concentrations of a substance in their environment are used to determine the lethality level. Fish are exposed for 96 hours while crustacean are exposed for 48 hours. While GHS does not define toxicity past 100mg/l, the EPA currently lists aquatic toxicity as practically non-toxic in concentrations greater than 100 ppm (EPA, 2014).

Exposure	Category 1	Category 2	Category 3
Acute	≤ 1.0 mg/L	≤ 10 mg/L	≤ 100mg/L
Chronic	≤ 1.0 mg/L	≤ 10mg/L	≤ 100mg/L

Source: EPA, 2014

When adequate toxicity data are available for more than one ingredient in the mixture, the combined toxicity of those ingredients may be calculated using the following additivity formulas (a) and (b), depending on the nature of the toxicity data:

***Based on acute aquatic toxicity*:**

$$\frac{\sum Ci}{L(E)C_{50m}} = \sum \frac{Ci}{n\ L(E)C50_i}$$

Where:

C_i = concentrationingredient i (weight percentage);

$L(E)C_{50i}$ = LC_{50} or EC_{50} for ingredient I, in (mg/l);

n = number of ingredients, and i is running from 1 to n;

$L(E)C_{50m}$ = L(E) C_{50} of the part of the mixture with test data;

The calculated toxicity may be used to assign that portion of the mixture an acute hazard category which is then subsequently used in applying the summation method;

***Based on chronic aquatic toxicity*:**

$$\frac{\sum Ci + \sum Cj}{EqNOEC_m} = \sum \frac{Ci}{n\ NOECi} + \sum \frac{Cj}{n\ 0.1xNOECCj}$$

where:

Ci = concentration of ingredient i (weight percentage) covering the rapidly degradable ingredients;

Cj = concentration of ingredient j (weight percentage) covering the

non-rapidly degradable ingredients;

NOECi = NOEC (or other recognized measures for chronic toxicity) for ingredient I covering the rapidly degradable ingredients, in mg/l;

NOECj = NOEC (or other recognized measures for chronic toxicity) for ingredient j covering the non-rapidly degradable ingredients, in mg/l;

n = number of ingredients, and i and j are running from 1 to n;

EqNOECm = Equivalent NOEC of the part of the mixture with test data;

The equivalent toxicity thus reflects the fact that no-rapidly degrading substances are classified one hazard category level more ‘‘severe’’ than rapidly degrading substances United Nations (2017).

2.9.5.4 Interactive effects.

Interactive toxicological effects may involve exposure of two or more toxicants either simultaneously or in sequence. Certain principles and definitions are very important in relation to interactive effects in the acute toxicology of chemicals. These interactive toxicological effects are well defined (www.cchs.ca/oshanswers/chemicals/synergism. htmi, 2014), as described (i) to (ii) bellow, retrieved 3.3.2014):-

2.9.9.5 Additive effects

The observed effect of exposure to a mixture of A, B, C, etc was exactly that expected for the situation in which exposure has been to an equivalent dose of A or B or C, etc alone; i.e. when the combined effect of two or more chemicals is equal to the sum of effect of each agent given alone (they do not interact in a direct way); for example: 2+2 = 4. This effect is the most common when two chemicals are given together. Subject to the proviso that the characteristics of the dose response lines for A, B, C, etc do not differ

significantly from each other, than a reciprocal rule can be applied to the data for the acute toxicity of mixtures of toxicants as follows for both LD_{50} and LC_{50}:

$$\frac{a}{AX100} + \frac{b}{BX100} = \frac{1}{LC_{50}\text{ of mixture}}$$

Where a, b, etc are percentages of A, B, etc in the mixture and A, B etc are LC_{50} values for the components. An alternative formula that may be used is:

$$\frac{100}{\frac{a}{A} + \frac{b}{B} + \frac{c}{C}} = LC_{50}\text{ of mixture}$$

2.9.5.6 Greater than additive effects

If the toxic effects observed for a two-component mixture A and B is greater than the effect that would be achieved by either A or B alone at an equivalent higher dose then the results is described as a synergistic effect. There are three special forms of synergistic effects:

Potentiation: Potentiation is the case in which A and B has toxicological activities that are quite different from each other but one increases the toxic effect of the other. That is this effect results when one substance that does not normally have a toxic effect is added to another chemical, it takes the second chemical much more toxic; for example: 0+2>2, not just 2. By Mosby's Medical Dictionary, Potentiation is defined as a synergistic action in which the effect of two drugs given simultaneously is greater than the sum of the effects of each drug given separately

(Mosby's Medical Dictionary, 2012). In medical-dictionary.thefreedictionary.com/potentiation).

Synergism: The word Synergism comes from the Greek word "Synergos" meaning working together.It differs to the interaction between two or more "things" when the combined effect is greater than if you added the "things" on their own (a type of "when one plus one is is greater than two" effect). In toxicology, synergism refers to the effect

caused when exposure to two or more chemicals at a time results in health effects that are greater than the sum of the effects of each agent given alone, for example: 2+2 >>4 (may be 10 times or more).

Antagonistic effects: Antagonistic effects are the converse (opposite) of synergism. That is the toxic effect of A and B together is less than that expected for the equivalent higher doses of either A or B alone or it is the situation where the combined effect of two or more compounds is less toxic than the individual effects; for example: 4+6 < 10. For example, in human medicine, antagonistic effect is the effect between the opposing actions of insulin and glucagon to blood sugar level. While insulin lowers blood sugar glucagon raises it. Thus, regulating the major physiological function of these two chemicals is crucial in order to keep up a healthy level of glucose in blood. Antagonistic effects are the basis of many antidotes for poisoning or for medical treatments. Murmu *et al.* 2015) have made a study on the antagonistic effects of two drugs in man i.e. Ondansetron and tramadol.

2.9.9.7 Effective levels.

In acute toxicology tests involving whole atmosphere exposures (e.g aquatic toxicology and inhalation toxicology), the effects levels are expressed in terms of concentrations rather than doses and the conventional nomenclature becomes EC (Effective Concentration) and LC (Lethal Concentration) rather than using doses (i.e. LC_{50} corresponds to LD_{50} with the dose being expressed as a concentration in the whole atmosphere surrounding the animal). The tests in common applications are:-

(i) LC_{50} : When animals are exposed to chemicals by the air they are breathing or the water they (fish) are living in, the dose the animals received is usually not known. For these situations the lethal concentration 50 (LC_{50}) is usually determined, that is the

concentration of chemical in the air or water that causes death to 50 percent of the animals. When reporting an LC_{50}, it is imperative that the time of exposure be indicated.

(ii) LD_{50}: this is lethal dose envisioned by (Saganuwan (2015) and is the standard measure of the toxicity of a material that will kill half of the sample population of a specific test animal in a specified period through exposure via ingestion, skin contact, or injection. LD50 is measured in micrograms (or milligrams) per 100grams of body weight (for smaller animals) or per kilogram of body weight (for bigger test subjects) of the body weight of the test animal ; lower the amount, more toxic the material. If a large number of doses is used with a large number of animals per dose a sigmoid dose-response curve is observed. A normally distrusted sigmoid curve approaches a response of 0 percent as the dose is decreased and approaches 100 percent as the dose is increased but theoretically never passes If a large number through 0 and 100 percent (www.business dictionary.com/definition/lethal-dose-50 -LD50-html 2013).

2.10 Various doses encountered in toxicology:

Four doses apply in toxicology:

2.10.1 Threshold dose (TD)

The minimum or the smallest dose of a substance which causes adverse change in an organism, as determined by the most sensitive biochemical and physiological methods even if no external indication of such a change is present, is known as the threshold dose (www.preservearticles.com/2012021423360/what-are-the-indices-of-toxicity.html, 2012).

2.10.2 Threshold limit (TL): The maximum concentration of a chemical allowable for a repeated exposure without producing adverse effects. It is based on survival as an effect Stedman's Medical Dictionary (2011).

2.10.3 Effective dose (**ED)**: The dose that produces a specific effect; when followed by a subscript (generally "ED_{50}"), it denotes the dose having such an effect on a certain percentage (50%) of the test animals; ED_{50} is the median effective dose.

2.10.4 MED or MLD: sometimes the 50% effective dose or 50% lethal dose is written as MED (Median Effective Dose) or MLD (Median Lethal Dose). This is the dose required to achieve 50% of the desired response in 50% of the population (Katzung *et al*. 2012).

2.11 Dose-response relationship

The dose-response relationship, or exposure-response relationship, describes the change in effect on an organism caused by differing levels of exposure (or doses) to a stress or (usually a chemical) after a certain exposure time. It can also be defined as the systemic description of the magnitude of the effect of a drug as a function of the dose (very low to very high. The relationship of dose to response can be illustrated as a graph called dose response curve which can be used to plot the results of many experiments (Murad, 2013). The characteristics of exposure and the spectrum of effects come together in a correlative relationship customarily referred to as the dose response relationship. This relationship is the most fundamental and pervasive concept in toxicology. Indeed, an understanding of this relationship is essential for the study of toxic materials.

0-1= no adverse effect level
2-3 = linear portion of the curve
4 = maximal response or effect

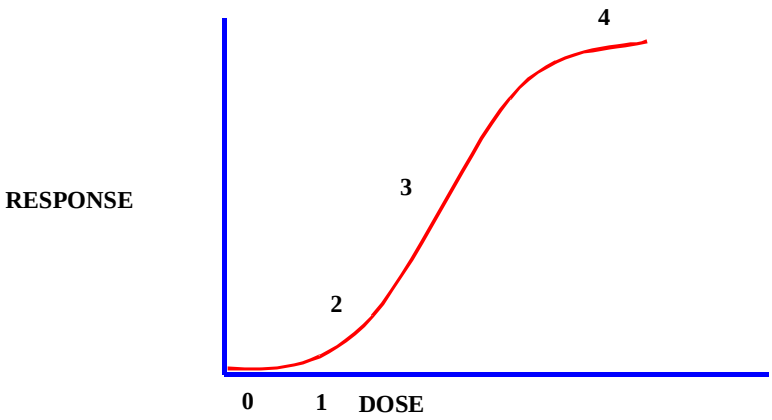


Figure 2.8 The relationship between the dose of a chemical (dependent variable) and the response produced (independent variable).

Source: Gomenou (2016)

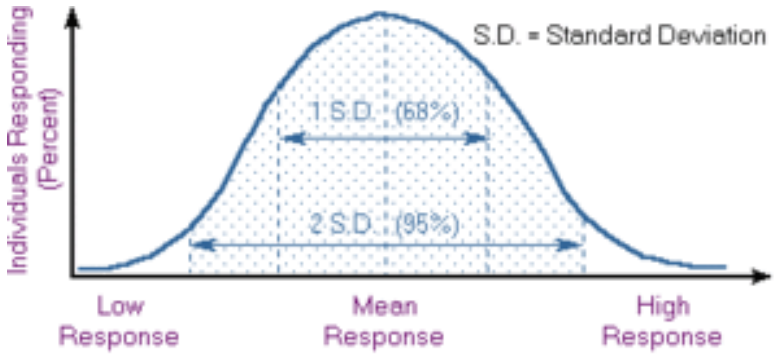


Figure 2.9 Some individuals are susceptible and others resistant.

Source: Gomenou (2016)

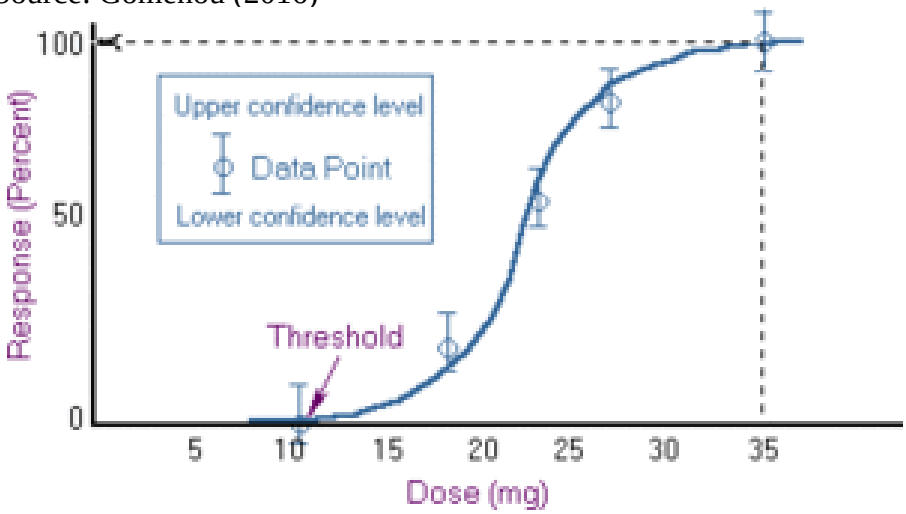


Figure 2.10 The dose response curve normally takes the form of sigmoid curve.

Source: Gomenou (2016)

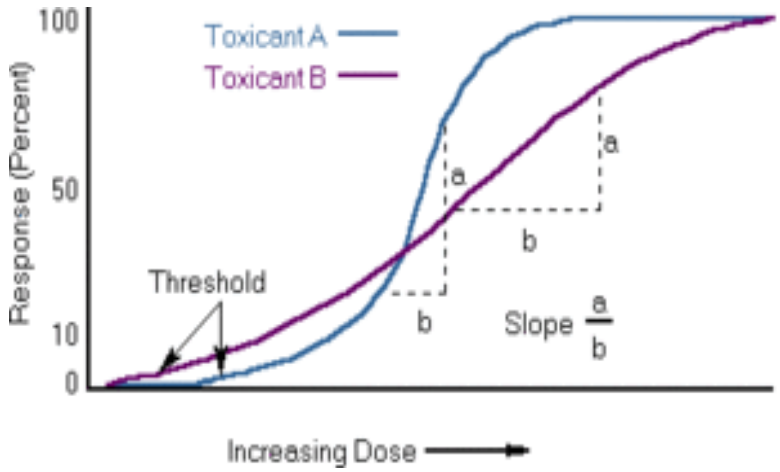


Figure 2.11 Predicting the toxicity of a substance at specific dose levels. Toxicity may not be realized at the threshold point also the percentage of population per unit change in dose (i.e. the slope).

Source: Gomenou (2016)

Assumptions:

A number of assumptions must be considered before the dose-response relationship can be appropriately used:

First: that the response is due to the chemical administered. In its most strict usage the dose-response relationship is based on the knowledge that the effect is a result of a known toxic agent(s).

Second: that the response is, in fact related to the dose. Perhaps because of the apparent simplicity of this assumption, this assumption is often a source of misunderstanding. The assumption is really a composite of three others that will recur frequently:-

(a) There is a molecular or receptor site (or sites) with which the chemical interacts to produce the response.

(b) The production of response and the degree of response are related to the concentration of the agent at the reactive site.

(c) The concentration at the site is, in turn, related to the dose administrated thus, the Numerical and graphic dimensions of the dose-response relationship include assumptions that:

●The response is a function of the concentration at a site.

●The concentration at the site is a function of the dose, and

● Response and dose are causally related.

Third: that one has both a quantifiable method of measuring and a precise means of expressing the toxicity.

A great variety of criteria or end-point of toxicity could be used. The ideal criterion would be one closely associated with the molecular events resulting from exposure to the toxin. Although many end-points are qualitative and they are often indirect measures of toxicity ToxTutor: In https://toxtutor.nlm (Wagner *et. al.,* 2017). The qualitative response to toxicant under conditions of acute exposure has been classified in to four types by Eke (2016).

2.11.1 Toxicity index

The index uses LC_{50} (concentrations at which 50% mortality occur in test organisms) or EC_{50} (concentration at which 50% of the test organisms exhibit a response; typically this involves an effect on behavior, such as immobilization in cladocerans). The LC_{50} and EC_{50} are referred to as toxicity concentrations, or endpoints. This PTI is the sum of toxicity quotients for each pesticide measured in a water sample. The PTI can be calculated using the following formula:

$$PTI = \sum_{I=1}^{n} \frac{C_i}{EC_{xi}}$$

Where C_i is the concentration of compound "i"

n is the number of compounds detected

EC_{xi} is the effect endpoint associated with compound "i" (eg.LC_{50} or EC_{50})
Nowell *et al*. (2014).

2.12 Status of the test animal

2.12.1 Individual and population risks

Each individual in any biological group differs in some way from all other members of the group even through the differences may be very small in some cases. For predictive toxicity testing the trend is to utilize homogeneous animal population and then to maintain them under near ideal conditions before and during the exposure to the toxicant. Ideal conditions may be affected by a number of factors: **(i) Physical factors:** temperature, Relative Humidity, ventilation, lighting, noise, **(ii) Chemical factors:** bedding, diet, water, **(iii) Disease causing agents**, (iv) **Time factors:** age, sex, size, frequency of sampling, diurnal variations, time to sample, (v) **Husbandry and manipulation factors:** consistent routines, stress, changes due to anaesthetics, animal density factors, and **(vi) Pain and distress** (University of Alaska, Centre for Research Services, IACUC Training: Factors influencing research animals,(www.uaf.edu/ iacuc/training/model-1-basic-concepts/factors-influencing-resea/, retrieved May 6, 2012).

2.12.2 Size

The results of acute toxicity tests are generally expressed in terms of either body weight or body surface-area and, of these two, bodyweight is more commonly used. Brodsky (2018) and Callaghan (2010) state that drug administration is based on total body weight and other authorities state that the starting dose, frequency of dose titration, and the optimal full replacement dose should be based among other factors body weight (www.globalrph.com/thyroid-calc.htm 2012). Although mosquito larvae have been intoxicated taking into account body weight in 1mg/100g body mass this would not be undertaken in this proposed research work.

2.12.3 Age

Age variation may give rise to differences in susceptibility to acute intoxication by different toxicant and there is no simple rule for relating age to toxic response for example younger larval instar (1st and 2nd instar) provide more sensitive targets, but tend to be less handy and unable to survive much more than 24h without food while the 3rd and early 4th instar enables good survival in controls and consistent assay results (Lacey, 2012).

Adult mosquitoes intoxication has been conducted with natural oil extracts in their different ages: 3-5 day old *Anopheles stephensi,* using leaf extract of *Eucalyptus globules* and other 6 natural oils for mosquito repellency (Shooshtari *et.al*. (2013); 7 days old *Anopheles arabiensis* Patton using leaf extracts of *Juniperus procera* also for mosquito repellency (Karunamoorthi *et. al*. (2014); and 6-10 days old *Anopheles gambiae s.s.* **Giles**for the establishment and estimation of dose response lines and effective dose of the 2 individual formulations (8% and 10%) of *Eucalyptus globulus,Ocimum bacilicum* (Sweet basil), *Cymbopogon citrates* (Lemon grass), *Citrus sinensis* (Sweet orange) *Azadirachta indica* (Neem) and *Hyptis snaveolens* (Scent leaf) (Lawal *et. al*. (2012).

On the other hand mosquito larvae have been tested according to the ages recommended by WHO standard procedure WHO (2005) using natural oil extracts: third and fourth instar larvae of *A. Darlingi* and *Aedes aegypti* respectively using *Copaifera multijuga* ethanolic extracts (Trindade *et. al*. (2013); third instar larvae of *A. Gambiae*s.s. **Giles**using oil from Indian borage (Kweka *et. al*. (2012); third instar larvae of *Aedes albopictus* (Diptera: Culicidae) using essential oils of *Citrus sinensis* L, *Eucalyptus camaldulensis* L. (Bilal *et. al*. (2012); fourth instar of *Aedes aegypti* using extract oil from *Citrus sinensis* (Warikoo *et. al*. (2012) and third or early fourth instar larvae intoxicating them with leaf extract of *Acalypha ciliate* (Aboaba *et. al*. (2012).

2.12.4 Those requiring modification before becoming fully toxic.

Sometimes lethal redistribution is associated quite simple with the change in body weight before death Australian Pesticides and Veterinary Medicines Authority (2016) (*Sharp et al*. 1972).The efficient design of acute toxicity studies takes into account the optimal numbers of animals to be involved at each dose level in order to achieve the desired degree of precision. This quantity will vary between different investigations and is, in part a function of the slope (β) of the achieved log dose –probit regression line.

By using the equation:

$$V(m) = 1 / \beta^2 \{1\sum_t(nz^2 / PQ) + (m-\bar{e})^2 / (\sum_t(nz^2 / PQ)(x-\bar{e})^2\}$$

V(m) = variance of the estimated log LC_{50}

n = number of larvae per treatment level

m = estimated log LC_{50}

β = slope of probit regression line

z = ordinance to the normal distribution corresponding to the probability level.

P = proportion killed on average at log-concentration x

Q = proportion not killed on average at x

$\bar{e}$ = mean log-concentration

x = log-concentration

t = number of test level

2.12.5 Tolerance, influence of formulations, and vehicle on acute toxicity

2.12.5.1 Tolerance

Tolerance is a state of decreased responsiveness to a toxic effect of a chemical resulting from prior exposure to that chemical or to a structurally related chemical. Two major mechanisms are responsible for tolerance:

One is due to a decreased amount of toxicant reaching the site where the toxic effect is produced (dispositional tolerance); and the other is due a reduced responsiveness of a tissue to the chemical comparatively less known about cellular mechanisms responsible for altering the responsiveness of a tissue to toxic chemical than known about dispositional tolerance.

2.12.5.2 Formulations

The acute toxicity of any toxicant to non-target species is also dependent on the form of its presentation (Shashibhushan *et al.* (2015) and the duration of exposure (ToxTutor In: https://toxtutor.nlm Accessed March 3 2017. Holland-Letz, & Schneider, 2015) investigated the toxicology of the bipyridylium herbicide diquat using fathead minrows (*Pimephales promelas*) and found that water hardness profoundly affected the results but duration of exposure was a critical factor. Water hardness has also been shown to affect the response by bish to intoxication by pyrethrum extracts and synthetic pyrethroids (Mack and Olson, 1A presentation of the influences of formulation on the acute toxicity of a toxicant is given in the scheme below):

Formalized presentation of the possible influences of formulation on the acute toxicity of toxicant (pesticide)

• Intrinsic acute toxicity of toxicant x

(=α)

• Formulation with ingredients y with own intrinsic acute toxicity

(= β)

•Formulation with acute toxicity (= γ)

• Toxicity of x diminished	Toxicity of x unchanged	Toxicity of x increased
(< α)	(α)	(> α)
• Rate of absorption of x	Additive action of y on	Rate of absorption of x

decreased (< α) toxicity of x (x3+y3) increased (>α)

($\gamma = \alpha + \beta$)

• Interaction of x and y to produce complex (x1y1) ($\gamma < \alpha + \beta$)

Interaction of x and y to produce complex (x2 y2) ($\gamma > \alpha + \beta$)

• Antagonistic action of y on toxicity of x (x1+y1) ($\gamma < \alpha + \beta$)

Synergistic action of y on toxicity of x (x2+y2) (• $\gamma > \alpha + \beta$)

The increase or diminish of acute toxicity by formulation is due to influences brought about by one or more of the following mechanisms:-

• Effect on rate of absorption: rooted in the toxicant's physical – chemical properties (Kissel (2011). Effect on bioavailability of the toxicant: due to changes in the physic-chemical properties of the toxicant in relation to the physiological milieu. In the case of solids an increase or a decrease in particle size can markedly cute the absorption characteristic from the respiratory tract and from the alimentary tract (Chiogna *et al*. (2016); even a toxicant absorption through the skin from the solidstate, e.g. dieldrin, the particle size maybe critical to the response. Other physical factors influencing bioavailability of toxicant include viscosity of suspensions (Revathi (2014) the presence of materials that influence surface tension and intentional or unintentional interactions of the toxicant with formulation components (Hoy et al. (2015).

• Synergistic, additive and antagonistic interactions of toxicant: the toxicity of a toxicant may be increased or decreased by use of formulation techniques (ToxTutor In: https://toxtutor.nlm Accessed March 3 2017; in practice it is not always possible to obtain optimum efficacy of the toxicant and achieve minimum toxicity.

2.12.5.3 Vehicle

The word ‘vehicle’ is a general term used to describe those constituents of toxicant formulations other than the major active components and may include solvents, carriers, surfactants etc. For organic toxicants, non-polar solvents are often required to dissolve the compound to assure uniform amounts on the toxicant (Meador et al. 2018).

The concentration of toxicant in relation to the vehicle can be very important. Absorption may be facilitated or diminished by change in toxicant concentration and there is no single correlation between concentration and uptake, the relationship is dependent on the individual chemical involved and the route of exposure. In their study, Nasuhoglu, *et. al*. (2016) conducted a study on the possibility of using organic solvents (OSs) to increase the susceptibility of bioluminescent micro-organisms in a bioassay for assessing the toxicity of chemicals dissolved in water. To conduct the tests acetonitrile, dimethyl sulphoxide, ethanol, methanol and isopropanol were used as Oss and Cd, Hg, and Zn as reference toxicants. The addition of Oss modified the toxicity of the three metals to *Vibrio harveyi*, according on the bioluminescence assay used. The sensitivity of the luminescence bioassay for Hg increased in the presence of the five Oss, thus indicating a greater toxic effect. However, the sensitivity of the assay for the other two metals, Cd and Zn increased or decreased (lesser toxic effect) depending on the concentration at which the Oss were used.

Whatever the route of exposure the vehicle can influence absorption but there is no single relationship between either the quantitative or the qualitative effect indifferent routes of uptake. The vehicle may be chosen to have an effect on the physiology of the target species, for example the vehicle may aid the penetration of insecticides through the insect cuticle. The influence of the same vehicle may be similar on the skin of non-target species and as a consequence the uptake of the pesticide by exposed non-target species may be

increased, thus increasing the hazard factors. A physic-chemical analysis of the percutaneous absorption process was devised by Berg (2014) and this included the value of activity coefficients. Later, Higuchi and Kinkel (2015) Higuchi and Kinkel (1965) published a more detailed discussion of the derivation of solvent-solvent and solvent-solute interactions, with particular reference to the antichilinesterase agent sarin application in bio-pharmaceutics (Xu *et al.* (2016). Higuchi formula can be expressed as:

$Q = hCo\ [1 - 8/\ \pi\ 2 \sum \infty\ m=o\ 1/\ (2m+1)^2\ exp\ \{-Dv\ (2m+1)^2\pi^2t\ /4h^2\}]$ When,

Q = amount of toxicant absorbed at time t per unit area of exposure

Co = initial concentration of penetrating toxicant

Dv = diffusion constant of toxicant in solvent.

t = elapsed time of application

h = thickness of applied phase

and the formula can be modified for toxicants that are present as suspensions in a vehicle (i.e. concentration is in excess of solubility):

$Q= [(2Co -Cs)\ (Cs\ Dvt)]\ ½$

If the solubility of the toxicant in the vehicle is very small (i.e. Co»Cs, the modified Higuchi equation can be simplified to:

$Q= (2CoCsDvt)\ ½$

Some toxicants may be present in significant amounts as vapours. The characteristic of percutaneous penetration of vapours are poorly defined.

2.13 CHEMICAL COMPOSITION OF THE ESSENTIAL FLOWER AND LEAF OIL EXTRACTS

2.13.1 *Chrysanthemum cinerariifolium* oil chemical composition

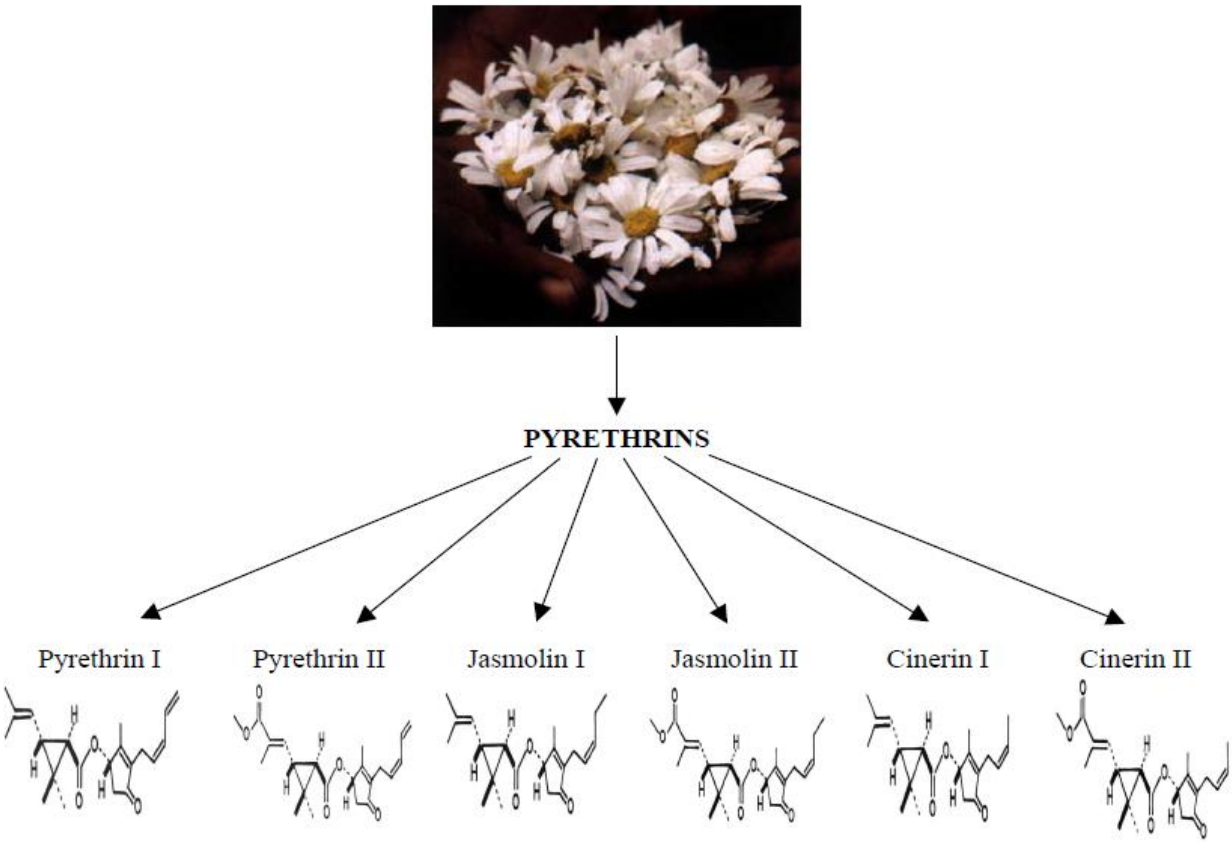


Figure 2.12: Composition of Pyrethrins

Source: Gunasekara (2005).

Pyrethrum extract is defined as a mixture of three naturally – occurring closely related insecticidal esters of chrysanthemic acid, pyrethrins I (pyrethrin I, cinerin I and Jasmolin I) and the three corresponding esters of pyrethrin acid, pyrethrin II, cinerin II and Jasmolin II. In the United States, the pyrethrum extract is standardized as 45-55% w/w total pyrethrums. The typical proportion of pyrethrins I to II is 0.2:2.8, while the ratio of pyrethrins: cinerins: jasmolins is 71:21:7 (USEPA, 2006).

Pyrethrum content is determined as the sum of pyrethrin I content (pyrethrin I, cinerin I and jamoslin I) and pyrethrins II content (Pyrethrin II, cinerin II and Jasmolin II). The active ingredient in pyrethrum extract consists of a mixture of four compounds in approximately the following percentage: pyrethrum 1, 40%; pyrethrin II, 36%; cinerin 1, 12% and cinerin II, 12%. The current commercial product is a more or less purified form of the mixture highly purified separate isomers although available for research purposes are too expensive for practical use (WHO 2018; WHO 2015; European Commission, Health and Consumers Directorate-General 2013; Doccola and Wild 2012.

2.13.2 Structure of Pyrethrins

The six related esters are represented by the following structures (WHO 2018; European Commission, Health and Consumers Directorate-General, 2013

Cinerin I $C_{20}H_{28}O_3$, molecular wt. 372.44

Cinerin II $C_{21}H_{28}O_5$, molecular wt. 360.43

Pyrethrin I $C_{21}H_{28}O_3$, molecular wt. 328.43

Pyrethrin II $C_{22}H_{28}O_5$, molecular wt. 372.44

Jasmolin I $C_{21}H_{30}O_3$

Jasmolin II $C_{22}H_{30}O_5$

2.13.3 Chemical name of pyrethrins

Chemical names have been suggested to some pyrethrins as shown below (WHO 2018).

Pyrethrin I: Pyrethrin ester of Chrisanthemummonocarboxylic acid.

Pyrethrin II: Pyrethrolone ester of chrysanthemomdicarboxylic acid monmethyl ester

Cineri I: 3(2-Butenyl) – 4- methyl-2-OXO-3-cydopenten – 1-yl ester of chysonthemumdicarboxylic acid monomethyl ester.

Other chemical names have been suggested as follows:

Pyrethrin I: (z) (s) – 2 – methyl-4-oxo-3-(penta 2, 4-dienyl) cyclopent-2-enyl (IR) – trans-2, 2-dimethyl – 3- (2-methylprop-1-enyl) cyclopropanecarboxylate.

Pyrethrin II: (z) – (s) – 2-methyl-4-oxo-3-(penta-2,4-dienyl) cyclopent – 2-2 enyl (E) – (IR) – trans-3-(2-methoxy carbon ylprop-I-enyl) – 2, 2-dimethylcyclopropanecarboxylate.

Cinerin II: (z) – (s) -3 – (but – 2enyl)-2 methyl-4-oxocyclopent-2-enyl(E) – (IR) – trans-3(2-methoxycarbonylprop-1-enyl)-2, 2-dimethylcyclopropanecarboxylate.

Jasmolin I: (z) – (s) – 2-methyl-4-oxo-3 (pent-2-enyl) cyclopent-2-enyl (IR) – trans-2, 2-dimethyl-3-(2-methylprop-1-enyl) – cyclopropanecarboxylate.

Jasmolin II: (z) – (s) – 2 methyl-4-oxo-3-(pent-2-enyl) cyclopent-2-enyl(E) – (IR) – trans-3-(2-methoxycarbonylprop-1-enyl) -2, 2-dimethyleyeclopropanecarboxylate.

2.13.4 *Eucalyptus camaldulensis* essential oil chemical composition

Zareen *et al.* (2016) performed chemical analysis to identify chemical composition of *Eucalyptus comaldulensis*. They identified 26 compounds in the essential oil of *Eucalyptus camaldulensis* of which 1,8-cineole was the most abundant (69.46%) followed by Y-Terpine (15.10%) and thirdly α-pinene (5.47%). Other compounds, whose figures in bracket are percent were in low level concentrations: α-Thujene (0.12), Camphene (0.03), β-pinene (0.21), β-myreen (0.30), β-Ocinene(0.01), Terpinolene (0.53), 1-Terpineol (0.03), Limonene oxide (0.01), α-Terpineol (1.29), Trans-carveol (0.02), Geranial (0.04), α-Terpienyl Acetate (1.31), α-Gurjunene (0.34), Aromadendrene (1.72), -Selinene (0.06), Y-Cadinene (0.05), D Cardinene (0.07), α – Calacorene (0.03), Epi Globulol (0.29), Globulol (2.00), Viridiflorol (0.61), β-Eudesmol (0.23) and α-Cadinol (0.05).

A few other researchers have attempted to find chemical composition of *Eucalyptus camaldudensis*. Siramon *et al.* (2013) six compounds in 3 clone samples i.e. p-cymene, y-Terpinene, 1.8-cineole, Terpenen -4-01, α – Pinenene and Terpineol Ghalem *et al*. (2014) states that *E. camaldulinsis* attributed high presence of 1.8 – cineole (15-78%), in

Mostenegro Khubeiz *et al.* (2016) found that *Eucalyptus camaldulensis* can be classified I the chemotype with low 1.8-cinede and high p-cymene (17.38-28.60%) and cryptone ratio (4.97 – 7.25%).

Other species of *Eucalpytus* have also been analyzed further chemical composition I their chemical analysis for chemical composition of essential oil of three species of *Eucalyptus* namely *E. dives*, *E. staigeriana* and *E. oilda*, Sebei *et al.* (2015) identified 24 compounds for *E.dives*, 29 compounds for *E. staigeriana* and 20 compounds for *E. Oilda*. Those with higher concentrations were: *E. dives* – piperitoe (40.5%), α-phellandrene (17.4%) p-cymene (8.5%), Terpin – 4-01 (4.7%); *E. staigeriana* – 1.8 – cineole (34.8%), Neral (10.8), Geranial (10.8), α-phellandrene (8.8%), and methyl geranate (5.2%), *E. Oilda-E-methylcinnamate* (99.4%).

Juan *et al*. (2011), gives results of chemical composition of thirteen species of *Eucalyptus* concentrations in brackets expressed a percent: α-Thujene (0.6 in 5 species), α-pinene (5.4 in 12 species), camphene (1.6 in 5 species), β-pipene (0.1 in 8 species), myrcene (0.2 in 7 species), 1.8 – cineole (58.9 in 11 species), Y-Terpinene (2.8 in 10 species), para-cymene (2.1 in 10 species), α – Terpineol (2.7 in 13 species), Aronomadendrene (2.1 in 7 species) and globulol (1.6 in 9 species). Elaissi *et al*. (2012), identified 144 compounds in 8 eucalyptus species essential oils representing 87.40 to 99.37% of the total oil content where 1.8 cinede was the main compound (4.5 $\pm$1.61 – 70.4 $\pm$ 2.5%).

2.13.5 *Nicotiana tabaccum* essential oil chemical composition

The active principle ingredient of tobacco is the alkaloid Nicotine, which is produced in the roots, but stored in the leaves. Nicotine with the chemical formula $C_{10}H_{14}N_2$ and molecular wt 162, 234 mol-1 is one of the few liquid alkaloids and is the chief addictive ingredient in tobacco. Other components found in the essential oil are beta-damascenone, megastigma trienone, oxo-edulan and 4-oxo-beta-ionone. (www.bojensen.net/essential

oils Eng./essential oils/29.htm 2014. Nicotine is also called 3-(1-methyl-2-pyrrolidnyl) pyridine according to IU/PAC. Nicotine also curtains Oleoresin (0.89%), phenols and flavonoids.

Loughrin *et al.* (1990) gives tobacco leaf composition as (E) – β-ocimene, (z) – 3-hexenyl acetate, (z)-3-hexen-1-01, linalool, β-caryophyllene, (E) – β-farnesene, Solanone, methyl salicylate, nicotine and neophytadiene. These comprised 50% of the GC peaks and weight of the total estimated volatiles.

2.14 Toxicity of *Chrysanthemum cinerariifolium*, *Eucalyptus camldulensis and Nicotiana tabaccum* essential oil extracts

2.14.1 Pyrethrins

The term "pyrethrins" refers to the natural insecticides derived from Chrysanthemum flowers; "pyrethroid" are the synthetic chemicals, and "pyrethrum" is a general name covering both compounds. The Environmental Protection Agency (EPA) of the United State of America classifies pyrethrin as a Restricted Use Pesticide (RUP). Restricted use Pesticides may be purchased and used only by certified applicators. Pyrethrin is one of the liquid esters derived from pyrethrum (feverfew) ($C_{21}H_{28}O_3$ or $C_{22}H_{28}O_5$), that are used as insecticides while Permethrin is a synthetic pyrethrin (C_{21} $H_{20}C_{12}O_3$) . In other words it is a man-made poison that is a copy of two poisons found in plants.

The pyrethrum chemistry identifies complete the structures and stereochemistry of six natural pyrethrin esters: Pyrethrin I and II, cinerin I and II and jasmolin I and II in a collective name "rethrins"). Pyrethrins I, cinerin I, and jasmolin I are esters of chrysanthemic acid whereas pyrethrin II, cinerin II and jasmolin II are esters of pyrethric acid.

2.14.2 Pyrethrum Extract

Pyrethrum extract is a mixture of three naturally occurring, closely related insecticidal esters of chrysanthemic acid (pyrethrins I) and three closely related esters of pyrethric acid (pyrethrins II). It contains not less than 45.0 percent and not more than 55.0 percent of the sum of pyrethrins I and II in a mixture consisting of approximately 20 to 25 percent (w/w) light isoparaffins. The ratio of pyrethrins I to pyrethrin II in the extract is not less than 0.8 and not greater than 2.8. It may also contain 3 to 5 percent butylated hydroxytoluence as an antioxidant and 23 to 25 percent phytochemical extracts containing triglyceride oils, terpenoids, and carotenoid plant colours It contains no other added substances. Such extracts contain about 30 percent by weight a mixture of six components in about the following amounts: pyrethrin I (11.4%), cinerinI (2.2%), jasmolin I (1.2% pyrethrin II (3.5% and jasmolin II (1.2%) (Wong and Glinski (2017)

Well after their use as insecticides, their chemical structures were determined by Herman Staudinger and Lavoslav Ruzicka in 1924. Pyrethrin I and pyrethrin II are structurally related esters with a cytopropane core. Pyrethrin I is a derivative of (+)-transchrysanthemic acid (Merc Index; McGraw-hill Ryeson Chemistry). Pyrethrin II is closely related, but one methyl group is oxidized a carboxymethyl group the resulting core being called pyrethic acid. Knowledge of their structures open the way for the production of synthetic analogues, which are called pyrethroids. In terms of their biosynthesis, pyrethrins are classed as terpenoids, being derived from dimethylallyl pyrophosphate, which combine by the action of the enzyme chrysanthemyl diphosphate synthase.

2.14.3 Toxicity of pyrethrins

Mann and Kaufman (2012) suggested use of pyrethrum for the impregnation of bed nets and for personal protection due to its high toxicity and excite-repellency against

insecticide –resistant mosquitoes. Despite its long history of use, few cases of specific resistance against insect vectors to pyrethrum, pyrethrum extract is still used in antimalarial programmes in India Rawani *et al*. (2013). Pyrethrins are used in many varieties of insecticides, fogging products, and some pet products, and have been used as an insecticide for over 100 years. In the 1800s it was known as "Persian insect powder", "Persian pellitory" and "Zacherlin". They affect the flow of sodium out of the nerve cells in insects, resulting in repeated and extended firings of the nerves, causing the insects to die (The use of pyrethrum for flea control in dogs and cats (en.wikipedia.org/wiki/Pyrethrum 2013). Piperonyl butoxide, a synergist, is often used in combination with pyrethrin, making the more effective by not allowing the insect's system to detoxify the pyrethrin (http:www. askheexterminator.com /Pesticide/ Pyrethrin.shtnl, 2013). Although pyrethrum is potent insecticide, it also is an insect repellent at lower concentrations. Pyrethrin and the synergists are biodegradable and rapidly integrate in sunlight and air, thus assuring no excessive build-up of insecticides dispensed in the area being treated.

The natural pyrethrins are contact poisons which quickly penetrate the nerve system of the insect. A few minutes after application, the insect cannot move or fly away. But, a "knockdown dose" does not mean a killing dose. The natural pyrethrins are swiftly detoxified by enzymes in the insect. Thus, some pests will recover. To delay the enzyme action so a lethal does is assured, organophosphates, carbomates, or synergists may be added to the pyrethrins.

In their use overdose and toxicity can result in a variety of symptoms, especially in pets, including drooling, lethargy, muscle tremors, vomiting, seizures and death (Permethrin and Pyrethrin toxicity in dogs). Toxicity symptoms in humans include asthmatic breathing, sneezing, nasal stuffiness, headache, nausea, incoordination, tremors,

convulsions, facial flushing and swelling and burning and itching sensations (Pyrethrins: Cornell University). Piperonyl butoxide (PBO) has a distinct health risk when it becomes airborne and pregnant women are exposed during the third trimester. This leads to delayed mental development in young children. A 2011 study found a significant association between PBO, measured in personal collected during the third trimester of pregnancy, and delayed mental development at 36 months. Children who were more highly exposed to PBO personal air samples ($\geq$ 4.34 ng /m^3) scored 3.9 points lower on the Mental Development Index than those with lower exposures (Horton *et. al.* 2011). The lead researcher stated regarding PBO, "This drop in IQ points is similar to that observed in lead exposure.

Information on the natural pyrethrins toxicity to mosquito larvae is lacking. However, Azab, *et. al.,* (2017) states that when they applied 225 mg pyrethrins per 100c.c. kerosene i.e. an extract of *Chrysanthemum cinerariifolium* diluted with kerosene gave complete kill of mosquitoes while extract of C. (P) roseum similarly diluted to contain 400 mg gave only 84% kill.

2.14.4 Documented cases of resistance to pyrethrins

There are no cases of insect resistance in regards to natural pyrethrins quoted in literature. However, a number of known cases of *Anopheles* mosquitoes and other mosquito species resistance to pyrethroids are more documented as opposed to pyrethrins in many countries which include the following: urban Benin (Ghabi *et al.* (2018); southern Benin (Ol'e Sangpa *et al.* (2017) ; Fodjo *et al.* (2018) west Kenya i.e. Gembe east and west, Mbita and four main western islands in the Suba county, Nyanza Province (Kawada (2017; 2018); cities of Douala and Yaouunde, Cameroon (Nwane *et al*. 2013; Acton, 2012); Nigeria (Ol'e Sangpa *et al.* (2017); north-western Tanzania (Protopoff *et al.* 2013); Ghana (Mitchell *et al*. 2013); south-west Ghana (Kudom *et al*. 2012; Awuah *et al.* (2016);

Tanzania, lower Moshi, northern Tanzania (Mahande *et al*. (2012); east of Tanzania (Nkya *et al*. 2014); eastern Uganda (Ondeto et al. (2017) ; South Africa (Djauaka et al. (2016) Hargreaves *et al.* 2000); Burkina Faso (Diabate *et al.* (2014) Diabate *et al*. 2004); Equatorial Guinea (Salgueiro *et al.* 2013) ; Angola (Wondji *et al.* (1212); Gabon (Diegbe *et al.* (2017); Irving and Wondji (2017) ; Ethiopia (Fettene *et al.* (2013); Cote d`voire (Aktar *et al*. (2009) and Congo Brazaville (Kaiser *et al*. 2010).

The above documented cases were investigated on major malaria vector species, *Anopheles gambiae s.s.***Giles**, *A. gambiae s.l*., *A. gambiae* M and S molecular forms, *A. arabiensis*, and *A. funestus*. Factors contributing to the mosquito resistance are broadly stated: the kdr west (kdrW) mutation specifically on the M molecular forms seems to be the major resistance mechanisms found in *A. gambiae s.s.* **Giles** from agricultural areas in Benin; due to presence of target site insensitivity resulting from kdr mutation and to increased metabolism through enzymatic activity (in both M and S molecular forms), thus showing presence of Ace 1^{R}mutation; in addition to kdrW detection of cytochrome P450s, CYP6P3 and CYP6M2 which were elevated in pyrethroid resistant populations (P450-related pyrethroid resistance seemed to be widespread in both *A . arabiensis* and *A. funestus*); *A. gambiae*s.s. **Giles**also showed resistance caused by high frequency of point mutations (L1O14S); cultivated sites showed larval tolerance of pyrethroids; the kdr-eastern (kdrE) variant was present in homozygous form in 97% of *A. gambiae s.s.* **Giles**; and use of single class of insecticide repeatedly without any other preference.

2.15 *Nicotiana tabaccum*

2.15.1 Toxicity of Nicotine

Toxicity of nicotine has not been widely tested on *A. gambiae* complex mosquito species. However, literature is available on its trial against other mosquito species. Yongxing *et al.*, (2009) applied nicotine against *Culex quinquefasciatus* 2^{nd} and 4^{th} instars 24 h

exposure. They found that the LC_{50} and LC_{90} 100% mortality was achieved by nicotine concentration of 0.05mg/L.

In another study the effects of the various aqueous extracts of *Datura stramonium* (Jimson weed) and *Nicotina tabaccum* on the mortality of culicine and *anophelene* larval species were that the effect of Tobacco leaf recorded the highest mortality rate with 80 and 100% concentrations having 100% death rate. In *Datura stramonium* extract treatment, there was less than 50% mortality of larvae of culicine species for the first 24h, but at 100%, there was 100% death rate. On *Anopheline* species, there was more than 70% mortality rate with 100% concentration recording 90% mortality rate, both 20% and 60% killed more than 50% of the larvae. By 72h, all tested concentrations of both the leaf and root extracts of Tobacco and Jimson's weed killed 100% of the tested larvae population. A gradient of increasing mortality with increasing concentrations was observed in all treatments (Ileke *et al.* (2015). One of the most important early uses of tobacco was as a hallucinogen in shamaristic rituals (Borio (2018). It was also used for medical reasons, including the treatment of rheumatic swelling, skin diseases, and scorpion stings (Kshetrimayum, (2017).

Nicotine, the active pesticide ingredients in tobacco, is not only very toxic to insects but also to animals. The LD_{50} of nicotine is 50mg/kg for rats and 3mg/kg for mice. It has been ascertained that 40-60mg (0.5-1.0mg/kg) can be a lethal dosage for adult humans (http⁄⁄www.bambooweb.unfolb/viewtopic.php^2 = 14ft=1382), retrived October 7, 2010). However, nicotine breaks down more quickly in the environment than some chemically manufactured pesticides, potentially making it a good biodegradable option. Many pesticides/insecticides do not degrade as quickly or to the same extent, easily accumulate more persistent concentrations within higher organisms, pollute water supplies and damage entire ecosystems (http⁄⁄en.wikipedia.org/wiki/Nicotine2013). Tobacco is an

insect eating plant. It has developed sticky nicotine-rich leaves that trap, kill and dissolve insects. Dead bees have been found on a tobacco leaf that was 25% dissolved. Tobacco usually traps smaller insects such as borg larvae (http⁄⁄www.linda-goodmA.com/ubb/Forum3/HTML/002473.html2013).

2.16 Toxicity of *eucalyptus* leaf essential oil extract

Attempts have been made experimentally by various researchers in search of effective method of controlling mosquito breeding using eucalyptus essential oil extracts from leaf, bark and seed. Other than *Anopheles stephensi* Liston (Diptera: culicidae), the rest of the *Anopheles gambiae* complex species, the malaria vectors, seem to have been excluded from studies carried out and instead more work has been done on *Aedes albopictus* (Diptera: Culicidae), *Aedes aegypti*, *Culex quinquefasciatus* and *Culex pipiens* probably because the *Anopheles gambiae* complex may not be available in the countries of the researchers. However, Taher *et al.* (2012) applied *Eucalyptus* leaf extract oil and El-Maghraby *et al*. (2012) applied Eucalyptol (Eucalyptus oil fraction; which is *Eucalyptus* oil commercial grade from the local market mixed with the crude oil with pet. Ether(60-80) and fractionated through silica gel column (70 mesh), the first fraction was eucalyptol). They applied 200ppm and 91.45 ppm respectively against *Culex pipiens* 3rd instar larvae. A total mortality for 12-18h was established at 200 ppm and 50% mortality at 91.45 ppm. The following are the attempts:

2.16.1 *Eucalyptus camaldulensis*

Zareen *et al.* (2016) carried out bioassays using *Eucalyptus camaldulensis* essential leaf oil extract on *Anopheles stephensi* larvae. Clear dose-response relationships were established with the highest dose of 320 ppm and the essential oil extract resulted almost 100% mortality in the population.

2.16.2 Essential oil from Eucalyptus *camaldulensis*

Essential oil from *Eucalyptuscamaldulensis*) was evaluated. The chemical compound of *Eucalyptus* essential oil was determined by gas chromatography coupled with mass spectrometry. Regression analysis revealed a statistically significant relationship between percentage of 1, 8-cineole and larval mortality after 24h. The results showed that this species of *Eucalyptus* had high values in yield of essential and in their chemical composition high content of 1, 8-cineole and additionary, this type of essential oil showed low larvicide effect against *Ae. aegypti*. Results in work suggested that when the essential oil of *Eucalptus* gets rich in 1, 8-cineole this diminishes its larvicidal effect on *Aedes aegypti*.

It is confirmed that *Eucalyptus* oil is rich in cineole and desirably *eucalyptus* oil according to the invention comprises cineole and preferably 1-8 ceneole in an amount of form approximately 35-90% by volume (http⁄⁄www.patentstorm.us/ patents/6251440/ description.html2012).

2.16.3 Characteristics of larval stage that make it possible for experimentation with these essential leaf extracts

Due to the absence of a siphon, the larvae of the anopheline mosquito tend to lie parallel to the water surface and are not subtended at an angle like the culicine mosquito larvae. They are surface feeders and invariably occupy the water surface. Among the stages of the life cycle of mosquitoes, the larval stage is the longest, lasting 1-3 weeks and hence a stable stage to handle (Asimeng, 2000). During the mating period and until the female can find vertebrate blood.

2.17 Protocal for mosquito rearing

2.17.1 Rationale for animal use and justification for animal species chosen.

2.17.1.1 Rationale

First, the larvae are part of the life cycle from which the adult mosquito will emerge. Importantly the best stage to break the life cycle of any water-based organism is the larval stage whereby the larvae are well exposed over the water surface and hence easy to handle and administer an insecticide. Second, there are four (4) larval stages (1^{st} to 4^{th} instars) with duration of larval period varying from 1-3 weeks before transforming into pupal stage which is adequate period to carry out toxicological tests and other general studies.

2.17.1.2 Justification for animal species chosen

The *Anopheles* mosquito larvae are easy to identify since they lie parallel to the water Surface and are not submerged at an angle like larvae of other species mosquito. With a little care they will be easy to draw from water especially when transferring them from rearing containers to bioassays containers. *Anopheles* mosquito larvae are surface feeders and are not cut off from entrapping a variety of food available on the water surface which renders them to insecticide uptake. Their complete contact with water also makes them suitable subjects for the lethal concentration test because water will be part of the dilution for testing just as water is the larvae's habitat.

2.18 Male - female ratio for mating

Feature of this strategy vector control is an important component of the World Health Organization (WHO) global strategy for malaria control. A particularly attractive feature of this strategy is the approach by researchers to control mosquitoes at larval stage. This initiative has been broadly ventured into using field collected larvae from water surfaces other than rearing adult mosquitoes to carry out bioassays on 3^{rd} or 4^{th} instar larvae by

researchers including Bossou *et al.* (2013); Sugumar *et al.*, (2014); Ghosh *et al.* (2012); Karthikeyan *et al*. (2012); and Singh *et al*. (2014). A better alternative to larval sampling is the catching of male and female adult *anopheline* mosquitoes in the field and bringing them to the laboratory for rearing and mating. This method is preferred since it aims at producing pure colony against the larvae sampling which can possibly include larval predators, disease which can be caused by introducing an unhealthy specimen into a colony and parasites likely to attack the larvae. There is no first hand rule for ratio of male and female mosquitoes to be caged for mating. Matter and Defoliant 1984, have suggested 2:1 (male: female) ratio which is optimal for insemination.

Boyer *et al*. (2012) have given a ratio of 20 males and 20 females during their catch of the first and second field sampling while Kweka (2012) suggests adult sex ratio of 1:3 (female to male). Further in a natural case rearing mating experiment Pimid *et al.* (2015) reared *Aedes aegypti* using thirty 4-5 day old females and 40 males which gives a female: male ratio of 3:4. Perhaps use of the unique sex ratio was that by Olayemi *et al.* (2011) and Oliva *et al*. (2011) in which while the researchers conducting laboratory experiments on reproductive performance of *Anopheles gambiae s.s.* **Giles** and *Anopheles arabiensis*, (Diptera:Culicidae), they used a sex ratio of 1:1 (male:female). Diabate *et al*. (2011) used a sex ratio of 3:1 (male: female); for *A. arabiensis* Munhenga *et al.* (2016) applied a ratio of 1:1:1 (irradiated males: wild males: wild virgin females) and both Maiga *et al.* (2014) and Jayaprakash and Karthikeyan (2014) have used a male female ratio of 1:2 while conducting the insemination of *A. gambiae*s.s. **Giles** and *An. stephensi* respectively. An interesting ratio is one used by Oliver*et al*. (2012); they had a total of 2,000 mosquitoes which were divided into cages consisting of 100 females and 100 males to allow mating.

2.18.1 Adult mosquito rearing

Mosquitoes may be reared either in an insectary or directly in the field. An insectary is a place in which living insects are kept and propagated. The insectary may be a separate building, a room or section of a room, usually modified or remodified to suit the conditions required for rearing; or new facilities may have to be designed and built. One of the most efficient existing insectories is that designed by Davidson and his staff in the Ross Institute and described by Kamau *et al.* (2003). Whichever planned to bean insectary, it must comply with enviro nmental conditions pertaining to successful rearing of mosquitoes and these are temperature, humidity and lighting. Insectary's humidity and temperature system allows to closely monitoring the consistency of the climate inside the different environmental rooms, which helps stabilize mosquito rearing capabilities (Intellectual Ventures Lab, 2014). The most elaborate is a complex environmental chamber with programmed electronic controls of temperature, humidity, and photoperiod. The size of the insectary will regulate the type of temperature, and humidity control system required. Temperature and humidity controls are probably the most important factors in the successful rearing of mosquitoes (AMCA ,2011). For this work the adult mosquitoes will be reared in an insectary specially prepared for the expected experiments in Human Anatomy building, School of Medicine, Moi University. Mosquito rearing in the laboratory has been attempted by many researchers using appropriate diet and incorporating conducive insectary conditions. Carvalho *et al*. (2014) reared adult mosquitoes in a standard cage (30x30x30 cm) and fed them with 10% sucrose soaked in cotton pads and fresh animal or human blood and they gave a diet of ground fish food (Food A), ground cat food (Food B), cat food (Food C) for mosquito larvae while maintaining the insectary at 28^0C and 80% Relative Humidity and dark/photo period of 12:12.

Phasomkusolsil *et al*. (2013) fed adult mosquitoes in the manner adapted by Calvalho *et al.* (2014) and for larvae using ground fish 0.1g (1st and 2nd instar larvae, 0.3g (3rd instar) and 0.5g (4th instar larvae) at 08.00 and 16.00 each day. Khan *et al*. (2013) following same suite for adult mosquitoes but observed that fastest larval and pupal development and higher survival rates were recorded using a combination diet of bean, corn, wheat, chickpea, rice and bovine liver at 5 mg per day. In addition to this Phasomkusolsil *et al*. (2011) indicates that egg hatching is controlled by temperature;at a temperature of 23^0C, 66.1% hatched within 11.8 days, while at 30^0C, 74% of the eggs hatched within 8.1 days. Kivuyo et l. (2014)Das *et al*. (2007) recommends an insectary room temperature of 28^0C, a RH of 80%, mosquito cages, 10% sterile sucrose solution and 12 hour day/night cycle. Gentile *et al.* (2015) Khan (2010) in his diet frofiles he used three types of combined diet; Diet 1(IAEA): bovine lever, tuna meal, vita mix (5+5+4.6) gm respectively; Diet 2 (Khan D1): wheat, corn, bean, chickpea, rice, bovine liver (2+3+2+3+3+3.6) gm respectively; and Diet 3 (Khan D 2): wheat, corn, bean, chickpea, rice, bovine liver, vita mix (2+2+2+2+2+2+2.6) gm respectively.

It has also been reported that during rearing female mosquitoes they can be fed on IV Lab's artificial diet which is protein-rich to provide the females with the nutrients needed to develop viable eggs (Intellectual Ventures Laboratory, 2014) while Sawadogo *et al.* (2017) recommends light regime LD 12:12 h photoperiod including dusk (1h) and dawn (1h). They kept adult mosquitoes in cages of 30x30x30 cm feeding them with 10% (w/v) sucrose solution with 0.2% methylparaben and females were blood-fed weekly on defibrinated bovine blood. They reared larvae in plastic trays (40x29x8 cm) feeding them with finely ground Floating Blend. However, for *Aedes polynesiensis* Chambers *et al.* (2011) performed their assays by maintaining larvae on 60 g/L liver powder solution and

adults were maintained on 10% sucrose with ambient temperature ranging from 23-31^{0}C and a relative humidity of at or above 80% using a humidifier (Chambers *et al*. (2011).

2.18.2 Swarming and mating behaviour of *anophelines*

Anopheles mosquitoes mating occur during early evening and it occurs in swarms. *Anopheles* male aggregate just before dusk and commence swarming at the onset of sunset. Swarming males use their erect anternal fibrillae to detect a nearby female mosquito wing beat frequencies. Males harmonize their wing beat with those of females as they near, possibly as a form of species recognition, before mating commences. In many species copulation is initiated in flight as males and females meeting within the swarms. Once a male *Anopheles* has grasped a receptive female, it reorientates itself so it is in the venter-to-venter position allowing the reproductive organs to meet. After coitus commences, the male moves into an end-to-end position with the female as the pair falls. Corpulation may continue for a short period of time after alighting but in most genera it is a very quick process which ceases before the pair reaches the ground (M4).

An important element of mating in the malaria vector *A. gambiae*s.s. **Giles** in nature is the crepuscular mating aggregation (swarm) composed almost entirely of males, where most coupling and insermination is generally believed to occur (Butail *et al*. (2013). In the laboratory, in an artificial ‘‘dust’’, Manoukis *et al*. (2014) observed that male *A. gambiae s.s.* swarmed over a black marker on the floor of their 1-2m cube cage. In contrast to the males, **Giles** females made only short flights over the marker, performing brief turning movements at its edge. It is proposed that swarming brings about the aggregation necessary before short-range attraction can take place, and that in nature, *anopheline* mosquitoes orientate visually first to an arena and then to a marker within the area. Female behaviour can be interpreted as a process of scanning possible swarm sites until mating is achieved and some report suggests indoor mating in Africa *anophelines*

(Diabate and Tripet (2015). *A. gambiae s.s.***Giles** has been *shown* in the laboratory to mate within the first hour of darkness, during a peak in flight activity.

Mosquito swarms are poorly understood mating aggregations. *A. gambiae*s.s.**Giles**, are known to depend on environmental conditions, such as the presence of a marker on the ground, and they may be highly relevant to reproductive isolation. In Mali results indicated that swarms in this species are approximately spherical, with an unexpectedly high density of individuals close to the swarm centriod. This high density may be the result of individual males maximizing their probability of encountering a female or a product of mosquito orientation through cues within the swarm. Results also suggest a difference in swarm organization between putative incipient species of *A. gambiae s.s***. Giles** within increasing numbers of males. This may be related to a difference in marker use between these groups, supporting the hypothesis that swarming behaviour is a mechanism of mate recognition and ultimately reproductive isolation Shishika *et al.* (2014).

EMBO (2014) presents molecular identification of 1145 males collected from 68 swarms in Doneguebougou, Mali. That is studied swarming behaviour of the molecular forms and investigated the role of swarm segregation in mediating assortative mating. They found evidence of clustering of swarms composed of individuals of a single molecular form within the village. Tethered M and S females were introduced into natural swarms of the M form to verify the existence of possible mate recognition operating within swarm. Both M and S females were inseminated regardless of their form under these conditions, suggesting no within-mate recognition. The result provides evidence that swarm spatial segregation strongly contributes to reproductive isolation between the molecular forms in Mali.

A study by Shishika *et al.* (2014) suggests that *A. gambiae s.s.* **Giles** mates in flight at particular mating sites over specific land markers known as swarm makers. The swarms are composed of males; females typically approach a swarm, in leave in copula. This study looked at the variation in mating success between swarms – based on major jekking models known as the female prevalence model, the hotspot model, and the hotshot model. The study found substantial variation in swarms. A strong correlation between the swarm size and mating success of individual males did not increase with swarm size. For the spatial distribution of swarms, the result revealed that some display sites were more attractive to both males and females and those females were more attracted to large swarms. While the swarm markers we recognize help us in localizing swarms, they did not account for the variation in swarm size or in the swarm mating success, suggesting that mosquitoes probably are attracted to these markers, but also perceive and respond to other aspects of the swarming site.

In an interesting study in mosquito swarming in Burkina Faso, West Africa, (Sawadogo *et al.*2013b) studied the M and S molecular forms of *A. gambiae* s.s. **Giles** which appear to have speciated in West Africa and the M form is now formally named *A.*coluzzii Coetzee and Wilkenson sp.n. and the S form retains the nominotypical name (abbreviated here to *A. gambiae*). They observed that *A. gambiae* started and stopped swarming earlier than *A. coluzzii* (3:35 ± 0.68) min:sec and 4:51 ± 1.21, respectively), and the mean duration of swarming was 23:37 ± 0.33 for *A. gambiae* and 21:39 ± 0.33 for *An. coluzzii*. Difficulties in establishing colonies of anopheline mosquitoes are often reported and attributed to the incapacity of male swarm formation in a confined space, concluded Sawadogo *et al.* (2017).

2.18.3 Egg oviposition

Anopheles mosquitoes lay eggs in singles unlike culicine mosquitoes which lay eggs stack together i.e. in "rafts". Kweka *et al.* (2011) at Insect Microbiology Lab, Michigan, conducted a range of experiments to elucidate those important factors favouring *A. gambiae s.s.* **Giles**female oviposition and observed that: oviposition traps could be designed to take advantage of attractant factors; egg output progressively diminished with diminishing moisture content of mud; contrast and darkness of substrate were very important in egg laying, and that *A. gambiae*s.s. **Giles** females could not discriminate colour (hue) of substrate but could not discriminate brightness and contrast; a dark and moist substrate was found to be the most stimulatory to oviposition; merely darkening the bottom of a dish of water increased egg laying by > 9 fold. When Walker performed experiment on heterotrophic bacteria cultured from *Anopheles gambiae*s.s. **Giles** larval habitats in Kenya to test for attractiveness, bacterial odours were repellent to gravid females; oviposition response was 8-fold greater when bacteria were absent compared to when bacteria were present in culture.

2.18.4 Laboratory bioassays

Laboratory bioassays are carried out prior to preparation and availability of test organisms. According to Sawadogo *et al.* (2017) and Chambers *et al*. (2011) adult male and female mosquitoes will be collected from the field in the appropriate ratio male: female and fed for mating. The number of eggs oviposited by various *Anopheles* species based on the diet is described by Phasomkusolsil *et al*. (2013) and the treatment given to eggs including placing them in pans under a constant temperature room at 25^0C until the eggs hatch is described by Fahmy *et al.* (2015); MR4 *Anopheles* Laboratory; Khan *et al.* (2013). A variety of mosquito species including *A. gambiae s.s.* **Giles** larval bioassays have been done extensively and is described by among others, Behbahani *et al.* (2014);

Bossou *et al*. (2013); MR4 *Anopheles* Laboratory; Karthikeyan *et al*. (2012), Narendhiran *et al.* (2014). In all the quoted authors above the results were subjected to probit analysis. Probit analysis is a type of regression used with dose -response or binomial variables. It stems from toxicology to show response of chemicals to living organism. Response is always binomial (e.g death/nor death) and relationship between the response and various concentrations is always sigmoid. One advantage of probit analysis is that it acts as a transformation from sigmoid to linear and then runs a regression on the relationship. Another advantage of probit analysis is that it's easier to generate and also easier to write an MCMC samplers. It is more robust than other analyses such as student T with 7 degrees of freedom. This makes it more robust and good. The bioassays were conducted in accordance with the Nair *et al.* (2014) guidelines and as conducted by diagnosis, prevention and control.

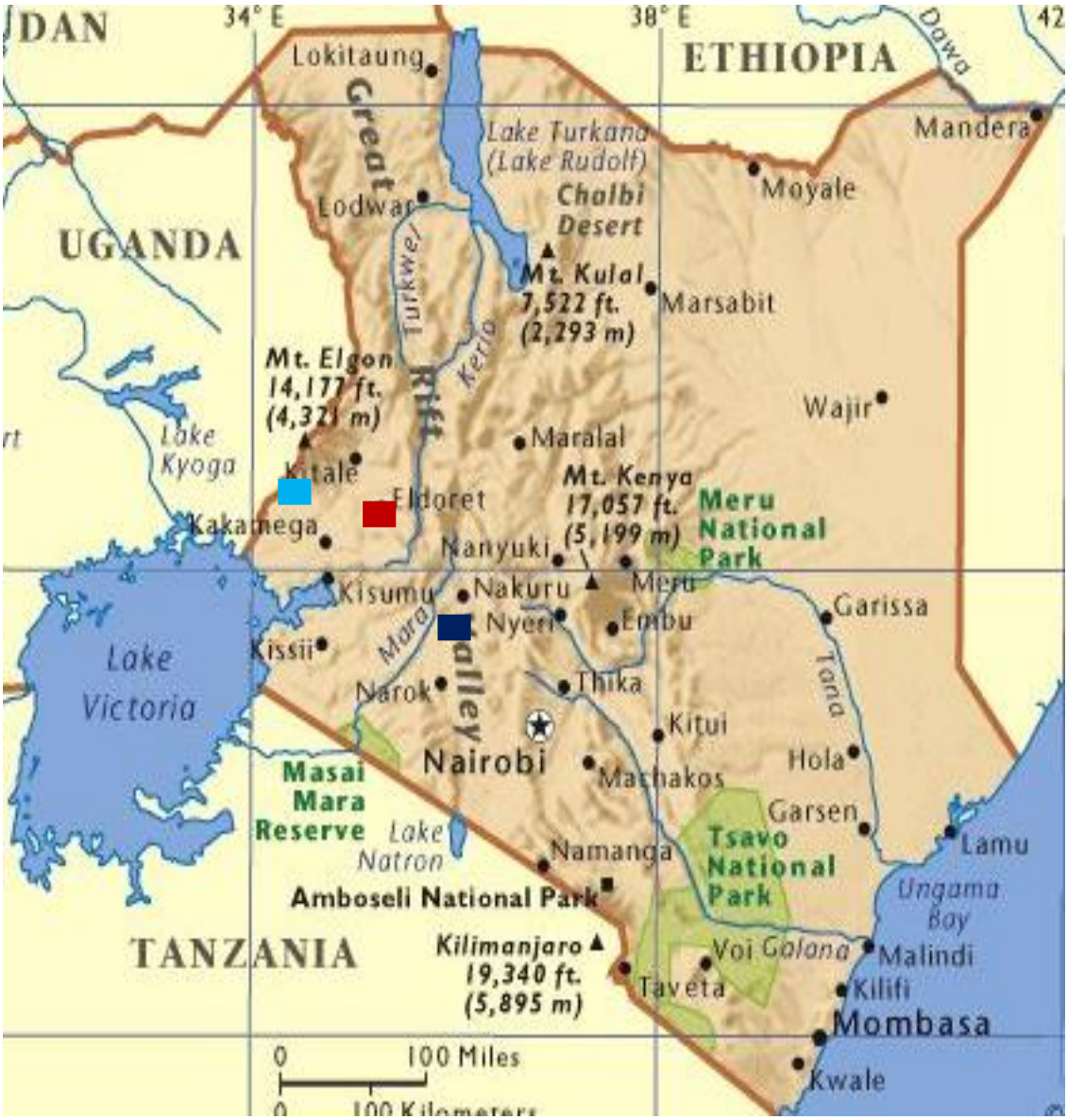
CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The study was carried out at Moi Teaching and Referral Hospital (MTRH) located in Eldoret Municipality of Uasin Gishu County in Rift Valley province of Kenya. The municipality is 47 km^2 in size and lies about 300 km north-west of Nairobi and 65 km north of Equator. The town is located at Latitude 0^0 31' 13"North and Longitude 35^0 16' 11"East (https://latitute.to/map/ke/kenya/cities/eldoret) Retrieved May 20, 2015. The town is at an altitude of between 1840- 2750 km above sea level. The climate is temperate throughout the year with evenly distributed rainfall; the rainy season occurring between March and September and the dry season between October and February.Hence the rainfall is bimodal.The annual precipitation range between 1000-1250 mm of which the months of April and May reads the highest precipitation. Average temperatures are 18^0C with a maximum and minimum of 25^0 and 9.8^0C respectively and a humidity of about 60%. Eldoret is surrounded by swampy areas which favour the breeding of malaria vectorsalmost throughout the year.

The study site was one of the unused rooms in the Department of Human Anatomy, School of Medicine, Moi University. This was located at the coordinates of 0^0 31' 13' North and 35^0 16' 11" East as indicated in the Figure 3.1 below.



Collection site for *Nitotiana tabaccum*

Study Site

Collection site for *Chrysanthemum cinerariifolium and Eucalyptus camaldulensis*

Fig. 3.1 Map of Kenya showing position of study site and location of sample collection.

3.2 Study Design

The study design was experimental i.e. laboratory-based to determine the toxic efficacy of natural essential oil flower and leaf extracts from *Chrysanthemum cinerariifolium, Eucalyptus camaldulensis* and *Nicotiana tabaccum* as larvicides. The laboratory bioassays were carried out following WHO (2013) standard procedures to establish the dose-mortality relationship and to calculate lethal concentrations LC_{50} and LC_{90} (concentrations involving death of 50% and 90% of the tested population). The laboratory bioassays were carried out on the 3rd*Anopheles gambiae* instar larvae upon which ranges of concentrations of each extract of the plants were administered. The 3rd instar larvae of *A. gambiaes.s.* **Giles** was preferred for testing because at this stage the larvae are very active and feed greedly and therefore would not likely miss feeding on larvicides (Asimeng, 2000).

3.3 Study population

All the 14,380 larvae that survived to 3rd instar were the study population.

3.4 Sample size

(i) Adult mosquitoes

Adult mosquitoes collected from the field in the ratio of 450:150 (3:1) (male:female) for laboratory rearing formed a first sample size.

(ii) Larvae

Two thousand four hundred seventy five (2,475) larvae at third instar level were used as sample size for all the objectives of the study i.e. extract efficacy, synergism, antagonistic, resistance ratio and persistence.

3.5 Sample collection and extraction of essential oils from the plants

3.5.1 Sample collection

3.5.2 Collection of vegetative material

Mature *vegetative material of Chrysanthemum cinerariifolium, Eucalyptus camaldulensis* and *Nicotiana tabaccum* were obtained in separate occasions from the field. *Eucalyptus camaldulensis* (Red gum) and *Chrysanthemum cinerariifolium* (Pyrethrum) were obtained from Kiambereria location, Molo an area located at 0^o 15' 0" South, and 350 44' 0" East (https://latitute.to/map/ke/kenya/cities/molo) Retrieved May 20, 2015. The area of collection of the two plant materials was at a distance of 110 km east of Eldoret, 220 km west of Nairobi and 57.6 km west of Nakuru.

Nicotiana tabaccum (Tobacco) was collected from Malakisi, Bungoma County an area lying at the coordinates of $0^0 40' 52.\ 48''$North and $34^0\ 25'\ 16.36''$ East (https://latitute.to/map/ke/kenya/cities/malakisi) Retrieved May 20, 2015. Malakisi is at a distance of 126.2 km East of Eldoret and 22.2 km from Malaba, the Kenya-Uganda boarder.

The three vegetative material were collected in different days because of the distance by region in which they were located.

Twenty kg of mature *Chysanthemum cinerariifolium* flowers were bought from a farmer who was picking *C. cinerariifolium* flowers for drying and marketing while mature leaves from *Eucalyptus camaldulensis* were freely given. The eucalyptus leaves were harvested together with their short branches so that it could be easy to dry them by hanging them using the tree branches. Twenty kg of green fresh leaves of *Eucalyptus camaldulensis* were collected. The pyrethrum flowers and eucalyptus leaves were collected on 24th November, 2008.

Twenty kg of *Nicotiana tabaccum* mature leaves were bought from a farmer in Malakisi, Bungoma. The leaves of *Nicotiana tabaccum* were carefully plucked from *Nicotiana tabaccum* trunks ensuring that petioles were not detarched from the leaves. Twenty kg of each plant material were preffered because complete dry flowers of *Chysanthemum cinerariifolium* weigh one quarter of the green flowers (Gachie, 2018). This meant that 20 kg of *Chrysanthemum cinerariifolium* was to weigh 5 kg when dry. It was presumed that the dry leaves of *Eucalyptus camaldulensis and 20kg of Nicotiana tabaccum* could as well weigh as those of *Chrysanthemum cinerariifolium* when dry and therefore a decision was made that 20 kg of all the vegetative materials be collected so as to have 5 kg dry material, enough for keeping in stock. All the collected material were placed in new clean gunny bags separately and transported to an airy open storage facility in Eldoret for drying.

3.5.3 Flower and leaf preparation

The vegetative materials were washed thoroughly with distilled water and they were hanged away from the sun in an airy store and allowed to dry for three weeks. Using the short branches holding the leaves of *Eucalyptus camaldulensis*, the branches were tied with a string and hang in the store for drying naturally. Since the leaves of *Nicotiana tabaccum* were broad and long a string was pierced through the petioles of the leaves and leaves were hang in the store for drying naturally. Flowers of *Chrysanthemum cinerariifolium* were pretty small measuring 3 cm with the petals but when they dry the petals detach from the stigma and leaving the small flower heads containing stigma, style and pistil as a single small noddle drying down to 1 cm in size (Gachie (2018). With the realization that the flower heads were to be the main content of pyrethrin and were likely to drop to waste as they dried, the fresh flowers were put in nylon bags of 0.4 cm mesh

and hanged the mesh at a rafter for the flowers to dry naturally. All the materials that were exposed for drying were monitored regularly until they completely dried.

3.5.4 Grinding of the flowers and leaves into powder

3.5.5 Grinding of *Chrysanthemum cinerariifolium* flowers

The dry flowers of *Chrysanthemum cinerariifolium* and the dry leaves of *Eucalyptus camaldulensis* and *Nicotiana tabaccum* were alot and to make work easier and faster two methods were used to grind the dry materials. One was by grinding using a blender (Kanchan, Tycoon, Japan), and the other was by manual grinding using mortar and pestle. The latter method was applied as a final method after the blender more so because the final product became smoother than that of the blender alone. The mortar was made of steel, borrowed from School of Engineering (Department of Production Engeering i.e. Mechanical Engineering) Moi University, Eldoret. The mortar which had no serial number nor make was a bowl-shaped equipment and heavy to lift. The pestle was made of steel, and had a rounded tip. Three types of pestles were available for use and were rough, medium rough, and fine pestles. First, 500 gm of *Chrysanthemum cinerariifolium* were placed in the blender and the first grinding of the flowers was done using the blender. The flowers were then re-grinded using mortar, first by rough pestle, then by the medium rough pestle and finally ground them using finepestle. This process enabled the achievement of fine *Chrysanthemum cinerariifolium* powder which was placed into amber or blue bottles and sealed. The powder was labeled indicating the botanic name, date ground, where green flowers collected, name of collector , date collected, and intended use of the powder. The powder was stored at 4^0 C until in use laiter.

3.5.6 Grinding of *Eucalyptus camaldulensis* leaves

Before grinding the dry leaves of *Eucalyptus camaldulensis* the blender and the mortar were cleaned with hot water and left to dry. This was purposely to remove any traces of

the powder of *Chrysanthemum cinerariifolium* which could contaminate the powder of *Eucalyptus camaldulensis.* Once dried, 500 gm of *Eucalyptus camaldulensis* dry leaves were placed in the blender and ground to fine powder. The fine powder was again regrinded using mortar and pestles in the manner described for *C. cinerariifolium*.The powder was labeled and stored in a similar process as described of *C. cinerariifolium* above.

3.5.7 Grinding of *Nicotiana tabaccum* dry leaves

Similarly to avoid contamination of *Nicotiana tabaccum* powder the blender and the mortar were cleaned and left to dry before grinding and storing the leaves of *Nicotiana tabaccum* in a similar process as described of *Chrysanthemum cinerariifolium. above.*

3.6 Extraction of essential oils from the plants

Extraction of the oils from the plants were carried out in the order of the solvents hexane, ethanol, methanol, DCM, ethyl acetate and aqueous. Extraction of oil using any other order could have been proper as well. It did not matter which solvent was first in use and which one was used last.

One hundred grams of each plant powder was soaked into 200ml of hexane in a stoppered bottle for 3 days (72 hours) at room temperature. This was placed on electric shaker (ROTA PXL 772-WT/002, England) as also used by Uthayarasa *et al.* (2010) Uthayarasa *et al*. (2010). The electric sharker was to promote complete dissolution. The soaking was carried out systematically, extract after extract in the order of *C.cinerariifolium, E. camaldulensis* and *N. tabaccum*. This consitituted 3 extracts of hexane. These three mixtures were filtered using Whatman N.o. 1 filter paper (M/s Glassil Scientific Industries, India) and filtrate was collected into a conical flask.

After filtration there remained a residue in the flasks called concentrates into which again 200 ml of hexane was added purposely to ensure maximum extraction of the oil was

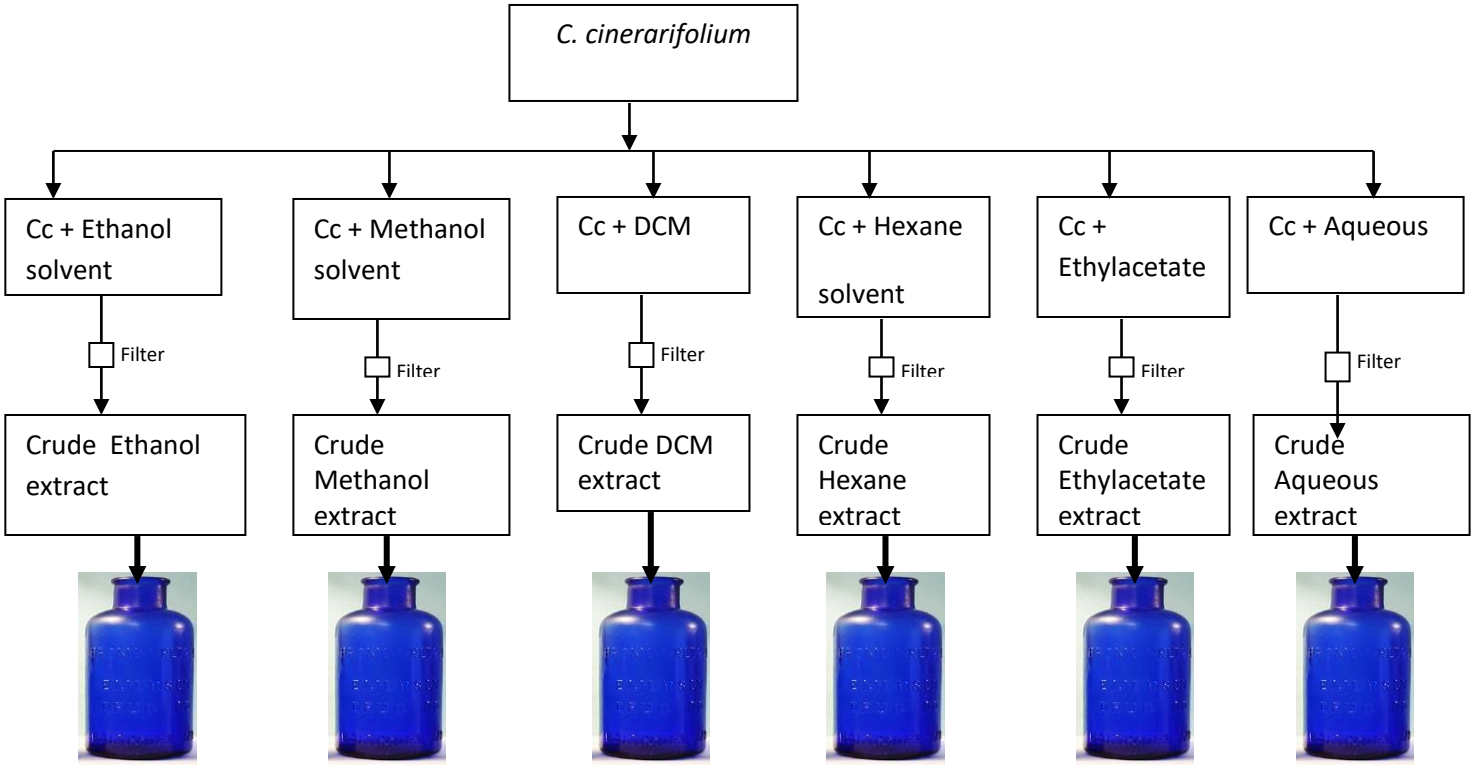
achieved. This procedure of adding 200 ml of hexane to the residues was repeated three times, a period by which all the oil was confirmed to have been extracted. It was observed that at the end of the 3rd filtration a clear filtrate was ensuing an indication that oil extraction was exclusively done.

Once extraction using hexane was over, oil extraction using other solvents namely ethanol, methanol, DCM, ethyl acetate and aqueous was carried out in a similar method as used for hexane extraction. The soaking of these five powders took 15 days.

From the six solvents, a toral of 18 extracts were obtained from the 3 plants. This was 6 extracts per plant. The 18 extracts were labelled indicating plant species, date of collection, by whom plants were collected, date of collection ands place of plant origin. The products were then stored at 40C in air tight amber or blue bottles until use.

It is important to state that the six solvents used in the extraction of oils in this study were the only ones available in the market during the period of study and therefore there was no choice other than using them.

3.6.1 Flow chat of the extraction process of the essential oils.



Amber bottles for extract storage 4^0C.

Fig. 3.2 Flow diagram of extraction process of the essential oils.

The crude extracts for *E. camaldulensis* and *N. tabaccum* were prepared in a similar method as indicated in the flow diagram representing C. cinerariifolium Fig.... above.

3.6.2 Concentrating the extracts

For better keeping and loger lasting of the extracts they were concentrated into powder.

The extracts were dried under reduced pressure using a rotary evaporator (BUCHI, Rotavapor, and R114) Uthayarasa *et al.* (2010). All extracts were stored at 4^0C in air tight amber or blue bottles not to be accessed by light throughout the study period. These dried filtrates (solutes) were labelled, plant species clearly indicated, date of collection, time and place of origin were shown and by whom they were collected.

3.7 Preparation of stock standard solution (SSS) of the flower and leaf extracts.

From each exract powder, 100g of the powder (solute) was weighed on weighing paper or small container and then transferred to a volumetric flask (‘‘vol flask’’). A funnel was used because of the slim neck of the vol flask.

A small quantity (200 ml) of distilled water as solvent was added to the vol flask and then contents were swirled gently until the solute was completely dissolved. More solvent was added until the meniscus of the liquid reached the calibration mark on the neck of the vol flask (a process called ‘’diluting to volume’’ or ‘’adding to the mark’’). The vol flask was then capped and inverted several times until the contents were mixed and completely dissolved. In this way, 1,000 L SSS of each extract was made. A total of 18 SSS were made which were accordingly labelled and stored as described under other extracts above ready for use latter in the making of working concentrations.

3.8 Preparation of *C. cinerariifolium* ethanol, methanol, DCM, hexane, ethyl acetate and aqueous working concentrations 50, 100, 150, 250 and 300 ppm.

From the standard stock solutions test concentrations of 50,100,150,200,250 and 300 ppm of the crude extracts were prepared in distilled water for use in larval bioassays (Zuhara *et al*, 2014, Kumar *et al*. (2012); Uthayarasa *et al*. Karthhikeyan *et al*. 2012; and Nganjuwa et al. 2015).

By use of micropipettes Gilson P-10, P-20, P-100 and P-200, concentrations of 50, 100, 150, 200 and 300 ppm of each plant and of each extract solvent were sepaterately drawn from the 1000 ppm concentrate of stock solution and put into 1 L volumetric flask. Deionized water was used to add up to the mark. The mark was the line at the top of the volumetric flask marking 1 L volume, and any measure beyond or below the line would be obsolete.

Mixtures were shaken gently side by side while avoiding their spillage. The use of pipettes was in accordance with the concentration volume drawn. For example 50 ppm was drawn using P-20 twice and P-10; 100 ppm was acquired using P-100 only one draw; 150 ppm (P-100, P-20 drawn twice , P-10); 200 ppm (P-200 drawn once); 250 ppm (P-200, P-20 x2, P-10) and 300 ppm (P-200 and P100).

All the prepared working concentrations were placed in air tight amber or blue bottles, labelled to show plant species, concentrations, source of plants, date of preparation and preparer's name and address. The concentrations were stored at 40 C as working concentrations ready for use in larval bioassays.

The working concentrations for *E. camaldulensis* and *N. tabaccum* plants using the six solvents was carried out in the same procedure as the one described for *C. cinerariifoliumin* in sub-section 3.0.4(i) above.

3.9 Field sampling of adult male and female *Anopheles* gambiae mosquitoes for laboratory rearing

From the previous researchers (cited below), it is generally stated that there is no first hand rule for ratio of male and female mosquitoes to be caged for mating:

Boyer *et al*.(2012) have given ratio of 20 males and 20 females; Kweka (2012) suggests 1:3 (male:female); Pimid *et al.* (2015) Wa Nazni (2009) reared Aedes aegypti ratio of 4:3 (male:female); Mamaret *et al.* (2017) Olayeni *et al.* (2011) and Oliva *et al.* (2011) at the ratio of 1:1 (male:female); Ng'habi *et al*. (2005) ratio of 3:1 (male:female); for A. arabiensis Hasan *et al.* (2010) applied a ratio of 1:1:1 (irradiated males:wild males:wild virgin females); Hewell and Benedict (2009) and Jayaprakash and Kathikeyan (2014) have used a male female 2:1; Oliver *et al.* (2012) a ratio of 100 males and 100 females; WHO (2013); WHO, (2005) recommends a ratio of 3:1 (male: female); Godfrey and Warren (2016) a ratio of 3:1 (male:female) Galizi *et al.* (2014) Loinibos and Escher

(2008) Loinibos and Escher (2008) used a ratio of 3:1 (male:female) and Madakachery *et al* (2013) had a ratio of 3:1 (male:female). In this research a ratio of 3:1 (male:female) was used for reasons stated below in the third paragraph.

General distinguishing features of *Anopheles* male and *Anopheles* female mosquitoes were of great value in identifying the species for collection. The distinguishing features used by White and Kaufman (2014) and Gillies and Coetzee (1987 were applied to identify and collect the mosquitoes. These features are as shown in Table 3.8.

Using backpack battery-powered hand-held aspirator (DUpTOk Aspis 2211XL, UK) , Maia *et al.* (2011), Maia *et al.* (2011) and as conducted by Olayemi *et al.* (2011), and Pimid *et al.* (2015) WA Nazni *et al*. (2009) a hand-held mouth aspirator (in case the battery operated aspirator broke down), adult biting male and female *Anopheles gambiae* **Giles** mosquitoes in the ratio of 3:1 (male:female) were collected from their various resting places (wetlands, shrubs, banana plantations, maize farms, residential houses, social halls, and other similar places) in Langas area of Eldoret municiality. Care was taken not to collect female *A.gambiae s.s.***Giles** whose spermathecae appeared enlarged as these mosquitoes could be engorged with eggs and therefore likely to ovipost eggs in the cages earlier than the others. Care was also taken not to collect any other species of mosquito such as *Culex* or *Aedes.* The males and females of *A. gambiae s.s.***Giles** were collected into separate glass jars capped with a net cloth to allow oxygen for the mosquitoes. These were transported to the laboratory for rearing. There were no extra numbers of both *Anopheles gambiaes.s.***Giles** sexes collected as any extra number did not have any value for rearing.

Collection of the adult mosquitoes started at 8.30 am and lasted until 5.00 pm the time by which 450 *Anopheles gambiae s.s.* **Giles** males and 150 *Anopheles gambiae s.s.* **Giles**

females were collected. The purpose of the adult mosquito collection was to enable grow pure colonies of mosquitoes for bioassays.

In one field trip 450 male and 150 female mosquitoes were collected. Ten field trips were made and a total of 4500 males and 1500 females were collected. This number and ratio of mosquito collection were acceptable for some reasons: the number was manageable in collection and rearing, it was also high enough to maintain good numbers in case of any eventualities such as deaths occurring among the mosquitoes. Importantly this number was large enough to engage mating in case some male and female mosquitoes developed impotence. Males were more in the ratio than females because of the fact that it is the males that swarm in order to attract females to join the swarm for mating. Care was taken not to collect female *A.gambiae s.s.***Giles** whose spermathecae appeared enlarged as these mosquitoes could be engorged with eggs and therefore likely to ovipost eggs in the cages earlier than the others. Care was also taken not to collect any other species of mosquito such as *Culex* or *Aedes.* General distinguishing features of *Anopheles* male and *Anopheles* female mosquitoes were of great value in showing the species for collection. The distinguishing features used by White and Kaufman (2014) and Gillies and Coetzee, 1987 were applied to identify and collect the mosquitoes. These features are as shown in Table 3.1.

Fig. 3.3. Adult female African malaria mosquito, *A. gambiae* (Differentiating features) *Anopheles gambiae* Giles. Photograph by Lyle Buss, University of Florida.

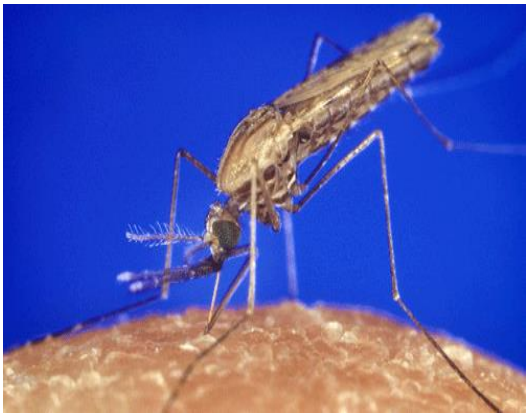


Fig. 3.4 Female *A. gambiae* **Giles** taking ablood meal. Photograph by James Gathany

Table 3.1 Features of differentiating male and female *Anopheles gambiae* mosquito

Female *Anopheles gambiae*	Male *Anopheles gambiae*
Abdominal segments have laterally projecting turfts of scales on segment II-VII.	Abdominal segments do not have these turfts.
Hind tarsus has at least 2 segments which are entirely pale.	Hind tarsus not have segments.
Hind tarsus 5 is entirely dark, while tarsus 4 is white.	Hind tarsus are not coloured.
Legs are speckled, sometimes sparsely.	Legs are not speckled.
Wings are entirely dark or may have pale sports confined to costa and vein 1.	Wings are not dark nor have pale sports on costa and vein 1.
Wing without a pale spot on basal half of costa.	Wing with at least 1 pale sport on basal half of costa.
Palps have dark apex.	Palps apex is pale.
Palps have 4 pale bands.	Palps have 3 pale bands.
Wing has pale interruption on 3rd main dark area of vein 1, sometimes fused with preceding pale area.	3rd main dark area has no pale area.
Wing with 2 pale spots on vein 5.1. Proboscis: longer tha pulps	Wing with 1 pale spot on vein 5.1. Proboscis: shorter or equal to pulps

The collection of these mosquitoes was carried out as follows: Each of the seven of us had either backpack battery-powered hand-held aspirator or a hand-held mouth aspirator. The collectors were also provided with magnifying glasses. While mosquitoes were on the landing surfaces (walls, tree trunks, short shrubs, grass, banana plantations, wet grounds, and eaves etc.) during the day they were quietly resting and this factor enabled easy collection of the adult mosquitoes. Without causing any disturbance to the resting mosquito, a magnifying glass of the power x50 and x100 magnification were used to observe the *Anopheles gambiae s.s.***Giles** mosquito features easily at a distance of 0.46 m (1ft 6 inches) from the mosquito landing surface and the mosquito was sucked at a much closer distance of half to one inch. The males and females of *A. gambiae s.s.***Giles** were collected into separate glass jars capped with a net cloth to allow oxygen for the mosquitoes. These were transported to the laboratory for rearing. There were no extra numbers of both *Anopheles gambiaes.s.***Giles** sexes collected as any extra number did not have any value for rearing.

Collection of the adult mosquitoes started at 8.30 am and lasted until 5.00 pm the time by which 450 *Anopheles gambiae s.s.***Giles** males and 150 *Anopheles gambiae s.s.* **Giles** females were collected. Since the area of collection was a wetland and a suitable breeding place for mosquitoes, and the population of the targeted species of mosquito was of great numbers, the targeted numbers of both male and female *A.gambiae s.s.***Giles** was easily met.

Collection of the mosquitoes was done once a day and once in a month between the months of January 2009 and December 2009. Collection was executed within the first three days of the month to allow ample time for eggs oviposition and larvae development.

3.9.1 Flower and leaf preparation

The vegetative materials were washed thoroughly with distilled water and they were hanged away from the sun in an airy store and allowed to dry for three weeks. Using the short branches holding the leaves of *Eucalyptus camaldulensis*, the branches were tied with a string and hang in the store for drying naturally. Since the leaves of *Nicotiana tabaccum* were broad and long a string was pierced through the petioles of the leaves and leaves were hang in the store for drying naturally. Flowers of *Chrysanthemum cinerariifolium* were pretty small measuring 3 cm with the petals but when they dry the petals detach from the stigma and leaving the small flower heads containing stigma, style and pistil as a single small noddle drying down to 1 cm in size (Gachie (2018). With the realization that the flower heads were to be the main content of pyrethrin and were likely to drop to waste as they dried, the fresh flowers were put in nylon bags of 0.4 cm mesh and hanged the mesh at a rafter for the flowers to dry naturally. All the materials that were exposed for drying were monitored regularly until they completely dried.

3.10 Laboratory rearing of adult male and female *Anopheles gambiae s.s.* **Giles** mosquitoes.

Table 3.2 shows the mosquito culture programme for ten field trips of adult mosquito collection. The mosquitoes were distributed into 3 cages in a combination of 150: 50 (males: females) per cage. Laboratory conditions were well maintained throught the rearing period (Temperature, 28 ± 2^{o} C, Relative Humidity (RH) 75 ± 5^{0} and 12:12 dark: photo period). The mosquitoes were fed accordingly and allowed to mate.

Table 3.2. Adult *A. gambiae s.s.***Giles** Laboratory rearing programme

Field trip	Date	Adult *Anopheles gambiae* Male : Female ratio (3:1)					
		Cage 1		Cage 2		Cage 3	
		Male	Femle	Male	Femle	Male	Femle
1	1.1.2009	450	150	450	150	450	150
2	1.2.2009	450	150	450	150	450	150
3	1.3.2009	450	150	450	150	450	150
4	1.5.2009	450	150	450	150	450	150
5	1.6.2009	450	150	450	150	450	150
6	1.7.2009	450	150	450	150	450	150
7	1.9.2009	450	150	450	150	450	150
8	1.10.2009	450	150	450	150	450	150
9	1.11.2009	450	150	450	150	450	150
10	1.12.2009	450	150	450	150	450	150

In all mosquitoes including *A. gambiae s.s.***Giles,** it is the males that congregate in large swarms in order to attract females to fly into the swarm to mate. In this case the males were more than the females in number in order to prevail in swarming. This was the reason why the ratio 3:1 (450:150; male:female) was used in this study and as also reported in the previous studies (WHO (2013) ; Godfrey and Warren, 2016;; Madakachery *et al.* 2013). Mosquito rearing cages are manufactured in many sizes Maiga *et al.* (2017); Ethiopian Public Health Instittute (EPHI) (2017); Panigrahi et al. 2014; Kivuyo *et al.* (2014) Das *et al*. 2007; Imam *et al.* 2014; and Carvalho *et al.* 2014).

The adult mosquitoes were then transferred into three locally wooden-made, collapsible, screened cages 30x30x30 cm; 30cm x30cm x30cm and 30cm x30cm x46cm; (WHO, 2005) in the ratio of 3:1 (males: females) for mating as reported in previous studies (WHO,2005, 1967; Godfrey and Warren, 2016; Lounibos and Escher, 2008; Madakachery *et al.* 2013). Mosquito rearing cages are manufactured in many sizes (Paulson, 2005; Benedict *et al.* 2009; Spitzen and Takken, 2005; Panigrahi et al. 2014; Kivuyo *et al.* (2014); Das *et al*. 2007; Imam *et al.* 2014; and Carvalho *et al.* 2014). The commonly used cage measures 30cm x 30cm x30cm. The rearing cages used in this study

were locally made as it was difficult to obtain the World Health Organization (WHO) or Control of Diseases Centre (CDC) or Kenya Medical Research Institute (KEMRI) standard cages. Three sizes were made locally for the study: one cage of the size 30.0cm x30.0cm x46cm and was marked cage No.1 and two cages each of the size 30.0cm x30.0cm 30.0cm and were marked cage Nos 2 and 3 for the study. Cage No 1 was easy to make and took 1 month because it had large members of the framework which were easy to join and nail without cracking. Cages No 2and 3 were the hardest cages to make because their sizes 30cm x30cm x30ccm required thin, slim, tender and small surface area and the two cages took 8 months to make as its weak framework got broken regularly as nailing was being done. All standard cages have only one chamber and the locally made cages that were used consisted of one chamber each. The reason why 3 cages were used was that two cages were reserve cages in case if one cage was used could not raise enough larvae for bioassays. That meant that larvae in cages 1 to 3 were summed up and the total larvae was the number of larvae that was available for the tests. However, the cages were not for comparison purposes i.e. which cage raised more larvae than the other and vise visa. Similarly larvae from the 3 cages were treated similarly during tests; they were not given any preference in terms of which cage they were reared.

Each rearing cage was provided with a marker (cue/attractant) and an infusion for the purpose of making the cages attractive for the mosquito habitation Milke and Mairelli (2012); Madakachery *et al.* 2013). A marker was an indicator to naturalize the adult mosquitoes such that they felt that they were in a natural environment. For this marker little brown sand on black polyethene paper was spread on cages' floor and green grass grown in little tins were placed at the inside corners of the cages. Cue or attractants consisted of chicken chaw and moistened dry roadside grass both mixed together. The

purpose for the cue or attractant was to emit carbon dioxide, a smell which attracts mosquitoes to enable them move nearer to the target to bite.

Three cages were used for the rearing and each cage contained 150 males and 50 females a number for one trip collection for mating. The mosquitoes were continuously fed with 10% sucrose solution with 0.2% methylparapen socked in cotton wool. The sucrose solution was for survival and flight of both mosquitoes during the mating period and until the female could be fed on vertebrate blood. Female mosquitoes were fed on bovine blood (collected from Eldoret slaughterhouse on a daily basis in order to have fresh blood) in order to energize female mosquitoes and enable them to oviposit. Bovine bloob was boiled, cooled and stirred every 10 to 15 minutes using a hooked rod to prevent blood cloting.

Mosquitoes were maintained at temperatures ranging from 26^0C to30^0C (or at 28 ± 2^0C) with relative humidity ranging from 60% to 80% (75+5% and 12:12h (dark : light) photoperiod standard for colony production and in readiness for mosquito mating to commence Okal *et al.* (2015) Okal *et al.* (2015). Since a dehumidifier was not available, humidity was maintained by use of wet towels hung out on cages. The towels were placed over 4 sides of the cages and left one side of the cages open. Towels prevented excess dripping. These towels were not allowed to dry out Baughman *et al.* (2017); Wijegunawardana *et al*. 2015). Lighting regulation was by Fluorescence light (Wijegunawardana *et al.* 2015).

Adults male and female *Anophelesgambiae* mosquitoes are normally reared in prepared ovitraps (plastic basins of unchlorinated spring, river, well water or alternative to this deionized water) Diabate and Tripet (2015). Spring water is cheap and in the case of these study 60 litres of spring water was purchased from Kiringet Mineral Water Factory, Molo and used as a supplement to distilled water (expensive to buy). On arrival in Eldoret the

water was filterd using a cotton cloth, boiled and cooled before it was put in 1 litre bottles and stored at 4^0C for use in mosquito rearing. However, when deionezed water was available it was used for mosquito rearing. The basins were placed in screened cages with a fine net to retain the male and female mosquitoes in the basins. However, the screens were incorporated with a stockenett at a top corner tied with a rubber band for cages access by hand insertion. In each basin suitable infusion (cue) which comprised aged chicken chow (mature) and roadside grass or hay were placed inside the cages for oviposition, attractancy or lures, Diabate and Tripet (2015); Godfrey and Warren, (2016); Madakachery *et al*. (2013).

Fig 3.5. Cage 1. Locally made cage for the study(30x30x46cm) Coutersy of Seme Capentry Workshop,Eldoret, Kenya.

Fig.3.6 Cage 3. Locally made cage for the study (30x30x30cm) Coutersy of Seme Capentry Workshop, Eldoret, Kenya

Fig 3.7. Cage 3. Locally made cage for the study (30x30x30cm) Coutersy of Seme Capentry Workshop, Eldoret, Kenya

Three cages were used for the rearing and each cage contained 450 males and 150 females a number for one trip collection for mating. The mosquitoes were continuously fed with 10% sucrose solution with 0.2% methylparapen socked in cotton wool. The sucrose solution was for survival and flight of both mosquitoes during the mating period and until the female could be fed on vertebrate blood. Female mosquitoes were fed on bovine blood (collected from Eldoret slaughterhouse on a daily basis in order to have fresh blood) in order to energize female mosquitoes and enable them to oviposit. Bovine bloob was boiled, cooled and stirred every 10 to 15 minutes using a hooked rod to prevent blood cloting.

Mosquitoes were maintained at temperatures ranging from 26^0C to30^0C (or at 28 ± 2^0C) with relative humidity ranging from 75% to 80% (75+5% and 12:12h (dark : light) photoperiod standard for colony production and in readiness for mosquito mating to commence Baughman *et al.* (2017); Okal *et al.* (2015). Since a dehumidifier was not available, humidity was maintained by use of wet towels hung out on cages. The towels were placed over 4 sides of the cages and left one side of the cages open. Towels prevented excess dripping. These towels were not allowed to dry out (Wijegunawardana *et al*. (2015). Lighting regulation was by Fluorescence light (Wijegunawardana *et al.* 2015).

Adults male and female *Anopheles gambiae* mosquitoes are normally reared in prepared ovitraps (plastic basins of unchlorinated spring, river, well water or alternative to this deionized water) (Diabate and Tripet (2015); (Godfrey and Warren, (2016). Spring water is cheap and in the case of these study 60 litres of spring water was purchased from Kiringet Mineral Water Factory, Molo and used as a supplement to distilled water (expensive to buy). On arrival in Eldoret the water was filterd using a cotton cloth, boiled and cooled before it was put in 1 litre bottles and stored at 4^0C for use in mosquito

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3.10.1 Eggs oviposition

Colonies were maintained and all experiments were carried out at a constant temperature of 28 ± 2^0C and 75 ± 5% Relative Humidity.

A method for *Anopheles* mosquito egg management carried out by Olayem and Ande (2009) was adapted. A small filter paper wrapped in a conical shape was put in a small beaker containing distilled water, and made sure that filter paper got moist. The purpose of the filter paper insertion was to prevent eggs from sticking to the walls of the beaker. Since *A. gambiae* s.s. **Giles** and *A. arabiensis* prefer laying in dark, the sides of beakers were lined with black paper. The beaker was then kept inside the cage overnight for the mosquitoes to lay eggs. The mosquitoes were fed with blood and were able to lay eggs 2 days after blood feed. The filter paper containing eggs was placed in plastic tray with 300 ml distilled water. A pinch of brewer's yeast was added to the tray and eggs were allowed to hatch to larvae during the next days. The eggs were transferred from the filter papers with the aid of mounting needles into the plastic bowls (5 cm height and 30 cm diameter, where they were held for 24 to 72 hours for hatching details as described below:

Day 1: The caged mosquitoes in addition to being fed on 10% sucrose solution the female mosquitoes were given a blood meal (blood collected from a slaughterhouse, treated and

stored for continuous use or else blood was collected daily from the slaughter house.) to lay eggs (Kivuyo et al 2014).

Deionized water was added to a rearing pan so that water covered completely bottom of pan and avoided adding too much water. A yeast suspension was added to each pan to a final concentration of 0.2% e.g. in 300 ml of water 3 ml of a 2% w/v yeast solution was added. Pan was swirled until yeast dispersed throughout the pan area (Kivuyo *et al.* (2014)

Day 3: It took 2 days for females to lay eggs after a blood meal. A small filter paper wrapped in a conical shape was put in a small beaker containing deionized water, making sure that filter paper got moist. The beaker was kept inside the cage overnight for the mosquitoes to lay eggs. Egg count was made by using a dissecting microscope (Owiti and Misire (2017); Impoinvil *et al.* (2007); Aiku *et al*. (2006); Pammplona *et al.* (2009); Fritz *et al*. (2008); Li *et al.* (2007): Nazni *et al*. (2009); Ellis, 2008; and Farida *et al.* 2011).

Day 4: The egging paper (filter paper) containing the mosquito eggs was held by the edge to avoid touching the eggs and gently rinsed them and released approximately 500 eggs into plastic trays (30x35x5 cm) and added 1 litre deionized water into the trays. The pans were labelled and covered to prevent contamination. *Anopheles* eggs were counted using a dissecting microscope and allowed to hatch to larvae within 24-48 hours (Impoinvil *et al*. 2007; (Kivuyo *et al.* (2014).

Next day the pans were uncovered without disturbing eggs to see if there were 1st instar larvae present (they are very small and hard to see, hence lighted area was used to check).

Adult *Anopheles gambiae s.s.***Giles** mosquito lay 50 to 200 eggs per oviposition (CDC, 2015).

Day 5: Eggs hatched into larvae. The larvae were counted and recorded.

3.10.2 Larvae rearing

Day 6, 7 and 8: Larvae were carefully fed for their growth and development by adding a pinch of ground fish food meal (0.1 g for 1st and 2nd instar larvae and 0.3 g of brewer's yiest for 3rd instar larvae at 8:00 and 16:00 each day) to each tray (Damdangdee *et al*., (2013). They were also fed every day with two tablets of finely ground brewer's yeast, and ground fish meal and monitored for density and population. By the 8th day the larvae had fully developed into 3rd instar and they had to be tested quickly before day 10 when they were to be in their 4th instar.

Since it was not feasible to reserve the larvae for the next experiments, ten trips of adult mosquito collection from the field was necessary to grow the larvae for the accomplishment of the study.

3.10.3 Larval bioassays

The larval bioassays were conducted in accordance with WHO, (2013) procedural instructions for determining susceptibility or resistance of mosquito larvae to insecticides and as also conducted by Sengottayan *et al*. (2007), Uthayarasa *et al.* (2010), and Singh *et al.* (2014). A total of 25, 3rd instar larvae (WHO, 2013) were picked at random and were used for bioassays per concentration for all the experiments. The number of larvae picked for use is in accordance with the previous workers WHO, (2013); Govindarajan and Karuppannan, (2011); Idris *et al.* (2013) Bossou *et al.* (2013); Zareen *et al.* (2016); Khanasi *et al*. (2012); and Elumalai *et al*. (2012).

Concentrations for *C.cinerariifolium* were first prepared then for *E. camaldulensis* and finally made concentrations for *N. tabaccum*. Six 500 ml capacity beakers were prepared and concentrations of 50, 100, 150, 200, 250 and 300 ppm were placed into the beakers, each concentration in separate beaker. Into these concentrations 200ml of distilled water was added. These beakers then represented six different concentrations for one solvent

eg. ethanol solvent. Other concentrations were made using methanol, DCM, hexane, ethyl acetate and aqueous and this completed the total 36 concentrations for *C. cinerariifolium*. Similarly, other 36 concentrations were made for each of the other two plants, *E. camaldulensis* and *N. tabaccum*. In all 108 concentrations were prepared for the three plants.

Control experiment was prepared and consisted of a mixture of acetone and DMSO (1:1v/v) while alternatively 200 ml of dechlorinated water with 2 ml of acetone and another set of beakers with dechlorinated water only served as a complementary control. For each concentration three replicates were run at the same time.

By use of a mouth aspirator, a batch of 25 3rd instar larvae were collected at random one by one from rearing tray. These were placed into a 0.5 ml cup of distilled water to maintain them alive as the rest of the larvae were being collected to make 25 in number for immersion into the beaker of the concentration. The larvae were dipped into the beakers at the rate of 25 3^{rd} instar larvae per beaker and per crude flower and leaf concentration.

Twenty five larvae per beaker were appropriately recommended for three reasons: First, larvae crowding will negatively influence normal feeding rates of the larvae. Secondly, the number of larvae per container will influence the amount of toxic moieties that will be available to each individual and thirdly, the amount of toxin per larvae will also be a function of the volume of water used for the bioassay. The recommended amount of water is 5 ml per larvae although because of evaporation and other factors 200 ml is generally agreed upon as suitable for bioassays (Ayorinde *et al.* 2015).

Each experiment set-up was maintained at ambient temperature (28 ± 2^0C, 75±5% Relative Humidity and a photo period of 12:12h i.e. 12h light and 12h darkness). Since a dehumidifier was not available wet towels were hang on cages to control humidity

(Wijegunawardana, 2015). The larvae were fed with dry baker's yeast powder on the water surface (50 mg/L) throughout the experiment periods. Dry baker's yeast was preferable because it easily floated on water for the larvae to feed on. The larvae were exposed to different concentrations of the oils and mortality was observed in 24 hours.

By observation, larvae that did not respond by a wiggling movement while the side of the beaker was tapped with a stirring rod was regarded as dead; or when larvae prodded with a wooden applicator stick, or stimulated with a Pasteur pipette and those that did not move away from the stimulus or show a wiggling motion were recorded as dead. Similarly larvae that did not respond when prodded with a needle at their cervical region and did not rise to the surface were regarded dead as well. Further careful observation was done to identify the moribund larvae which were counted and added to the dead ones for the calculation of the percentage mortality. Mortality was recorded 24 h post-treatment and mortality data was subjected to Probit regression analyses (Norusis, 2008) to determine 50% (LC_{50}) and 90% (LC_{90}) of lethal concentration. The percentage mortality was calculated using Dawider (2013) formula.

3.11 Inclusion criteria

All *Anopheles gambiae* mosquito larvae that attained 3rd instar level.

3.12 Exclusion criteria

(i) Any living aquatic organism that was not bearing the characteristics of *Anopheles gambiae*mosquito larvae and might have entered the breeding cages by chance.

(ii) Any larvae that showed no life at the time of commencement of bioassays.

(iii) All larvae that were past the 3rd instar i.e. 4th instar and pupa.

3.13 Ethical issues

(i) Legal authority to conduct research was sought from research and ethics committee of the

The Institute of Tropical Medicine and Infectious Diseases/Kenya Medical Research Institute (KEMRI), Nairobi and Institutional Research and Ethics Committee (IREC), Eldoret.

(ii) A qualified competent research assistant was approved to participate in the research.

(iii) The research explained clearly and truthfully the purpose of the research and anticipated benefits (especially to the management of Malakisi tobacco growers and the homes from which adult mosquitoes were collected)and reassurance that there was no danger involved with the procedures.

(iv) Assurance of confidentiality of the findings and intended use of research findings.

(v) People in whose area of jurisdiction such as wetlands, compounds, cattle sheds, social halls, and other adult mosquito attractants and rest places were asked for permission for the researcher to collect adult male and female *Anopheles gambiae* mosquitoes for the purpose of laboratory rearing.

3.14 Study variables, outcome variables and operationalization

3.14.1 Study variables

(a) Observe individual extract efficacy against the larvae

(b) Observe whether the intraction of two extracts yields to greater effect than that expected given their individual activities (Synergism).

(c) Observe whether the intraction of two extracts is less than that expected given their individual activities (Antagonism).

(d) Observe the effect of the diagnostic dose against diagnostic time comparatively for susceptible and fied strains of the larvae.(Resistance Ratio- RR).

(e) Observed the biodegradation of extracts with time when applied in stagnating waters (Persistance)

3.14.2 Outcome variables

(a) Successful rearing of adult mosquitoes.

(b) Results of each objective.

- LC_{50} of extracts.
- Synergistic activities of extracts
  - Antagonistic activities of extracts.
  - Larvae resistance to extracts.
  - Period taken for extracts to biodegrade.

3.14.3 Expected Results

(a) To obtain safe , effective and potentially economic plant extracts to replace the chemical and other synthetic insecticides.

(b) To obtain useful data base of biological activities of natural oil extracts from local plants for further development for pest/insect control.

(c) To be plant-based insect vector of disease control prototype system and be able to apply for further research for human health.

(d) To develop and make value-added to human labour as insecticidal agents in the replacement of environmental manipulation and environmental modification which are labour-intensive and economically expensive.

Table 3.3 . Operationalizational activities

Concept variable	**Measurement:** howinformation will be collected	**Operationalization**: How information will be interpreted	**Outcomes:**
Concentration formulation of herbicides	Make various dilutions of *C.cinerariifolium, E. camaldulensis and N. tabaccum.*	Percent by volume	LC_{50}
Intoxication of larvae by, *C.cinerariifolium,E. camaldulensis and N. tabaccum* oils.	Count number of dead 3rd instar larvae	Total number dead	50% population larval mortality
C.cinerariifolium,E. camaldulensis and *N. tabaccum* disintegration with time: Exposure to light Exposure to darkness	Measure hourly and daily residuals of extracts to observe their disintegration and complete disappearance.	*C.cinerariifolium* concentration in parts per million (ppm)	Residual concentration at end of each hour /day measured in ppm

3.15 Data management

3.15.1 Data storage

Two types of data were gathered during this study. These were data collected from the field during adult mosquito collection and data collected in the laboratory when performing experiments. Both data were stored in Microsoft Excel Spreasheet programme, flash discs and hard written copy in analysis book

3.15.2 Data analysis

Data was analysed using computer soft ware Standard Package for Social Sciences (SPSS) version 12. Data was entered into excel spread sheet for asnalysis before it was and exported to SPSS for storage. Descriptive statistics were generated for various variables and reported as frequency distributions, means and proportions. Measures of dispersion for contineous variables were measured and presented as standard deviations and standard mean errors.

The analysis of whether combinations of extracts were synergistic or antagonistic was calculated using isobolograph through plots of any two treatment combinations. The

isobole method was conducted considering, for example concentration for *DCM C. cinerariifolium* and Methanol of *E. camaldulensis* producing the same level of effect, were plotted as the intercepts for each axis. The intercepts were joined by a straight dashed line which formed a linear curve which represented the zero-interaction isobole or expected isoeffective dose combinations, or the combinations of DCM C. *cinerariifolium* and Methanol of *E. camaldulensis* giving the same effect as either DCM C. *cinerariifolium* or Methanol of *E. camaldulensis* .Thus a liear relationship of the treatment combinations was obtained.

If combinations were neither synergistic nor antagonistic at ratio 100 ppm:100 ppm (1:1) concentrations of either treatments were estimated. This was then compared to observed combinations that killed 100% larvae. The solid, concave up curve below the zero-interaction isobole represented the hypothetical LC_{50} values resulting from the synergistic effects of the combinations of the two extracts i.e. they were more potent than one would have expected based on their individual effective doses. On the other hand, the solid concave down curve above the zero-interaction isobole represented the hypothetical LC_{50} values resulting from the antagonistic effects of the combinations of the two extracts, that is they were less potent in combination than one would have expected based on their individual effective doses.

The above described method was validated by using the Synergistic Factor (SF) method of the two combining extracts in which SF is defined as the ratio of the theoretical lethal concentration value to the observed lethal concentration value. This was thus calculated: LC_{50} value of the extract alone divided by LC_{50} value of the combined plant extracts. When the ratio was > 1, the toxin interaction was considered synergistic because toxicity exceeded the value predicted from individual additive toxicity. When the ratio was < 1,

the interaction was considered antagonistic, wheras a ratio of one indicated that the values were additive. The greater the value, the higher the synergistic activity.

The analysis methods described above were repeated for all treatment combininations and subjected them to mean comparison using analysis of variance (ANOVA) which determined the significance of the data.

The resistance ratio (RR) had no units. This was simply determined by calculation: LC_{50} of field strain divide by LC_{50} of laboratory strain then categorizing RR into 3 levels: Slightly resistant ($1 \leq RR \leq 5$); moderately resistant ($5 \leq RR \leq 10$); and highly resistant ($RR > 10$) (Rodnguez *et al.* 2007). This is to say value of RR greater than 1 is an indication of resistance and value less than or equal to 1 are considered susceptible. However, the results were also counterchecked using WHO (2013) method of determining RR at the prescribed diagnostic dose and diagnostic time and this was:

100 – 98% mortality = Susceptable; 98 – 90% mortality = possibly Resistant and < 90% = confirmed Resistant (more testing required). Results were presented in tables and line graphs. Acute oral toxicity was analysed through Probit-log to obtain percent mortality.

Persistence was determined by testing the larvae using the highest concentration of each plant that exhibited 100% larval mortality. These were *C. cinerariifolium* (164.86 ppm), *E. camaldulensis* (168.65 ppm) and *N. tabaccum* (189.58 ppm). The solutions of these concentrations were sampled once daily to the level of 100 ppm, sampled twice daily to the level of 50 ppm, sampled hourly to the level of 20.00 ppm and finally sampled twice half-hourly to the level of 0.00 ppm. The intervals of sampling were undertaken in order to maintain the trend and accuracy in the extracts biodegradation and importantly to avoid missing the end point of extracts expirely from the solution. All samples were analysed using Gass Chromatography- Mass Spectrometry (GC-MS) (Pelkin Elmer SQ 8 GC/MS) method (Hashmi *et al.* 2013). The length of time each concentration took to be zero ppm

was recorded as the period of the extract's biodegradation. The zero ppm point denoted the point at which the concentration in the solution was nil. The period of time taken for complete degradation of the extract was calculated from its initial concentration in ppm to zero ppm concentration which gave the period in hours or days.

Associatiation of factors was examined using Chi-square test procedures and significancy levels considered at p = 0.05%. Time was measured in cummulative hours during whole period of experiments and treated as a contineous variable in this study. Time was an important factor for rating performance, goals or targets. It was also used to prioratize activities that were delicate and important such as the development of *A. gambiae s.s.* **Giles** larvae of which its instars development stages take very short time.

3.15.3 Larvae used in bioassays.

Table 3.5 shows the number of experiments that were performed, number of larvae used per experiment and the total lavae used. It was observed that a total of 2475 larvae were used. These larvae were 3rd instar larvae obtained from the batches of cages (3 cages) as the maturity of the larvae got fully developed. Three cages were preffered so as to cater for any cage(s) that failed to hatch larvae. The number of larvae survival to 3rd instar were 14, 380, adequate for bioassays.

Table 3.4. Number of larvae used in bioassays for all experiments.

Experiment	Number of tests	Number of Larvae per test	Total Larvae
Individual crude leaf extracts efficacy	18	25	450
Synergistic activities	30	25	750
Antagonistic activities	30	25	750
Resistance ratio	3	25	75
Total larvae			2475

3.16 Bioassays programme.

The experiments were restricted to the time of larval maturity since the 3rd instar larvae were to mature to 4th instar within a period of 2 to 3 days (Kivuyo *et al.* (2014). It was impelling that the bioassays had to be performed for all concentrations within that limit of time. The concentration testing were carried out in two sessions, morning (concentrations 50 to 150 ppm) and afternoon (200 to 300 ppm). Every concentration was set up with a difference of 30 minutes from the other as an allowance for checking larval mortality of each concentration at 24h exposure.

The 10th trip of adult mosquito collection from the field in December 2009 was used to raise larvae for re-testing the highest crude leaf extract of each plant. This was intended to re-confirm the earlier highest flower and leaf concentrations of each plant that caused 100% larvae mortality. The extracts re-tested were DCM of *Chrysanthemum cinerariifolium* (164.86 ppm); DCM *Eucalyptus camaldulensis* (168.65 ppm); and ethanol of *Nicotiana tabaccum* (189.58 ppm) in order to compare with the preceding tests.

3.1.2 Testing for the efficacy of individual extracts of *Chrysanthemum cinerariifolium*, *Eucalyptus camaldulensis* and *Nicotiana tabaccum* upon 3rd instar larvae of *A. gambiae s.***s. Giles**

Each plant species was tested using all the extracts of the six solvents as indicated in Table 3.6. Each individual extract shown in Table 3.6 was tested against the larvae. Fig. 3.5 shows experimental design for individual extracts of the three plants to determine extracts efficacy. Under the individual extracts tests 18 experiments were performed.

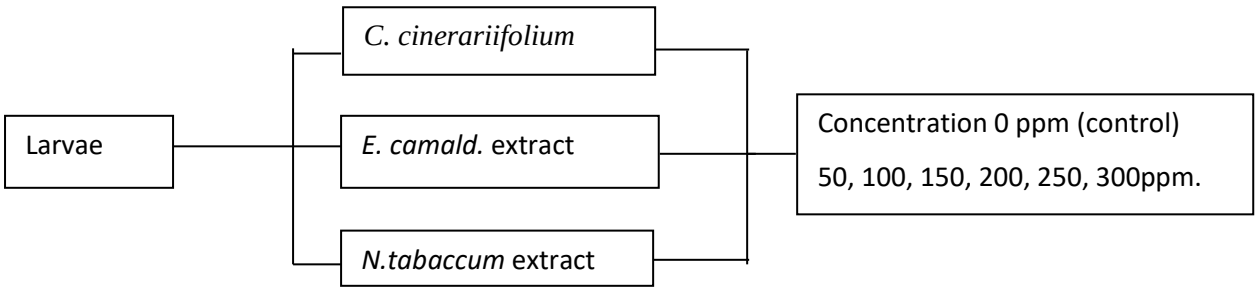

Fig. 3.8 Experimental design for individual extracts of the three plants to determine extracts efficacy.

3.16.1 Experimental procedures.

(**i) Experiment 1:** Crude flower and leaf extracts combination of *Chrysanthemum cinerariifolium and Eucalyptus camaldulensis* in the ratio of 1:1(v/v) to determine synergestic effects.

The larval bioassays were conducted in accordance with WHO, (2013) and cited by Nair *et al.* (2014): Procedural instructions for determining susceptibility or resistance of mosquito larvae to insecticides. A total of 25, 3rd instar larvae (WHO, 2013) were picked at random and were used for bioassays per concentration for all the experiments. The number of larvae picked for use was in accordance with the WHO, (2013) and previous workers (Govindarajan and Karuppannan, 2011; Bossou *et al.* (2013) Idris *et al*. 2008; Khanasi *et al*. 2012; Elumalai *et al*. 2012) and as also was conducted by Sengottayan (2007). Uthayarasa *et al.* (2010) and Singh (2014).

Six 250 ml capacity beakers were prepared and into each beaker 200 ml of distilled water was provided. These beakers represented six diferrent concentrations namely 50, 100, 150, 200, 250, and 300 ppm of one crude leaf extract. Into each beaker of 200ml, various concentrations ranging from 50 to 300 ppm were placed. For the three plants 36 concentrations were made ready for testing..

Control experiment was prepared and consisted of a mixture of acetone and DMSO (1:1v/v) while alternatively 200 ml of dechlorinated water with 2 ml of acetone and another set of beakers with dechlorinated water only served as a complementary control. For each concentration three replicates were run at the same time.

Table 3.5. Individual Crude leaf extracts for larvicidal efficacy tests.

Plant species	**Combination extracts**	**Ratio (v/v)**	**ppm Conc.**
C. cinerariifolim (Cc):	Ethanolof *Cc* + Aqueous of *E. camaldulensis(Ec)*	1:1	100:100
	Methanol of *Cc* +Ethyl acetate of *Ec*	1:1	100:100
	DCM of *Cc* + Hexane of *Ec*	1:1	100:100
	Methanol of *Cc* + Aqueous of *Ec*	1:1	100:100
	DCM of Cc + Aqueous of *Ec*	1:1	100:100
	DCM of *Cc* + Methanol of *Ec*	1:1	100:100
	DCM of *Cc* + Ethyl acetate of *Ec*	1:1	100:100
	Methanol of *Cc* + Ethyl acetate of *Ec*	1:1	100:100
	Methanol of *Cc* + Hexane of *Ec*	1:1	100:100
	Ethyl acetate of *Cc* + Hexane of *Ec*	1:1	100:100
	Ethanol of *Cc* + Methanol of Ec	1:1	100:100
	Hexane of Cc + Ethanol of Ec	1:1	100:100

By use of a mouth aspirator, a batch of 25 3rd instar larvae were collected at random one by one from rearing tray and placed into a 0.5 ml cup of distilled water to maintain them alive as the rest of the larvae were being collected to make 25 in number for immersion into the beaker of the concentration. The larvae were dipped into the beakers at the rate of 25 3^{rd} instar larvae per beaker and per extract concentration. Twenty five larvae per beaker were appropriately recommended for two reasons: One, larvae crowding would negatively influence normal feeding rates of the larvae. Secondly, the number of larvae per container would influence the amount of toxic moieties that was to be available to each individual. The amount of toxin per larvae was also to be a function of the volume of water used for the bioassay; the recommended amount of water was 5 ml per larvae although because of evaporation and other factors 250 ml was generally agreed upon as suitable for bioassays (WHO, 2013).

Each experiment set-up was maintained at ambient temperature (28 ± 2^0C, 75±5% Relative Humidity and a photo period of 12:12h i.e. 12h light and 12h darkness). Since a dehumidifier was not available wet towels were hang on cages to control humidity Baughman *et al.* (2017) (Trembley, 1944 and Wijegunawardana, 2015).

The larvae were fed with dry baker's yeast powder on the water surface (50 mg/L) throughout the experiment periods. Dry baker's yeast was preferable because it easily floated on water for the larvae to feed on.

The larvae were exposed to different concentrations of the oils and mortality was observed in 24 hours. By observation, larvae that did not respond by a wiggling movement while the side of the beaker was tapped with a stirring rod was regarded as dead; or when larvae prodded with a wooden applicator stick, or stimulated with a Pasteur pipette and those that did not move away from the stimulus or show a wiggling motion

were recorded as dead. Similarly larvae that did not respond when prodded with a needle at their cervical region and did not rise to the surface were regarded dead as well.

Further careful observation was done to identify the moribund larvae which were counted and added to the dead ones for the calculation of the percentage mortality. Mortality was recorded 24 h post-treatment and mortality data was subjected to Probit regression analyses Norusis, (2008) to determine 50% (LC_{50}) and 90% (LC_{90}) of lethal concentration. The percentage mortality was calculated using Dawidar *et al.* (2013) formula.

3.17 Synergistic activities:

Table 3.6 Synergistic activities on 3rd instar larvae of *A. gambiae* against *Chrysanthemum cinerariifolium* crude flower extracts in combination with crude leaf extracts of *Eucalyptus camaldulensis* in the concentration of 100 ppm of each extract; ratio 1:1 (v/v) Table 3.7 shows combination of two extracts for synergistic activities.

3.17.1 Experimental procedure:

Plants extracts combinations: (i) *Chrysanthemum cinerariifolium* and *Eucalyptus camaldulensis;* (ii) *Chrysanthemum cinerariifolium* and *Nicotiana tabaccum* and *(iii) Eucalyptus camaldulensis* and *Nicotiana tabaccum* all in the ratio of 1:1(v/v) or 100 ppm: 100 ppm to determine synergestic effects. Table 3.6 is a reflection of the extract combination (i) ,(ii), and (iii) above.

Table 3.6 Individual crude leaf extracts combination for synergistic activities.

Plant species	Combination extracts	Ratio (v/v)	ppm Conc.
C. cinerariifolim (Cc):	Ethanol of *Cc* + Aqueous of *Nicotiana tabaccum(Nt)*	1:1	100:100
	Methanol of *Cc* +Ethyl acetate of *Nt*	1:1	100:100
	DCM of *Cc* + Hexane of *Nt*	1:1	100:100
	Methanol of *Cc* + Aqueous of *Nt*	1:1	100:100
	DCM of Cc + Aqueous of *Nt*	1:1	100:100
	DCM of *Cc* + Methanol of *Nt*	1:1	100:100
	DCM of *Cc* + Ethyl acetate of *Nt*	1:1	100:100
	Methanol of *Cc* + Ethyl acetate of *Nt*	1:1	100:100
	Methanol of *Cc* + Hexane of *Nt*	1:1	100:100
	Ethyl acetate of *Cc* + Hexane of *Nt*	1:1	100:100
	Ethanol of *Cc* + Methanol of Nt	1:1	100:100
	Hexane of Cc + Ethanol of Nt	1:1	100:100

Fig. 3.8 is a replica of Table 3.6 but clearly showing out the combination extracts being tested using concentrations 50 to 300 ppm.

All the three tests were carried out in a similar procedure as those of the individual extracts efficacy tests. The individual crude leaf extract results were used to determine synergistic activities. The extracts were combined alternately ensuring that all extracts participated in activities.

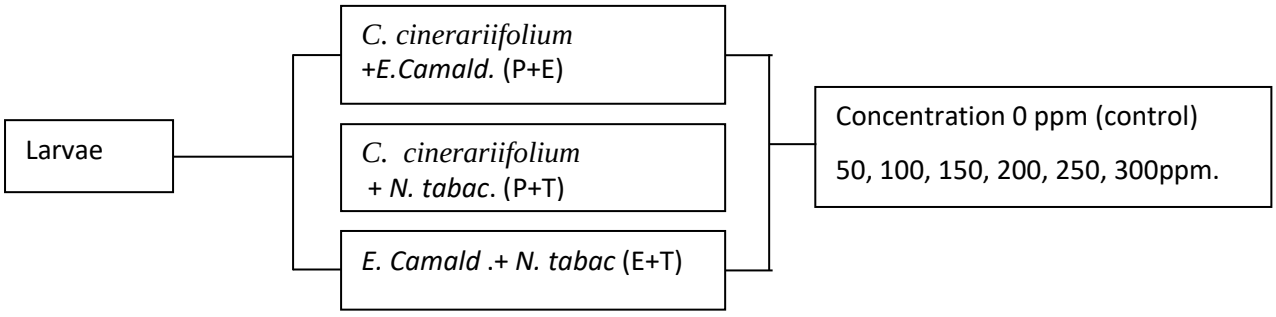

Figure 3.8. Arrangement for combined extracts tests.

Table 3.7 Extracts combination for synergistic activities

Plant species	Combination extracts	Ratio (v/v)	ppm Conc.
Eucalyptus Camaldulensis (Ec)	Ethanol of *Cc* + Aqueous of *Nicotiana tabaccum(Nt)*	1:1	100:100
	Methanol of *Cc* +Ethyl acetate of *Nt*	1:1	100:100
	DCM of *Cc* + Hexane of *Nt*	1:1	100:100
	Methanol of *Cc* + Aqueous of *Nt*	1:1	100:100
	DCM of Cc + Aqueous of *Nt*	1:1	100:100
	DCM of *Cc* + Methanol of *Nt*	1:1	100:100
	DCM of *Cc* + Ethyl acetate of *Nt*	1:1	100:100
	Methanol of *Cc* + Ethyl acetate of *Nt*	1:1	100:100
	Methanol of *Cc* + Hexane of *Nt*	1:1	100:100
	Ethyl acetate of *Cc* + Hexane of *Nt*	1:1	100:100
	Ethanol of *Cc* + Methanol of Nt	1:1	100:100
	Hexane of Cc + Ethanol of Nt	1:1	100:100

3.18 Antagonistic effects when the crude leaf extracts are alternately combined as P+E, P+T and E+T and administerd on *A. gambiae* s.s. mosquito larvae.

In antagonistic tests similar merthodology as that adapted for synergistic tests was used Similary the individual crude leaf extract results were used to determine antagonistic activities while the extracts were combined alternately as was the case of synergism tests and the extracts administerd to the larvae.

3.19 Determination of resistance ratio (RR) of the crude leaf extracts to the *A. gambiae s.s.* **Giles** larval.

In resistance ratio (RR) experiments, larvae from the field in Langas area of Eldoret were collected monthly between January and June 2010. These larvae were marked as

field generation larvae. Langas area was preferred for larvae collection because the area consists of wet lands characterized by water retaining plants namely reeds, papyrus, moss, and tall grass and therefore the area was suitable for mosquito breeding even in the dry seasons. Larvae were constantly available during dry and rainy seasons and they were collected for testing The laboratory reared larvae were marked as susceptible and were similarly tested for resistance ratio.The resistance ratio of the field generation larvae was compared with the resistance ratio of the susceptible larvae to find variations in their resistance as a function of their habitat (WHO, 2012; Grisales *et al*. 2013).

In January and February 2010 larvae were collected from the wetlands (swamps) since the two months were dry months. In March, April, May and June 2010, larvae were collected from drainage ditches, hoof prints, swamps, open containers (plastics and metalic), farrow ditches, and used tyres. These were months experiencing plenty of rainfall. Since WHO standard dipper was not available, *A. gambiae s.s* **Giles** larvae were collected using an improvised dipper as used by Wong *et al.* (2013) and later by Emedi *et al.* (2012). A ladle was made from an empty 350ml sized water bottle by longitudinally cutting an opening to make an oval hole half was long from the bottom. Larvae were collected randomly so that the 3rd instar could be separted from the rest in the laboratory. Also there was fear that selection of 3rd instar larvae was difficult since any slight disturbance of larvae makes them escape into bottom of pools. Larvae were collected, put in bowled-flat based cylinder, its mouth covered with stockenett and as many as possible larvae were collected. The larvae were dipped into 300ml bottles containing fresh water from the habitat of the larvae and transported them to the insectary for bioassays.

In the laboratory, the field generation larvae were identified and separated using taxonomic keys of Gillies and Coetzee (1987). For this identification a x50 or x100

magnifying glass was used in the observation of the prominent keys i.e dypeals,saddle hair, thorax, abdomen, mesople and mesopleural hair.

The field generation larval were fed on green algae collected from their habitat because this was one of the common foods for the larvae and an ubrupt change of food could affect the larvae. The larvae were maintained in similar rearing conditions as the laboratory species (28+20C, 75+5% Relative Humidity and 12:12h (Light: Dark periods). In the absence of a humidifier humidity was maintained by use of wet towels spread over rearing cages but leaving one side of the cage uncovered with the towels (Hashmat*et al.* 2014; Dassanayake *et al.* 2015; and Baughman *et al.* (2017).

Tests for RR were carried out following WHO (2013) proceduresa and following same procedures as those carried out for individual extracts, synergistic and antagonistic activities.

Overall resistance was determined as follows: The resistance ratio (RR) had no units. This was simply determined by calculation: LC_{50} of field strain divide by LC_{50} of laboratory strain then categorizing RR into 3 levels: Slightly resistant ($1 < RR \leq 5$); moderately resistant ($5 < RR \leq 10$); (RR>10) (Rodnguez *et al.* 2007). This can be interpreted that values of RR greater than 1 is an indication of resistance and values less than or to 1 are considered susceptible. The results were also counterchecked using WHO (2013) method of determining RR at the prescribed diagnostic dose and diagnostic time and this was: 100 – 98% mortality = Susceptable; 98 – 90% mortality = possibly Resistant and < 90% = confirmed Resistant (more testing required). Acute oral toxicity was analysed through Probit-log to obtain percent mortality.

3.20 Determination of *Chrysanthemum cinerariifolium, Eucalyptus camaldulensis* and *Nicotiana tabaccum* crude leaf extracts persistence.

All 18 crude leaf extracts of the three plants were in stock in their various concentrations to use. In persistence experiments it was not possible to test using all the 18 extracts. These were many and it was difficult to test them in totality in terms of time, cost and patience. The only persistence tests that have been done in the past studies were those of *Chrysanthemum cinerariifolium* (Chen and Casida, 1969) in which persistence for both dark and light periods were investigated. Persistence of the remaining 17 crude leaf extracts have not been investigated in the past hence leaving a big gap. However, it was to this investigator's discretion to use the highest toxic crude leaf extract of each plant to test for persistence.

The best crude flower and leaf extracts in toxicity of each plant selected for use in persistence experiments were methanol extract of *Chrysanthamum cinerariifolium* (164.86ppm), DCM extract of *Eucalyptus camaldulensis* (168.65 ppm) and ethanol extract of *Nicotiana tabaccum* (189.58ppm).

3.20.1 Experimental procedure:

Experiments for determination of extracts persistence as carried out in accordance with the methods used by Attia *et al*. (2015). One highest extract of each plant was placed in 300 ml disposable bowls seperately in their relevant concetrations. Hence DCM *Chrysanthamum cinerariifolium* (164.86 ppm), DCM extract of *Eucalyptus camaldulensis* (168.65 ppm) and ethanol extract of *Nicotiana tabaccum* (189.58 ppm) were all set separately in 300 ml beakers. Distilled water was added into each bowl to make 300 ml of water and concentration just to imitate stagnating water pools.

By use of a mouth aspirator 25 3rd instar larvae were collected and dropped into each of the three solutions. Observation for larval mortality was not a requirement for extract

persistence. To moniter the decline of extracts in the solution with time was the essence of the experiment. The larvae were an inclusion in this experiment, first, so as to allow some extract solution taken by the larvae as expected under normal mosquito control programme.

These solutions were taken outside the laboratory and exposed to the normal external environmental conditions (temperature, humidity, etc.) as it would occur in mosquito breeding places such as natural water pools. The concentrations were then monitored on how they reduced under these natural conditions to reach zero ppm with time. Monitoring was accomplished by taking 1ml of sample from each solution of *C. cinerariifolium*, *E.camaldulensis* and *N. tabaccum.*

By use of 1ml pipette a sample of each plant extract was taken and separately placed in 1ml sterile bottle. Sampling was done daily (when concentrations were between 164.86 and 100.00 ppm, 168.65 and 100.00 ppm and 189.58 and 100.00 ppm), twice daily (when concentrations were 100.00 and 50.00 ppm), once daily (when concentrations were 50.00 and 20.00), hourly (when the concentrations were 20.00 and .10.00 ppm) and half hourly (when concentrations were 10.00 and 0.00 ppm). However, extract which showed higher rate of degradation than others was sampled more at closer interval of time such as *C. cinerariifolium*. This was an up-down procedure observing how a concentration reduced from its initial high concentration to zero. One extract was done at a time to avoid accumulation of samples in the laboratory. Samples were labelled to indicate species of plant, time sample taken, date of sampling, name and address of sampler, and indication of what was to be analysed.

Samples were well packed to avoid exposure to the sun and transported them to laboratory for analysis using Gass Chromatography- Mass Spectrometry (GC-MS)(Pelkin Elmer SQ 8 GC/MS) analysis (Hashmi *et al.* 2013). The results of analysis showed the

actual concentration of the extract in the solution at the time of sampling. The zero ppm point denoted the point at which the concentration in the solution was nil. The period of time taken for complete degradation of the extract was calculated from its initial concentration in ppm to zero ppm concentration which gave the period in hour or days.

CHAPTER FOUR

4.0 RESULTS

4.1 Collection of adult male and female *Anpheles gambiae s.s.* Giles mosquitoes from the field for laboratory rearing.

A detailed description of the collection of adult male and female *Anopheles gambiae s.s.* **Giles** is in the methology section of this study. The number of adult mosquitoes collected in the ratio 3:1 (males: females) were 450: 150 in one trip of *A. gambiae s.s.* **Giles** per month and coillection continued for 10 months. A total number of 4500 and 1500 adult male and female respectively were collected in ten trips of the ten months between January 2009 to March 2009; May 2009 to July 2009; and September 2009 to December 2009). The adult mosquito collection from the field was successful.

From Table 4.1, it was observed that the average number of eggs oviposited in cages 1, 2 and 3 varied from 9,680 lowest (cage 1) in the month of February 2009, to 15,300 highest (cage 3) in the month of July 2009. This gave an oviposition of between 65 and 102 eggs per female *mosquito.* Statistically, the average egg oviposition in cages 1, 2 and 3 were 103,616 ± 94,770 SD (cage 1), 1,0842 ± 1,004 SD (cage 2) and 11,110 ± 1,007 (cage 3).

4.2 Eggs oviposited.

Table 4.1 shows the total number of eggs oviposited during the period January to December 2009.

Table 4.1 Eggs oviposited in each cage from the period January to December 2009.

Trip	Date	Cage 1	Cage 2	Cage 3	Total larvae
1	January 2009	9835	9935	10910	**30680**
2	February 2009	9680	10350	12225	**32255**
3	March 2009	9997	12116	9696	**31809**
4	May 2009	10050	13100	12783	**35933**
5	June 2009	10350	10610	10846	**31806**
6	July 2009	12150	11118	15300	**38568**
7	September 2009	10200	11366	11020	**32586**
8	October 2009	9776	9900	9880	**29556**
9	November 2009	9728	10110	10077	**29915**
10	December 2009	11850	9814	11318	**32982**
Total		**103616**	**108419**	**114055**	**326090**

4.3 Number of larvae hatched

Table 4.2 indicates the number of larvae hatched between the months of January 2009 and December 2009. It was observed that in the month of February, 2009 the lowest number of larvae (5085) were hatched while the highest number was in the month of May, 2009 (10,972 larvae hatched). In total 252,778 larvae were hatched in the period of 10 months. This was 77.5% of the eggs hatched.

Table 4.2 Number of larvae hatched in each of the three cages

Trip	Date	Cage 1	Cage 2	Cage 3	Total
1	January 2009	5520	5613	8421	**19,554**
2	February 2009	5085	8020	10221	**23,326**
3	March 2009	7993	9883	6003	**23,879**
4	May 2009	8770	12431	10972	**32,173**
5	June 2009	8103	8501	9112	**25,716**
6	July 2009	11021	9941	13122	**34,084**
7	September 2009	8831	8954	8650	**26,435**
8	October 2009	7221	7943	7885	**23,049**
9	November 2009	6544	8111	7994	**22,649**
10	December 2009	8993	6611	6309	**21,913**
Total		**78,081**	**86,008**	**88,689**	**252,778**

4.4 Larvae that survived to 3rd instar

Table 4.3 indicates larval survival to third instar. The total number of larval survival in 10 trips of egg hatching were 4,553 larvae (cage 1); 4,834 larvae (cage 2); and 4,993 larvae (cage 3). This gave a total of 14,380 larvae hatched in 3 cages in various months between January 2009 and December 2009 which accounted for 5.7% larval survival and 5.8 times more than the larvae required for bioassays.

Table 4.3 Larval survival to 3rd instar in each age

Field trip	Date	Cage 1	Cage 2	Cage 3	Total larvae in 3 cages
1	January 2009	800	1,092	960	**2,852**
2	February 2009	610	783	525	**1,918**
3	March 2009	507	640	701	**1,848**
4	May 2009	517	611	747	**1,875**
5	June 2009	583	608	818	**2009**
6	July 2009	221	251	380	**852**
7	September 2009	199	311	275	**785**
8	October 2009	396	142	124	**662**
9	November 2009	437	278	301	**1,016**
10	December 2009	283	118	162	**563**
Total		**4,553**	**4,834**	**4,993**	**14,380**

4.5 Efficacy of the oils of the three plants.

Table 4.4 shows results of efficacy of *C. cinerariifolium, E.camaldulensis* and *N. tabaccum* exracts applied individually to the larvae for 24 hours. The results showed that all the three crude leaf extracts of the three plants showed significant larvicidal activity at P = <0.05 level of significance. DCM flower extract of *C. cinerariifolium* was the most effective as it exhibited 100% mortality for LC_{50} at the highest concentration of 164.86 ppm after 24 hours of exposure. Of the determined larvicidal effect of the three plant leaf extracts against third instar larvae of *Anopheles gambiae*s.s. **Giles,** LC_{50} values of DCM extracts of *C. cinerariifolium* and E. *camaldulensis* were 164.86 ppm and 168.65 ppm respectively both achieving 100% larval mortality. The latter extract also exhibited 100% mortality when 168.65 ppm of the crude extract was applied. In addition to these two high

extracts, ethanol of *C.cinerariifolium*, ethanol of *N. tabaccum*, and methanol of ***E.*** *camaldulensis* gave 100% mortality of the larvae at 187.78 ppm, 189.58 ppm and 197.46 ppm respectively.

Other notable results were demonstrated by methanol of *C. Cinerariifolium*, which when 222.45ppm of the extract was applied gave 98% larval mortality; hexane of *E. camaldulensis* at 198.56 ppm resulted to 96% larval mortality; methanol of *N. tabaccum* at 224.35 ppm exhibited 96% larval mortality; Ethyl acetate of *N. tabaccum* at 201.52 ppm gave 96% mortality of the larvae; and when 210.15 ppm of *E. camaldulensis* ethanol was applied it gave 96% larval mortality.

The last performance amongst the leaf extracts were noted to be DCM of *N. tabaccum*, which at 229.72 ppm gave 88% mortality of the larvae; hexane of *C. Cinerariifolium* at 230.66 ppm exhibited 88% mortality of the larvae; aqueous of *C. Cinereriifolium* gave 84% larval mortality with the concentration of 247.84 ppm; aqueous of *N. tabaccum* at 258.42 ppm gave 84% mortality of the larvae and ethyl acetate of *E. camaldulensis at* 260.56 ppm resulted to 80% larval mortality (Table 4.5). From these results it was observed that all the crude leaf extracts activity on LC_{50}ranged from 164.86 ppm (DCM of *C. Cinerariifolium*) to 260.56 ppm (ethyl acetate of *E .camaldulensis*). Importantly, it was observed that all the plant crude leaf extracts indicated dose – dependent mortality of the tested larvae such that as the dose was increased larval mortality increased, however, with the increase in time of exposure also.Mortality of the larvae under these conditions was high and this indicated that all the crude leaf extracts had a good potential to be used as larvicides.

Table 4.4 Larvicidal efficacy of crude leaf extracts of *Chrysathemum cinerariifolium* (Asteraceae), *Eucalyptus camaldulensis* (Myrtaceae) and *Nacotiana tabaccum* (Tobacco) when independently applied against the malaria vector *Anopheles gambiae s.s.***Giles** (Diptera: Culicidae).

Plant	**Part used**	**Extract solvent**	**LC_{50}(ppm) fiducial limit**	**LC_{90}(ppm) fiducial limit**
C. cinerariifolium	Leaf	Ethanol	187.78 (179.78–196.53)	268.26 (247.89-298.53)
		Methanol	222.45 209.85-238.71	331.68 (304.33-371.87)
		DCM	164.86 (161.57-176.28)	255.17 (235.79-283.96)
		Hexane	230.66 (214.79-252.67)	364.86 (328.04-421.73)
		Ethyl acetate	227.56 (219.77-269.96)	347.38 (317.68-391.95)
		Aqueous	247.84 (233.37-267.72)	318.56 (292.26-356.96)
E. Camaldulensis	Leaf	Ethanol	210.15 (193.88-232.07)	335.58 (307.90-376.24)
		Methanol	197.46 (189.61-208.69)	329.68 302.40-369.52
		DCM	168.65 (152.44-176.95)	315.85 (292.84-348.10)
		Hexane	198.56 (181.66-220.45)	338.85 (310.91-379.91)
		Ethyl acetate	260.56 (240.77-289.96)	347.38 (317.68-391.95)
		Aqueous	259.58 (239.87-288.87)	390.48 (367.25-437.70)
N. tabaccum	Leaf	Ethanol	189.58 (181.50-298.42)	320.75 (294.27-359.41)
		Methanol	224.35 (211.73-240.86)	332.75 (305.31-373.07)
		DCM	229.72 (216.80-246.63)	342.64 (313.35-386.59)
		Hexane	235.85 (221.25-240.53)	314.70 (291.77-346.54)
		Ethyl acetate	201.52 (191.00-213.84)	322.84 (296.18-361.75)
		Aqueous	258.42 (238.79-287.58)	393.36 (350.44-462.19)

4.6 Larvae mortality monitored 6-hourly

Table 4.5 indicates larval mortality in every six hours. Ethanol and DCM of *Cc*, *Ec* methanol and DCM, and *Nt* ethanol exhibited the highest larvae mortality at 100%.The rest of the extracts' mortality ranged from 80% (ethyl acetate and aqueous of *Ec*). By comparison *Cc* demonstrated high values of mortality (ranges of 88% to 100%), followed by *Nt* (84% to 100%) and thirdly *Ec* (80% to 100%).

A major observation made was that the highest number of larvae mortality occurred between the hours of 15 and 24.

Table 4.5: Mortality of the Larvae versus all extracts monitored six hourly for 24 hours

		Conc. (ppm)	Exp. Larvae	No. of alive larvae at					% larvae alive at	% larvae mortalitya t
Plant	**Extract solvent**	**0(ctrl)**	**25**	**1h**	**6h**	**12h**	**18h**	**24h**	**24h**	**24h**
		0(check)	25							
Chrysanthemum cinerariifolium	**Ethanol**	50	25	24	24	22	21	20	82	20
		100	25	20	16	11	8	8	32	68
		150	25	21	15	10	6	3	12	88
		200	25	18	10	3	0	0	0	**100**
		250	-	-	-	-	-	-	-	
		300	-	-	-	-	-	-	-	
	Methanol	50	25	25	24	22	21	21	76	24
		100	25	23	20	20	19	16	64	36
		150	25	25	18	11	6	15	20	80
		200	25	21	17	13	7	3	12	88
		250	25	15	8	0	0	0	0	**98**
		300	-	-	-	-	-	-	-	-
	DCM	50	25	24	21	18	17	16	64	36
		100	25	18	15	10	8	6	24	76
		150	25	16	14	10	6	1	4	96
		200	25	1	0	0	0	0	0	**100**
		250	-	-	-	-	-	-	-	-
		300	-	-	-	-	-	-	-	-
	Hexane	**50**	25	24	22	22	20	21	84	16
		100	25	23	19	17	16	13	52	48
		150	25	24	22	18	15	12	48	52
		200	25	23	18	15	9	6	24	76
		250	25	20	0	0	0	0	0	**88**
		300	-	-	-	-	-	-	-	-
	Ethylacetate	50	25	25	23	21	20	19	76	24
		100	25	24	18	16	14	12	48	52
		150	25	23	17	13	8	10	40	60
		200	25	22	20	14	6	4	16	84
		250	25	21	17	14	8	2	8	92
		300	25	1	0	0	0	0	0	**92**
	Aqueous	50	25	25	24	24	22	21	84	16
		100	25	24	20	19	17	15	60	40
		150	25	23	20	16	14	12	48	52
		200	25	22	19	17	14	9	36	64
		250	25	21	17	14	7	0	0	84
		300	-	-	-	-	-	-	-	-

Nicotiana tabaccum	Ethanol	50	25	25	23	19	17	15	60	40
		100	25	24	22	18	14	10	40	60
		150	25	23	19	16	10	5	20	80
		200	25	20	16	0	0	0	0	100
		250	-	-	-	-	-	-	-	-
		300	-	-	-	-	-	-	-	-
	Methanol	50	25	25	23	19	15	11	44	56
		100	25	23	19	15	11	8	32	68
		150	25	22	17	15	9	4	16	84
		200	25	20	14	0	0	0	0	96
		250	25	-	-	-	-	-	-	-
		300		-	-	-	-	-	-	-
	DCM	50	25	25	21	19	15	11	44	46
		100	25	24	20	18	13	10	40	60
		150	25	23	20	19	10	7	28	72
		200	25	22	20	17	11	4	16	84
		250	25	20	14	8	0	0	0	88
		300	-	-	-	-	-	-	-	-
	Hexane	50	25	25	23	22	20	18	72	28
		100	25	23	20	16	7	10	40	60
		150	25	23	22	15	10	9	36	64
		200	25	21	19	14	10	8	32	68
		250	25	20	14	0	0	0	0	84
		300	-	-	-	-	-	-	-	-
	Ethyl acetate	50	25	25	23	19	13	9	36	64
		100	25	24	21	16	9	8	32	68
		150	25	24	20	15	8	7	28	72
		200	25	22	19	11	3	22	12	88
		250	25	20	17	0	0	0	0	96
		300	-	-	-	-	-	-	-	-
	Aqueous	50	25	25	23	21	20	19	76	24
		100	25	24	22	19	18	17	68	32
		150	25	23	20	18	16	14	56	62
		200	25	23	20	16	8	10	40	84
		250	-	-	-	-	-	-	-	-
		300	-	-	-	-	-	-	-	-

Eucalyptus camaldulensis	Ethanol	50	25	25	22	19	15	17	68	32
		100	25	23	19	16	13	10	40	60
		150	25	20	16	10	6	4	16	84
		200	25	20	15	9	5	1	4	96
		250	25	20	18	0	0	0	0	96
		300	-	-	-	-	-	-	-	-
	Methanol									
		50	25	25	22	19	17	16	64	36
		100	25	24	20	17	14	10	40	60
		150	25	23	16	10	6	4	16	84
		200	25	22	16	0	0	0	0	100
		250	-	-	-	-	-	-	-	-
		300	-	-	-	-	-	-	-	-
	DCM	50	25	23	20	17	15	14	56	44
		100	25	21	17	15	10	7	28	72
		150	25	18	16	12	7	2	8	92
		200	25	15	7	0	0	0	0	100
		250	-	-	-	-	-	-	-	-
		300	-	-	-	-	-	-	-	-
	Hexane	50	25	25	23	20	18	17	68	32
		100	25	24	22	19	14	12	48	52
		150	25	23	20	18	15	11	44	56
		200	25	20	15	9	0	0	0	96
		250	-	-	-	-	-	-	-	-
		300	-	-	-	-	-	-	-	-
	Ethylacetate	50	25	25	24	22	21	20	80	20
		100	25	25	23	20	17	15	60	40
		150	25	24	20	17	15	14	56	44
		200	25	22	19	17	9	11	44	56
		250	25	20	17	15	12	10	40	60
		300	25	18	15	6	0	0	0	80
	Aqueous	50	25	25	23	19	18	17	68	32
		100	25	24	20	16	15	14	56	44
		150	25	23	20	18	12	10	40	60
		200	25	22	19	18	9	9	36	64
		250	25	20	18	15	10	7	28	72
		300	25	20	17	0	0	0	0	80

4.7 Re-testing of the highest extracts

Retesting of the highest extract of each plant i.e. *C. cinerariifolium (*164.86 ppm*),* *E.camaldulensis* (168.65 ppm) and *N. tabaccum (*189.58 ppm*)* was carried out to compare with the preceding tests. The results were 164.86, 168.66 and 189.56 ppm respectively. results differed by small margins 0.00ppm (*C. cinerariifolium*), 0.01 ppm (*E.camaldulensis)* and *0.02 ppm (N. tabaccum*) giving minimal differences in their percentage 0.00%, 0.0059% and 0.01055% in the order of *C. cinerariifolium, E.camaldulensis*and *N.tabaccum.*

4.8 Regression equations of the individual extracts efficacy

These are as shown in table Table 4.6 and regression lines of intersection drawn from this table shown in Fig. 4.1.

Table 4.6 Regression equations of the individual extracts efficacy

Name of plant	**Extract solvent**	**LC_{50}(ppm)**	**Fiducial limits**		**Regression equation**	**Chi-square value (x^2)**
			Upper	Lower		
C. cinerariifolium	Ethanol	187.78	179.78	196.53	0.7470+1.7758x	4.5217
	Methanol	222.45	209.85	238.71	0.4684+1.9089x	10.6452
	DCM	164.86	161.57	176.28	0.7336+1.7893x	14.2584
	Hexane	230.66	214.79	252.67	0.5566+1.9098x	19.5759
	Ethylacetate	227.56	219.77	269.96	0.4899+1.9078x	19.5759
	Aqueous	247.84	233.37	267.72	0.6758+1.2123x	18.6202
E. Camaldulensis	Ethanol	210.15	193.88	232.07	0.7686+1.9694x	4.6621
	Methanol	197.46	189.61	208.69	0.4868+1.9227x	13.256
	DCM	168.65	152.44	176.95	0.6975+1.9567x	10.4532
	Hexane	198.56	181.66	220.45	0.7086+1.8685x	9.5033
	Ethylacetate	260.56	240.77	289.96	0.05886+1.7825x	14.0773
	Aqueous	259.58	239.87	288.87	0.05977+1.8365x	6.7556
N. tabaccum	Ethanol	189.58	181.50	298.42	0.7376+1.8898x	3.5463
	Methanol	224.35	211.73	240.86	0.4988+1.8985x	3.8642
	DCM	229.72	216.80	246.63	0.5878+1.9874x	15.5740
	Hexane	235.85	221.25	240.53	0.5663+1.9096x	4.6542
	Ethylacetate	201.52	191.00	213.84	0.6455+1.8990x	14.0773
	Aqueous	258.42	238.79	287.58	0.0608+2.1015x	6.4444

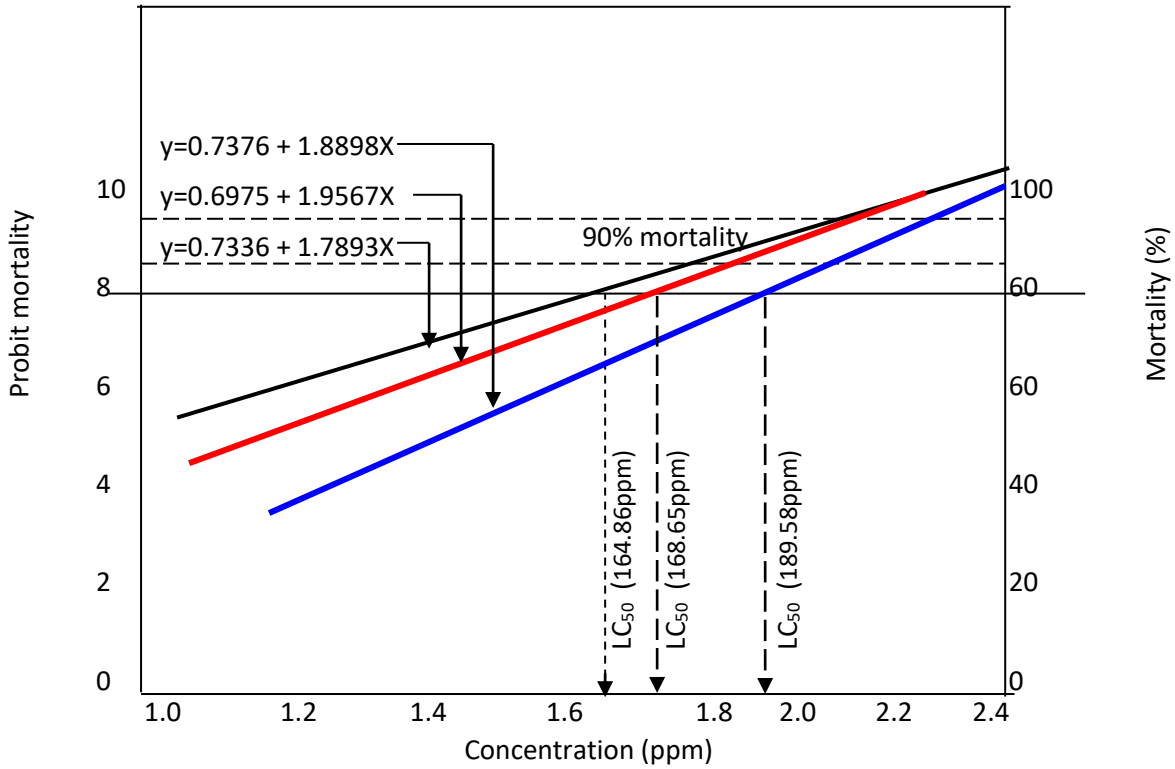

Figure 4.1. Regression lines (based on probit analysis) of log concentration of leaf extracts of *C.cinerariifolium* (0.7336 + 1.7893X), *E. camaldulensis* (0.6975 + 1.9567X) and *N. tabaccum* (0.7376 + 1.8898X) v/s percent mortality of third instar larvae of *A. gambiae s.s*. **Giles.**

Two issues arise from figure 4.1. First, the regressional lines for the three plants tended to converge at 100% mortality and all the three regression lines run through eight mark of probit mortality both which confirm a hundred percent larval mortality. This three regression line which adapts their regression equations conveniently locate the concentrations about which LC_{50} for the three plants occurred. From the diagram regressional line for *Chrysanthemum cinerariifolium* LC_{50} occurred at the concentration of 164.86 ppm, *Eucalyptus camaldulensis*LC_{50} occurred at the concentration of 168.65 ppm *and Nicotiana tabaccum* LC_{50} occurred at the concentration of 189.58 ppm. The three concentrations of the plants exhibited 100% larval mortality.

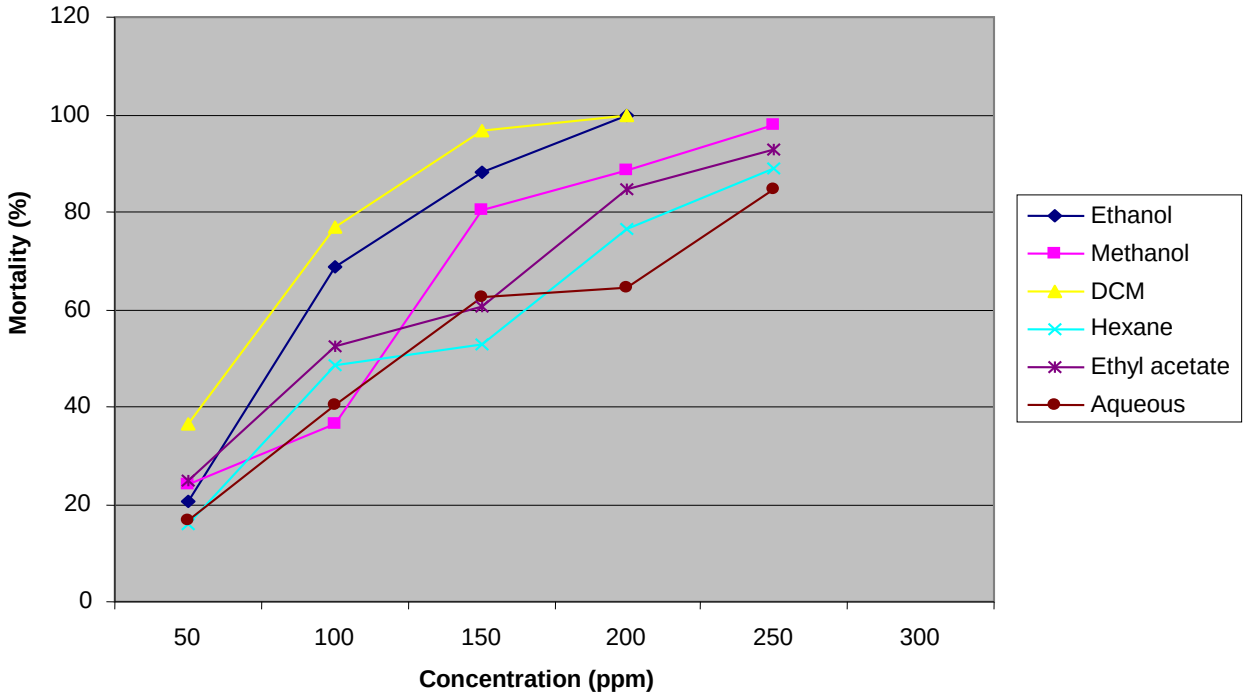

Fig 4.2. Comparison percent of six crude flower extracts of *Chrysanthemum cinerariifolium* against mortality of third instar larvae of *A. gambiae* s.s. **Giles** mosquito.

Notably DCM and ethanol extracts, of *Chrysanthemum cinerariifolium* (Fig. 4.2) exhibited the highest mortality (100%) while methanol, ethyl acetate, hexane and aqueous achieved mortality of 98.00%, 92.65%, 96.00% and 80.65% respectively on 24 hour larvae exposure. However, mortality for the six extracts of *C. Cinerariifolium* showed an upward trend with no indication of retracts by any factors. The DCM and ethanol extracts converge at a point 100% larval mortality as the highest toxic crude leaf extracts.

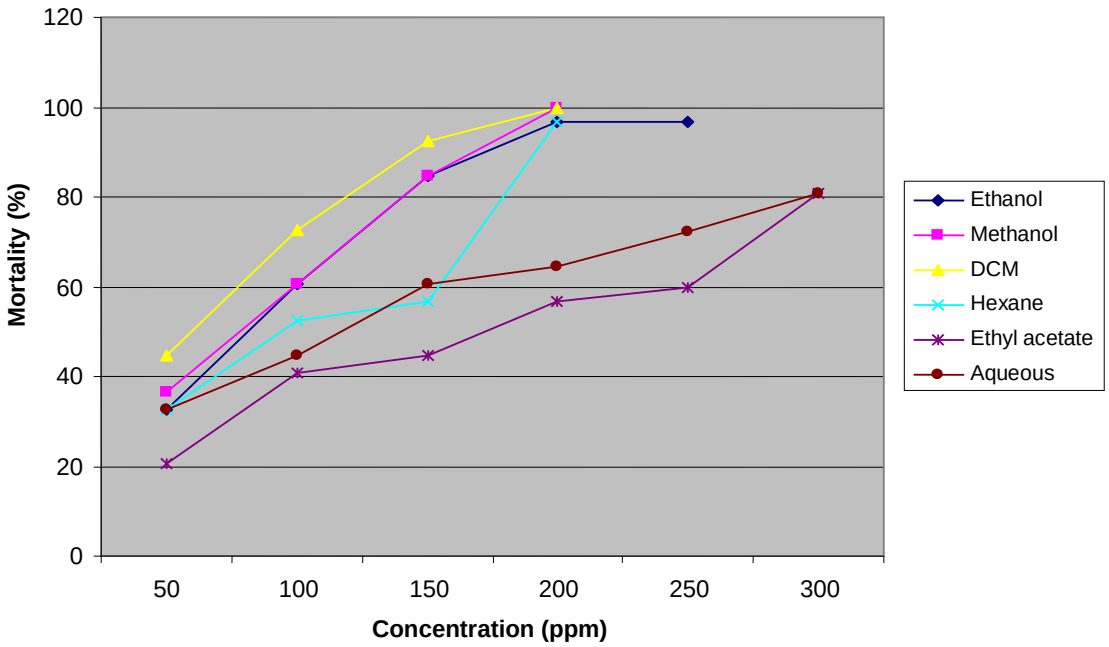

Fig 4.3 Comparison percent of six crude leaf extracts of *Eucalyptus camaldulensis* against mortality of third instar larvae of *A. gambiae* s.s. **Giles** mosquito.

Among the extracts used in this test, five extracts exhibited 100% larvae mortality on an exposure for 24 hours. These extracts were *C. cinerariifolium* (DCM and ethanol), *E. camaldulensis* (DCM and methanol) and *N. tabaccum* (ethanol). The next group which achieved high mortality were *C. cinerariifolium* (methanol, 98%), *E. camaldulensis* (ethanol and hexane each with a mortality of 96%) and *N. tabaccum* (methanol and ethyl acetate both with 96% larval mortality). Twenty three extracts yielded less than 50% larvae mortality, their effect in larvae mortality ranging from 16% (*C. cinerariifolium* hexane and aqueous) to 46% (*N. tabaccum* DCM).

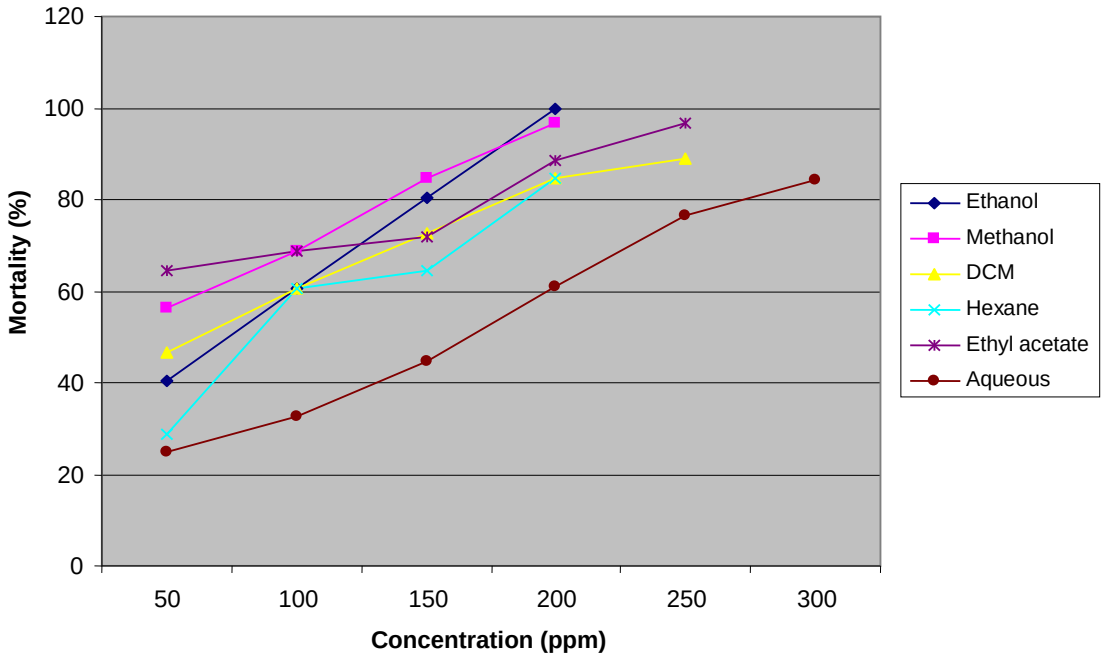

Fig 4.4 Comparison percent of six crude leaf extracts of *Nicotiana tabaccum* against mortality of third instar larvae of *A. gambiae* s.s. **Giles** mosquito.

Ethanol, with astraight line curve achieves 100% larvae mortality and closely followed by methanol which also indicates almost a straight line curve but managing 96.62% larval mortality. The larvae did not develop any resistance with these extracts but rather indicated a steady state (without decling) of larvae mortality. The other extracts namely ethyl acetate, DCM, hexane and aqueous contributed various mortalities: 88.76%, 88.55, 84.62 and 84.26% respectively. The larvae developed slight resistance to these four extracts. Ethanol extract of *Nicotiana tabaccum* was the only extract that caused 100% larval mortality with an extremely straight line curve, a demonstration of a constant upward and non-resistance in larval mortality. The same trend occurred for methanol extract. Ethyl acetate extract kept a low larvae mortality as evidenced at the

concentrations of between 50 ppm and 150 ppm. But it maintained an increased mortality from concentration 150 ppm to 250 ppm. DCM extract showed a straight line curve right from the 50 ppm concentration to 200 ppm concentration but indicated a slow larval mortality from the 200 ppm mark to 250 ppm. Hexane showed a strong larvae kill between 50 ppm and 100 ppm but slackened at two points of 100 and 150 ppm, however, improving into a traight curve between 150 and 200 ppm concentrations ending at a mortality of 84.62%. Finally, aqueous extract remained abrasive with its gentle S-curve which indicated an uprising mortality with little resistance, however, declined to give a final minimal mortality of 84.26% .

4.9 Synergistc effects of th crude leaf extracts when they are applied in the combination of P+E, P+T,and E+T and administered on the mosquito larvae.

By definition Synergistic interactions are those in which one of the combining extract will improve on the activity of the other weaker abstract such that the result of the weaker extract is better than the result when it acted alone.

Sixty extracts were made to combine in pairs to make thirty combinations for the synergistic tests. In these tests it was observed that when the extracts were combined in the ratio of 1:1 (v/v), (i.e. 100 : 100 ppm), 17 pairs acted synergistically and 2 pairs were neither synergistic nor antagonistic (Table 4.8). The synergists were identified, isolated and clearly tabulated.

Of the five highest (indicated by less concentrations) synergistic activities to note were the following extract combinations arranged in their order: *C. cinerariifolim* (Cc) DCM flower extract + *E. camaldulensis* (Ec) methanol leaf extract (DCM Cc was a synergist: DCM Cc improved Ec methanol from 197.46 ppm to 118.65 ppm); *C. cinerariifolim* DCM flower extract + *E. camaldulensis* ethyl acetate leaf extract (DCM Cc was a synergist: DCM *Cc* 164.86 ppm improved *Ec* ethyl acetate from 260.56 ppm to 121.45 ppm); *C.*

cinerariifolim DCM flower extract + *E. camaldulensis* hexane leaf extract (*Cc* DCM extract was a synergist: *Cc* DCM 164.86 ppm improved Ec hexane from 168.65 ppm to 127.85 ppm); *C. cinerariifolim* DCM flower extract + *N. tabaccum (Nt)* hexane (*Cc* DCM was a synergist: *Cc* DCM improved Nt leaf extract from 229.72 ppm to 130.78 ppm); and *E. camaldulensis* DCM leaf extract + *N. tabaccum* methanol leaf extract (*Ec* DCM was a synergist: *Ec* DCM 168.65 ppm improved *Nt* methanol 224.35 ppm to 149.35 ppm). All these combinations yielded 100% larval mortality at the synergistic concentrations of 118.65, 121.45, 127.85, 130.78 and 149.35 ppm respectively as shown in Table 4.13.

Statistically, these concentrations were significantly different from the individual exract concentrations, $P = < 0.05$. Consequently, a conclusion was made that the DCM extracts will be preferable for use as synergists in combination with the relevant extracts for the control of mosquitoes in the field.

Table 4.7 Individual extract combination for synergistic activities

Plant	Ratio	Combination solvent extracts	Individual LC_{50}ppm		Combination LC_{50}ppm	Synergistic Factor (SF)		effect
Chrysanthemum cinerariifolim+ Eucalyptus camaldulensis	1:1	Ethanol +Aqueous	187.78	210.15	152.85	1.2285	149	S
		Methanol +Ethyl acetate	224.45	260.56	189.54	1.736	1.3747	S
		DCM + Hexane	164.86	168.65	127.85	1.2894	1.3191	S
Chrysanthemum cinerariifolim+ Nicotiana tabaccum	1:1	Ethanol +Aqueous	187.78	189.58	146.64	1.2855	1.2928	S
		Methanol +Ethyl acetate	224.45	224.35	232.66	0.9561	0.9643	NS
		DCM + Hexane	164.86	229.72	130.78	1.2606	1.7565	S
Eucalyptus camaldulensis+ Nicotiana tabaccum	1:1	Ethanol +Aqueous	210.15	258.42	242.80	0.8655	1.0643	SA
		Methanol +Ethyl acetate	197.46	201.52	219.45	0.8998	0.9182	NS
		DCM + Hexane	168.65	235.85	135.55	1.2442	1.7399	S
Chrysanthemum cinerariifolim+ Eucalyptus camaldulensis	1:1	Methanol + Aqueous	224.45	259.58	201.55	1.1136	1.2879	S
		DCM + Aqueous	164.86	259.58	151.75	1.0864	1.7106	S
		DCM +Methanol	164.86	197.46	118.65	1.3895	1.6642	S
		DCM +Ethyl acetate	164.86	260.56	121.45	1.3574	2.1454	S
		Methanol +Ethyl acetate	224.45	260.56	230.50	0.9651	1.1304	S
		Methanol + Hexane	197.46	198.56	147.46	1.3391	1.3465	S
		Ethylacetate+ Hexane	227.56	198.56	236.65	0.9616	0.8390	NS
Chrysanthemum cinerariifolim + Nicotiana tabaccum	1:1	DCM +Methanol	164.86	224.35	225.80	0.7301	0.9936	NS
		DCM +Ethyl acetate	164.86	201.52	160.75	1.0256	1.2536	S
		Methanol + Aqueous	224.45	259.58	263.15	0.8453	0.9864	NS
		DCM + Aqueous	164.86	259.58	155.80	1.0582	2.6164	S
		Ethanol + Hexane	224.45	201.52	160.75	1.0256	1.2536	S
		Methanol + Hexane	224.45	235.85	231.65	0.9360	0.9924	NS
		Ethylacetate+ Hexane	227.56	235.85	241.45	0.9425	0.9768	NS
Eucalyptus camaldulensis + Nicotiana tabaccum	1:1	DCM +Methanol	164.65	224.35	149.35	1.1292	1.5022	S
		DCM +Ethyl acetate	164.65	201.52	211.55	0.7972	0.9526	NS
		DCM + Aqueous	164.86	259.58	158.43	1.0406	1.6385	S
		Methanol + Aqueous	222.45	259.58	264.65	0.8483	0.9808	NS
		Methanol + Hexane	197.46	235.85	220.75	0.8945	1.0684	SA
		Ethylacetate+ Hexane	260.56	235.85	262.60	0.9922	0.8981	NS
		Methanol + Ethanol	197.46	189.58	144.58	1.3643	1.3080	S

Key: S- Synergistic; SA- neither Synergistic nor Antagonistic; SF-Synergistic Factor; NS-Non-synergistic.

Table 4.8 The identified synergistic flower and leaf extracts of the three plants.

Plant	Ratio	Combination solvent extracts	Individual LC_{50}ppm		Combination LC_{50}ppm	Synergistic Factor (SF)		Effect
Chrysanthemum cinerariifolim + *Eucalyptus camaldulensis*	1:1	Ethanol +Aqueous Methanol +Ethyl acetate DCM + Hexane	187.78 224.45 164.86	210.15 260.56 168.65	152.85 189.54 127.85	1.2285 1.736 1.2894	149 1.3747 1.3191	S S S
Chrysanthemum cinerariifolim + *Nicotiana tabaccum*	1:1	Ethanol +Aqueous DCM + Hexane	187.78 164.86	189.58 229.72	146.64 130.78	1.2855 1.2606	1.2928 1.7565	S S
Eucalyptus camaldulensis+ *N. Tabaccum*	1:1	DCM + Hexane	168.65	235.85	135.55	1.2442	1.7399	S
Chrysanthemum cinerariifolim + *Eucalyptus camaldulensis*	1:1	Methanol + Aqueous DCM + Aqueous DCM +Methanol DCM +Ethyl acetate Methanol + Hexane	224.45 164.86 164.86 164.86 197.46	259.58 259.58 197.46 260.56 198.56	201.55 151.75 118.65 121.45 147.46	1.1136 1.0864 1.3895 1.3574 1.3391	1.2879 1.7106 1.6642 2.1454 1.3465	S S S S S
Chrysanthemum cinerariifolim + *Nicotiana tabaccum*	1:1	DCM +Ethyl acetate DCM + Aqueous Ethanol + Hexane	164.86 164.86 224.45	201.52 259.58 201.52	160.75 155.80 160.75	1.0256 1.0582 1.0256	1.2536 2.6164 1.2536	S S S
Eucalyptus camaldulensis+ *Nicotiana tabaccum*	1:1	DCM +Methanol DCM + Aqueous Ethylacetate+ Hexane Methanol + Ethanol	164.65 164.86 197.46	224.35 259.58 189.58	149.35 158.43 144.58	1.1292 1.0406 1.3643	1.5022 1.6385 1.3080	S S S

Table 4.9 Cc. + Ec Extract Combination for Synergistic Activities and Mortality Percent

Plant	Extract	Individual	Combination	% Mortality
Cc. + Ec	Ethanol + Aqueous	187.78 + 210.15	152.85 1852.90 152.85 152.92	100 100 100 100
	Methanol + Ethyl acetate	224.45 + 260.56	189.54 189.54 190.0 190.3	100 99.5 99.5 99.5
	DCM + Hexane	164.86+ 168.65	127.85 126.99 127.85 127.80	100 100 100 100
	Methanol + Aqueous	224.45+ 259.58	201.55 201.60 201.55 201.58	94 94 94 94
	DCM + Aqueous	164.86+ 259.58	151.75 152.00 151.75 151.70	100 100 100 100
	DCM + Methanol	164.86+ 197.46	115.65 118.62 118.65 118.68	100 100 100 100
	DCM +Ethylacetate	164.86+ 260.56	121.45 121.45 121.70 121.60	100 100 100 100
	Methanol +Ethylacetate	224.45+ 260.56	230.50 230.75 230.50 230.55	92 92 92 92
	Methanol + Hexane	197.46 + 198.56	147.46 147.46 147.50 147.48	100 100 100 100

In Cc. + Ec Extract Combination for Synergistic Activities and Mortality Percent, it was observed that mortality of the larvae ranged from 92% (Methanol + Ethylacetate) to 100% for mainly Cc DCM, Cc methanol and Cc ethanol as synergistic determinants. Exhibition of larvae mortality was generally high because of these synergists.

Table 4.10 Cc+ Nt Extract Combination for Synergistic Activities and Mortality Percent

Cc+ Nt	Ethanol + Aqueous	187.78+189.58	146.64 146.65 146.64 146.68	100 100 100 100
	Methanol + Ethyl acetate	224.45+224.35	232.66 232.66 232.68 232.69	80 80 80 80
	DCM + Hexane	164.86+229.72	130.78 130.80 130.81 130.78	100 100 100 100
	DCM + methanol	164.86+224.35	225.80 225.84 225.80 225.82	85 85 85 85
	DCM + Ethylacetate	164.86+201.52	160.75 160.75 161.00 161.50	100 100 100 100
	Methanol + Aqueous	224.45+259.58	263.15 263.00 263.10 263.15	64 64 64 64
	DCM + Aqueous	164.86+259	155.80 155.75 155.80 155.78	100 100 100 100
	Ethanol + Hexane	187.78+201.52	160.75 160.80 160.82 160.75	100 100 100 100
	Methanol + Hexane	224.45+235.85	231.65 232.00 231.70 231.65	90.5 90.5 90.5 90.5
	Ethylacetate + Hexane	227.56+235.85	241.45 241.45 241.42 241.10	72 72 72 72

In Cc+ Nt Extract Combination for Synergistic Activities and Mortality Percent, resulted to mortality ranging from 64% (methanol + aqueous) to 100% mortality contributed by Ethanol + Aqueous, DCM + Ethylacetate and ethanol + hexane. In these activities, it appeared that aqueous and hexane acted against synergism by reducing the activities of methanol and ethylacetate.

Table 4.11 Ec + Nt Extract Combination for Synergistic Activities and Mortality Percent

Ec +Nt	Ethanol + Aqueous	210.15+258.42	242.80 242.80 243.00 243.50	70 70 70 70
	Methanol +Ethylacetate	197.46+201.52	219.45 220.10 219.45 220.00	88 88 88 88
	DCM + Hexane	168.65+235.85	135.55 135.55 135.60 135.58	100 100 100 100
	DCM +Methanol	168.65+224.35	149.35 149.30 149.35 149.38	100 100 100 100
	DCM +Ethylacetate	168.65+201.52	211.55 211.58 211.55 211.70	90 90 90 90
	DCM + Aqueous	168.65+259.58	158.43 159.00 158.55 158.43	100 100 100 100
	Methanol +Aqueous	222.45+259.58	264.65 265.00 264.65 264.80	62 62 62 62
	Methanol + Hexane	197.46+235.85	220.75 220.70 220.73 220.75	83 83 83 83
	Ethylacetate + Hexane	260.56+235.85	262.60 262.58 262.60 262.59	65 65 65 65
	Methanol + Ethanol	197.46+189.58	144.58 144.58 144.60 144.59	100 100 100 100

In Ec + Nt Extract Combination for Synergistic Activities and Mortality Percent, resulted to a lowly range of 62% (methanol + aqueous) and rising to the pick of 100% mortality (DCM + Hexane, DCM +Methanol, DCM + Aqueous and Methanol + Ethanol). Similarly, aqueous, Ethylacetate and Hexane worked in disfavour of synergism.

Table 4.12 Analysis of Variance: Multiple Comparisons

Dependent Variable: Combination extract effect

LSD

(I) Plant extracts	(J) Plant extracts	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
DCM + Aqueous	DCM + Hexane	24.1775*	8.34505	.006	7.2362	41.1188
	DCM + Methanol	33.9000*	8.34505	.000	16.9587	50.8413
	DCM +Ethylacetate	30.2500*	8.34505	.001	13.3087	47.1913
	Ethanol + Aqueous	-2.0150	7.22702	.782	-16.6866	12.6566
	Methanol + Aqueous	-49.7700*	8.34505	.000	-66.7113	-32.8287
	Methanol + Ethyl ace	-59.4587*	7.22702	.000	-74.1304	-44.7871
	Methanol + Hexane	4.3250	8.34505	.608	-12.6163	21.2663
	Methanol +Ethylaceta	-78.7750*	8.34505	.000	-95.7163	-61.8337
DCM + Hexane	DCM + Aqueous	-24.1775*	8.34505	.006	-41.1188	-7.2362
	DCM + Methanol	9.7225	8.34505	.252	-7.2188	26.6638
	DCM +Ethylacetate	6.0725	8.34505	.472	-10.8688	23.0138
	Ethanol + Aqueous	-26.1925*	7.22702	.001	-40.8641	-11.5209
	Methanol + Aqueous	-73.9475*	8.34505	.000	-90.8888	-57.0062
	Methanol + Ethyl ace	-83.6363*	7.22702	.000	-98.3079	-68.9646
	Methanol + Hexane	-19.8525*	8.34505	.023	-36.7938	-2.9112
	Methanol +Ethylaceta	-102.9525*	8.34505	.000	-119.8938	-86.0112
DCM + Methanol	DCM + Aqueous	-33.9000*	8.34505	.000	-50.8413	-16.9587
	DCM + Hexane	-9.7225	8.34505	.252	-26.6638	7.2188
	DCM +Ethylacetate	-3.6500	8.34505	.665	-20.5913	13.2913
	Ethanol + Aqueous	-35.9150*	7.22702	.000	-50.5866	-21.2434
	Methanol + Aqueous	-83.6700*	8.34505	.000	-100.6113	-66.7287
	Methanol + Ethyl ace	-93.3587*	7.22702	.000	-108.0304	-78.6871
	Methanol + Hexane	-29.5750*	8.34505	.001	-46.5163	-12.6337
	Methanol +Ethylaceta	-112.6750*	8.34505	.000	-129.6163	-95.7337
DCM +Ethylacetate	DCM + Aqueous	-30.2500*	8.34505	.001	-47.1913	-13.3087
	DCM + Hexane	-6.0725	8.34505	.472	-23.0138	10.8688
	DCM + Methanol	3.6500	8.34505	.665	-13.2913	20.5913
	Ethanol + Aqueous	-32.2650*	7.22702	.000	-46.9366	-17.5934
	Methanol + Aqueous	-80.0200*	8.34505	.000	-96.9613	-63.0787
	Methanol + Ethyl ace	-89.7088*	7.22702	.000	-104.3804	-75.0371
	Methanol + Hexane	-25.9250*	8.34505	.004	-42.8663	-8.9837
	Methanol +Ethylaceta	-109.0250*	8.34505	.000	-125.9663	-92.0837
Ethanol + Aqueous	DCM + Aqueous	2.0150	7.22702	.782	-12.6566	16.6866
	DCM + Hexane	26.1925*	7.22702	.001	11.5209	40.8641
	DCM + Methanol	35.9150*	7.22702	.000	21.2434	50.5866
	DCM +Ethylacetate	32.2650*	7.22702	.000	17.5934	46.9366

	Methanol + Aqueous	-47.7550*	7.22702	.000	-62.4266	-33.0834
	Methanol + Ethyl ace	-57.4437*	5.90084	.000	-69.4231	-45.4644
	Methanol + Hexane	6.3400	7.22702	.386	-8.3316	21.0116
	Methanol +Ethylaceta	-76.7600*	7.22702	.000	-91.4316	-62.0884
Methanol + Aqueous	DCM + Aqueous	49.7700*	8.34505	.000	32.8287	66.7113
	DCM + Hexane	73.9475*	8.34505	.000	57.0062	90.8888
	DCM + Methanol	83.6700*	8.34505	.000	66.7287	100.6113
	DCM +Ethylacetate	80.0200*	8.34505	.000	63.0787	96.9613
	Ethanol + Aqueous	47.7550*	7.22702	.000	33.0834	62.4266
	Methanol + Ethyl ace	-9.6887	7.22702	.189	-24.3604	4.9829
	Methanol + Hexane	54.0950*	8.34505	.000	37.1537	71.0363
	Methanol +Ethylaceta	-29.0050*	8.34505	.001	-45.9463	-12.0637
Methanol + Ethyl ace	DCM + Aqueous	59.4587*	7.22702	.000	44.7871	74.1304
	DCM + Hexane	83.6363*	7.22702	.000	68.9646	98.3079
	DCM + Methanol	93.3587*	7.22702	.000	78.6871	108.0304
	DCM +Ethylacetate	89.7088*	7.22702	.000	75.0371	104.3804
	Ethanol + Aqueous	57.4437*	5.90084	.000	45.4644	69.4231
	Methanol + Aqueous	9.6887	7.22702	.189	-4.9829	24.3604
	Methanol + Hexane	63.7838*	7.22702	.000	49.1121	78.4554
	Methanol +Ethylaceta	-19.3163*	7.22702	.011	-33.9879	-4.6446
Methanol + Hexane	DCM + Aqueous	-4.3250	8.34505	.608	-21.2663	12.6163
	DCM + Hexane	19.8525*	8.34505	.023	2.9112	36.7938
	DCM + Methanol	29.5750*	8.34505	.001	12.6337	46.5163
	DCM +Ethylacetate	25.9250*	8.34505	.004	8.9837	42.8663
	Ethanol + Aqueous	-6.3400	7.22702	.386	-21.0116	8.3316
	Methanol + Aqueous	-54.0950*	8.34505	.000	-71.0363	-37.1537
	Methanol + Ethyl ace	-63.7838*	7.22702	.000	-78.4554	-49.1121
	Methanol +Ethylaceta	-83.1000*	8.34505	.000	-100.0413	-66.1587
Methanol +Ethylaceta	DCM + Aqueous	78.7750*	8.34505	.000	61.8337	95.7163
	DCM + Hexane	102.9525*	8.34505	.000	86.0112	119.8938
	DCM + Methanol	112.6750*	8.34505	.000	95.7337	129.6163
	DCM +Ethylacetate	109.0250*	8.34505	.000	92.0837	125.9663
	Ethanol + Aqueous	76.7600*	7.22702	.000	62.0884	91.4316
	Methanol + Aqueous	29.0050*	8.34505	.001	12.0637	45.9463
	Methanol + Ethyl ace	19.3163*	7.22702	.011	4.6446	33.9879
	Methanol + Hexane	83.1000*	8.34505	.000	66.1587	100.0413

Based on observed means.
The error term is Mean Square (Error) = 139.280.

*. The mean difference is significant at the 0.05 level.

Table 4.13 Synergistic plant extract combination showing percent larval mortality

Activity	Plant	Extract	Combination	% Mortality
Synergistic	Cc. + Ec	Ethanol + Aqueous	152.85	100.00
Synergistic	Cc. + Ec	Ethanol + Aqueous	185.29	100.00
Synergistic	Cc. + Ec	Ethanol + Aqueous	152.85	100.00
Synergistic	Cc. + Ec	Ethanol + Aqueous	152.92	100.00
Synergistic	Cc + Ec	Methanol + Ethyl acetate	189.54	100.00
Synergistic	Cc. + Ec	Methanol + Ethyl acetate	189.54	100.00
Synergistic	Cc. + Ec	Methanol + Ethyl acetate	190.00	100.00
Synergistic	Cc + Ec	Methanol + Ethyl acetate	190.30	100.00
Synergistic	Cc. + Ec	DCM + Hexane	127.85	100.00
Synergistic	Cc. + Ec	DCM + Hexane	126.99	100.00
Synergistic	Cc. + Ec	DCM + Hexane	127.85	100.00
Synergistic	Cc. + Ec	DCM + Hexane	127.80	100.00
Synergistic	Cc. + Ec	Methanol + Aqueous	201.55	98.00
Synergistic	Cc. + Ec	Methanol + Aqueous	201.60	98.00
Synergistic	Cc. + Ec	Methanol + Aqueous	201.55	98.00
Synergistic	Cc. + Ec	Methanol + Aqueous	201.58	98.00
Synergistic	Cc. + Ec	DCM + Aqueous	151.75	93.00
Synergistic	Cc. + Ec	DCM + Aqueous	152.00	93.00
Synergistic	Cc. + Ec	DCM + Aqueous	151.75	93.00
Synergistic	Cc. + Ec	DCM + Aqueous	151.70	93.00
Synergistic	Cc. + Ec	DCM + Methanol	115.65	100.00
Synergistic	Cc. + Ec	DCM + Methanol	118.62	100.00
Synergistic	Cc. + Ec	DCM + Methanol	118.65	100.00
Synergistic	Cc. + Ec	DCM + Methanol	118.68	100.00
Synergistic	Cc. + Ec	DCM +Ethylacetate	121.45	100.00
Synergistic	Cc. + Ec	DCM +Ethylacetate	121.45	100.00
Synergistic	Cc. + Ec	DCM +Ethylacetate	121.70	100.00
Synergistic	Cc. + Ec	DCM +Ethylacetate	121.60	100.00
Synergistic	Cc. + Ec	Methanol +Ethylacetate	230.50	97.00
Synergistic	Cc. + Ec	Methanol +Ethylacetate	230.75	97.00
Synergistic	Cc. + Ec	Methanol +Ethylacetate	230.50	97.00
Synergistic	Cc. + Ec	Methanol +Ethylacetate	230.55	97.00
Synergistic	Cc. + Ec	Methanol + Hexane	147.46	100.00
Synergistic	Cc. + Ec	Methanol + Hexane	147.46	100.00
Synergistic	Cc. + Ec	Methanol + Hexane	147.50	100.00
Synergistic	Cc. + Ec	Methanol + Hexane	147.48	100.00
Synergistic	Cc+ Nt	Ethanol + Aqueous	146.64	90.00
Synergistic	Cc+ Nt	Ethanol + Aqueous	146.65	90.00
Synergistic	Cc+ Nt	Ethanol + Aqueous	146.64	90.00
Synergistic	Cc+ Nt	Ethanol + Aqueous	146.68	90.00

Synergistic	Cc+ Nt	Methanol + Ethyl acetate	232.66	91.00
Synergistic	Cc+ Nt	Methanol + Ethyl acetate	232.66	91.00
Synergistic	Cc+ Nt	Methanol + Ethyl acetate	232.68	91.00
Synergistic	Cc+ Nt	Methanol + Ethyl acetate	232.69	91.00
Synergistic	Cc+ Nt	DCM + Hexane	130.78	98.00
Synergistic	Cc+ Nt	DCM + Hexane	130.80	98.00
Synergistic	Cc+ Nt	DCM + Hexane	130.81	98.00
Synergistic	Cc+ Nt	DCM + Hexane	130.78	98.00
Synergistic	Cc+ Nt	DCM + methanol	225.80	82.00
Synergistic	Cc+ Nt	DCM + methanol	225.84	82.00
Synergistic	Cc+ Nt	DCM + methanol	225.80	82.00
Synergistic	Cc+ Nt	DCM + methanol	225.82	82.00
Synergistic	Cc+ Nt	DCM + Ethylacetate	160.75	96.00
Synergistic	Cc+ Nt	DCM + Ethylacetate	160.75	96.00
Synergistic	Cc+ Nt	DCM + Ethylacetate	161.00	96.00
Synergistic	Cc+ Nt	DCM + Ethylacetate	161.50	96.00
Synergistic	Cc+ Nt	Methanol + Aqueous	263.15	78.00
Synergistic	Cc+ Nt	Methanol + Aqueous	263.00	78.00
Synergistic	Cc+ Nt	Methanol + Aqueous	263.10	78.00
Synergistic	Cc+ Nt	Methanol + Aqueous	263.15	78.00
Synergistic	Cc+ Nt	DCM + Aqueous	155.80	97.00
Synergistic	Cc+ Nt	DCM + Aqueous	155.75	97.00
Synergistic	Cc+ Nt	DCM + Aqueous	155.80	97.00
Synergistic	Cc+ Nt	DCM + Aqueous	155.78	97.00
Synergistic	Cc+ Nt	Ethanol + Hexane	160.75	96.00
Synergistic	Cc+ Nt	Ethanol + Hexane	160.80	96.00
Synergistic	Cc+ Nt	Ethanol + Hexane	160.82	96.00
Synergistic	Cc+ Nt	Ethanol + Hexane	160.75	96.00
Synergistic	Cc+ Nt	Methanol + Hexane	231.65	78.00
Synergistic	Cc+ Nt	Methanol + Hexane	232.00	78.00
Synergistic	Cc+ Nt	Methanol + Hexane	231.70	78.00
Synergistic	Cc+ Nt	Methanol + Hexane	231.65	78.00
Synergistic	Cc+ Nt	Ethylacetate + Hexane	241.45	69.00
Synergistic	Cc+ Nt	Ethylacetate + Hexane	241.45	69.00
Synergistic	Cc+ Nt	Ethylacetate + Hexane	241.42	69.00
Synergistic	Cc+ Nt	Ethylacetate + Hexane	241.10	69.00
Synergistic	Ec +Nt	Ethanol + Aqueous	242.80	91.00
Synergistic	Ec +Nt	Ethanol + Aqueous	242.80	91.00
Synergistic	Ec +Nt	Ethanol + Aqueous	243.00	91.00
Synergistic	Ec +Nt	Ethanol + Aqueous	243.50	91.00
Synergistic	Ec +Nt	Methanol +Ethylacetate	219.45	73.00
Synergistic	Ec +Nt	Methanol +Ethylacetate	220.10	73.00
Synergistic	Ec +Nt	Methanol +Ethylacetate	219.45	73.00

Synergistic	Ec +Nt	Methanol +Ethylacetate	220.00	73.00
Synergistic	Ec +Nt	DCM + Hexane	135.55	100.00
Synergistic	Ec +Nt	DCM + Hexane	135.55	100.00
Synergistic	Ec +Nt	DCM + Hexane	135.60	100.00
Synergistic	Ec +Nt	DCM + Hexane	135.58	100.00
Synergistic	Ec +Nt	DCM +Methanol	149.35	100.00
Synergistic	Ec +Nt	DCM +Methanol	149.30	100.00
Synergistic	Ec +Nt	DCM +Methanol	149.35	100.00
Synergistic	Ec +Nt	DCM +Methanol	149.38	100.00
Synergistic	Ec +Nt	DCM +Ethylacetate	211.55	66.00
Synergistic	Ec +Nt	DCM +Ethylacetate	211.58	66.00
Synergistic	Ec +Nt	DCM +Ethylacetate	211.55	66.00
Synergistic	Ec +Nt	DCM +Ethylacetate	211.70	66.00
Synergistic	Ec +Nt	DCM + Aqueous	158.43	97.00
Synergistic	Ec +Nt	DCM + Aqueous	159.00	97.00
Synergistic	Ec +Nt	DCM + Aqueous	158.55	97.00
Synergistic	Ec +Nt	DCM + Aqueous	158.43	97.00
Synergistic	Ec +Nt	Methanol +Aqueous	264.65	62.00
Synergistic	Ec +Nt	Methanol +Aqueous	265.00	62.00
Synergistic	Ec +Nt	Methanol +Aqueous	264.65	62.00
Synergistic	Ec +Nt	Methanol +Aqueous	264.80	62.00
Synergistic	Ec +Nt	Methanol + Hexane	220.75	100.00
Synergistic	Ec +Nt	Methanol + Hexane	220.70	100.00
Synergistic	Ec +Nt	Methanol + Hexane	220.73	100.00
Synergistic	Ec +Nt	Methanol + Hexane	220.75	100.00
Synergistic	Ec +Nt	Ethylacetate + Hexane	262.60	58.00
Synergistic	Ec +Nt	Ethylacetate + Hexane	262.58	58.00
Synergistic	Ec +Nt	Ethylacetate + Hexane	262.60	58.00
Synergistic	Ec +Nt	Ethylacetate + Hexane	262.59	58.00
Synergistic	Ec +Nt	Methanol + Ethanol	144.58	100.00
Synergistic	Ec +Nt	Methanol + Ethanol	144.58	100.00
Synergistic	Ec +Nt	Methanol + Ethanol	144.60	100.00
Synergistic	Ec +Nt	Methanol + Ethanol	144.59	100.00

Table 4.14 Multiple Comparisons (Synergistic)

Dependent Variable: Mortality

LSD

(I) Plant	(J) Plant	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Cc. + Ec	Cc+ Nt	9.2528*	2.65773	.001	3.9873	14.5182
	Ec +Nt	12.6028*	2.65773	.000	7.3373	17.8682
Cc+ Nt	Cc. + Ec	-9.2528*	2.65773	.001	-14.5182	-3.9873
	Ec +Nt	3.3500	2.58685	.198	-1.7750	8.4750
Ec +Nt	Cc. + Ec	-12.6028*	2.65773	.000	-17.8682	-7.3373
	Cc+ Nt	-3.3500	2.58685	.198	-8.4750	1.7750

Based on observed means.

The error term is Mean Square (Error) = 133.835.

*. The mean difference is significant at the 0.05 level.

```
UNIANOVA Mortality BY Extract
  /METHOD=SSTYPE(3)
  /INTERCEPT=INCLUDE
  /CRITERIA=ALPHA (0.05)
  /DESIGN=Extract.
```

There is a statistical difference within plants. ($p < 0.001$). In this plant multiple comparison, Cc + Nt and Ec + Nt were not significantly different ($p > 0.198$, hence, in terms of plants they were different. In plant mortality one way analysis of variance showed the same as two plants above and therefore there was a statistical significance.

4.10 Antagonistic effects when the flower and leaf extracts were alternately combined as P+E, P+T and E+T and administerd on *A. gambiae* s.s. mosquito larvae

One of the important activities of an antagonist is to lower the toxicity of an active substance. Table 4.15 indicates combining extracts for antagonistic activities and sums up three scenarios: antagonistic, not antagonistic, neither antagonistic nor synergistic. Table 4.16 shows extracts that were antagonists. The antagonism activities are explained in Table 4.17 under extracts serials number 1 to 10. It was observed that every antagonist concentration rendered the corresponding combining concentration weaker such that the concentrations could not achieve 100% larval mortality. It was also observed that four of *N.*

tabaccum extracts of *N. tabaccum* (Aqueous , ethyl acetate, hexane, methanol), acted as antagonists in various combinations while two of *E. camaldulensis* (ethyl acetate and hexane) and one extract of *C. cinerariifolium* (methanol) as well acted as antagonists.

Table 4.15 Combining extracts for antagonistic activities

	Ratio	Combination solvent extracts	Individual LC_{50}ppm		Combination LC_{50}ppm	SF		Effect
Chrysanthemum cinerariifolim + *Eucalyptus*	1:1	Ethanol +Aqueous Methanol +Ethyl acetate DCM + Hexane	187.78 224.45 164.86	210.15 260.56 168.65	152.85 189.54 127.85	1.2285 1.736 1.2894	1.3749 1.3747 1.3191	NA NA NA
Chrysanthemum cinerariifolim +Nicotiana tabaccum	1:1	Ethanol +Aqueous Methanol +Ethyl acetate DCM + Hexane	187.78 224.45 164.86	189.58 224.35 229.72	146.64 232.66 130.78	1.2855 0.9561 1.2606	1.2928 0.9643 1.7565	NA A NA
Eucalyptus camaldulensis.. + *Nicotiana tabaccum*	1:1	Ethanol +Aqueous Methanol +Ethyl acetate DCM + Hexane	210.15 197.46 168.65	258.42 201.52 235.85	242.80 219.45 135.55	0.8655 0.8998 1.2442	1.0643 0.9182 1.7399	SA A NA
Chrysanthemum cinerariifolim. + *Eucalyptus camaldulensis*	1:1	Methanol + Aqueous DCM + Aqueous DCM +Methanol DCM +Ethyl acetate Methanol +Ethyl acetate Methanol + Hexane Ethylacetate+ Hexane	224.45 164.86 164.86 164.86 224.45 197.46 227.56	259.58 259.58 197.46 260.56 260.56 198.56 198.56	201.55 151.75 118.65 121.45 230.50 147.46 236.65	1.1136 1.0864 1.3895 1.3574 0.9651 1.3391 0.9616	1.2879 1.7106 1.6642 2.1454 1.1304 1.3465 0.8390	NA NA NA NA SA NA A
Chrysanthemum cinerariifolim +Nicotiana tabaccum	1:1	DCM +Methanol DCM +Ethyl acetate Methanol + Aqueous DCM + Aqueous Ethanol +Hexane Methanol + Hexane Ethylacetate+ Hexane	164.86 164.86 224.45 164.86 224.45 224.45 227.56	224.35 201.52 259.58 259.58 201.52 235.85 2|35.85	225.80 160.75 263.15 155.80 160.75 231.65 241.45	0.7301 1.0256 0.8453 1.0582 1.0256 0.9360 0.9425	0.9936 1.2536 0.9864 2.6164 1.2536 0.9924 0.9768	A NA A NA NA A A
Eucalyptus camaldulensis+ *Nicotiana tabaccum*	1:1	DCM +Methanol DCM +Ethyl acetate DCM + Aqueous Methanol + Aqueous Methanol + Hexane Ethylacetate+ Hexane Methanol + Ethanol	164.65 164.65 164.86 222.45 197.46 260.56 197.46	224.35 201.52 259.58 259.58 235.85 235.85 189.58	149.35 211.55 158.43 264.65 220.75 262.60 144.58	1.1292 0.7972 1.0406 0.8483 0.8945 0.9922 1.3643	1.5022 0.9526 1.6385 0.9808 1.0684 0.8981 1.3080	NA A NA A SA A

Key: A – Antagonistic; SA- neither Synergistic nor Antagonistic: NA- Not Antagonistic.

Table 4.16 Extracts of the plants that were antagonists.

	Ratio	Combination solvent extracts	Concetration (LC_{50}ppm)		Combination (LC_{50}ppm)	SF		Effect
Eucalyptus camaldulensis + Nicotiana tabbacum	1:1	Methanol + Ethylacetate	197.46	201.52	219.45	0.8998	0.9183	A
Chrysanthemum cinerariifolium+ Nictiana tabbacum	1:1	DCM +Methanol	164.86	224.35	225.80	0.7301	0.996	A
Chrysanthemum cinerariifolium+ Nictiana tabbacum	1:1	Methanol + Hexane	225.45	235.85	237.65	0.9360	0.9924	A
Chrysanthemum cinerariifolium+ Chrysanthemum cinerariifolium	1:1	Ethylacetate +Hexane	227.56	235.85	241.45	0.9425	0.9768	A
Eucalyptus camaldulensis +Nicotiana tabbacum	1:1	DCM +Ethylacetate	168.65	201.52	211.55	0.7972	0.9526	A
Chrysanthemum cinerariifolium+ Nictiana tabbacum	1:1	DCM +Aqueous	222.45	259.58	263.15	0.8453	0.9864	A
Eucalyptus camaldulensis +Eucalyptus camaldulensis	1:1	Methanol +Aqueous	222.45	258.42	264.65	0.8483	0.9808	A

Table 4.17 Lowering of concentrations by antagonists

S/NO	Antagonistic crude leaf extract	Concentrations lowered (ppm)
1	*Aqueous* of *Nicotiana tabaccum*	Methanol of *Eucalyptus camaldulensis*from 222.45 ppm to 264.65 ppm
2	Ethyl acetate of *Nicotiana Tabaccum*	Methanol of *E. camaldulensis* from 197.46 ppm to 219.45 ppm
3	Methanol of *Nicotiana tabaccum*	DCM of *C. cinerariifolium* from 164.86 ppm to 225.80 ppm
4	Hexane of *N. tabaccum*	Ethyl acetate of *C.cinerariifolium* from 227.56 ppm to 241.45 ppm
5	Hexane of *Nicotiana tabaccum*	Methanol of *C.cinerariifolium* from 224.45 ppm to 231.65 ppm
6	Hexane of *E. camaldulensis*	Ethyl acetate of *N. tabaccum* from 260.56 ppm to 262.60 ppm
7	Methanol of *C. cinerariifolium*	DCM of *C. cinerariifolium* from 224.45 ppm to 232.66 ppm
8	Ethyl acetate of *Nicotiana tabaccum*	DCM of *E. camaldulensis* from 164.65 ppm to 211.55 ppm
9	Methanol of *Chrysanthemum cinerariifolium*	Ethyl acetate of *C.cinerariifolium* from 224.35 ppm to 232.66 ppm
10	Ethyl acetate of *Eucalyptus camaldulensis*	*Hexane of N. tabaccum from 235.85 ppm to 262.60 ppm*

Table 4.18 Antagonistic plant extract combination showing percent larval mortality

Activity	Plant	Extract	Combination	% Mortality
Antagonistic	Cc. + Ec	Ethanol + Aqueous	152.85	95.00
Antagonistic	Cc. + Ec	Ethanol + Aqueous	185.29	95.00
Antagonistic	Cc. + Ec	Ethanol + Aqueous	152.85	95.00
Antagonistic	Cc. + Ec	Ethanol + Aqueous	152.92	95.00
Antagonistic	Cc. + Ec	Methanol + Ethyl acetate	189.54	93.00
Antagonistic	Cc. + Ec	Methanol + Ethyl acetate	189.54	93.00
Antagonistic	Cc. + Ec	Methanol + Ethyl acetate	190.00	93.00
Antagonistic	Cc. + Ec	Methanol + Ethyl acetate	190.30	93.00
Antagonistic	Cc. + Ec	DCM + Hexane	127.85	90.00
Antagonistic	Cc. + Ec	DCM + Hexane	126.99	90.00
Antagonistic	Cc. + Ec	DCM + Hexane	127.85	90.00
Antagonistic	Cc. + Ec	DCM + Hexane	127.80	90.00
Antagonistic	Cc. + Ec	Methanol + Aqueous	201.55	82.00
Antagonistic	Cc. + Ec	Methanol + Aqueous	201.60	82.00
Antagonistic	Cc. + Ec	Methanol + Aqueous	201.55	82.00
Antagonistic	Cc. + Ec	Methanol + Aqueous	201.58	82.00
Antagonistic	Cc. + Ec	DCM + Aqueous	151.75	94.00
Antagonistic	Cc. + Ec	DCM + Aqueous	152.00	94.00
Antagonistic	Cc. + Ec	DCM + Aqueous	151.75	94.00
Antagonistic	Cc. + Ec	DCM + Aqueous	151.70	94.00
Antagonistic	Cc. + Ec	DCM + Methanol	115.65	97.00
Antagonistic	Cc. + Ec	DCM + Methanol	118.62	97.00
Antagonistic	Cc. + Ec	DCM + Methanol	118.65	97.00
Antagonistic	Cc. + Ec	DCM + Methanol	118.68	97.00
Antagonistic	Cc. + Ec	DCM +Ethylacetate	121.45	92.00
Antagonistic	Cc. + Ec	DCM +Ethylacetate	121.45	92.00
Antagonistic	Cc. + Ec	DCM +Ethylacetate	121.70	92.00
Antagonistic	Cc. + Ec	DCM +Ethylacetate	121.60	92.00
Antagonistic	Cc. + Ec	Methanol +Ethylacetate	230.50	80.00
Antagonistic	Cc. + Ec	Methanol +Ethylacetate	230.75	80.00
Antagonistic	Cc. + Ec	Methanol +Ethylacetate	230.50	80.00
Antagonistic	Cc. + Ec	Methanol +Ethylacetate	230.55	80.00
Antagonistic	Cc. + Ec	Methanol + Hexane	147.46	90.00
Antagonistic	Cc. + Ec	Methanol + Hexane	147.46	90.00
Antagonistic	Cc. + Ec	Methanol + Hexane	147.50	90.00
Antagonistic	Cc. + Ec	Methanol + Hexane	147.48	90.00
Antagonistic	Cc+ Nt	Ethanol + Aqueous	146.64	92.00
Antagonistic	Cc+ Nt	Ethanol + Aqueous	146.65	92.00
Antagonistic	Cc+ Nt	Ethanol + Aqueous	146.64	92.00
Antagonistic	Cc+ Nt	Ethanol + Aqueous	146.68	92.00

Antagonistic	Cc+ Nt	Methanol + Ethyl acetate	232.66	79.00
Antagonistic	Cc+ Nt	Methanol + Ethyl acetate	232.66	79.00
Antagonistic	Cc+ Nt	Methanol + Ethyl acetate	232.68	79.00
Antagonistic	Cc+ Nt	Methanol + Ethyl acetate	232.69	79.00
Antagonistic	Cc+ Nt	DCM + Hexane	130.78	83.00
Antagonistic	Cc+ Nt	DCM + Hexane	130.80	83.00
Antagonistic	Cc+ Nt	DCM + Hexane	130.81	83.00
Antagonistic	Cc+ Nt	DCM + Hexane	130.78	83.00
Antagonistic	Cc+ Nt	DCM + methanol	225.80	65.00
Antagonistic	Cc+ Nt	DCM + methanol	225.84	65.00
Antagonistic	Cc+ Nt	DCM + methanol	225.80	65.00
Antagonistic	Cc+ Nt	DCM + methanol	225.82	65.00
Antagonistic	Cc+ Nt	DCM + Ethylacetate	160.75	91.00
Antagonistic	Cc+ Nt	DCM + Ethylacetate	160.75	91.00
Antagonistic	Cc+ Nt	DCM + Ethylacetate	161.00	91.00
Antagonistic	Cc+ Nt	DCM + Ethylacetate	161.50	91.00
Antagonistic	Cc+ Nt	Methanol + Aqueous	263.15	55.00
Antagonistic	Cc+ Nt	Methanol + Aqueous	263.00	55.00
Antagonistic	Cc+ Nt	Methanol + Aqueous	263.10	55.00
Antagonistic	Cc+ Nt	Methanol + Aqueous	263.15	55.00
Antagonistic	Cc+ Nt	DCM + Aqueous	155.80	92.00
Antagonistic	Cc+ Nt	DCM + Aqueous	155.75	92.00
Antagonistic	Cc+ Nt	DCM + Aqueous	155.80	92.00
Antagonistic	Cc+ Nt	DCM + Aqueous	155.78	92.00
Antagonistic	Cc+ Nt	Ethanol + Hexane	160.75	68.00
Antagonistic	Cc+ Nt	Ethanol + Hexane	160.80	68.00
Antagonistic	Cc+ Nt	Ethanol + Hexane	160.82	68.00
Antagonistic	Cc+ Nt	Ethanol + Hexane	160.75	68.00
Antagonistic	Cc+ Nt	Methanol + Hexane	231.65	84.00
Antagonistic	Cc+ Nt	Methanol + Hexane	232.00	84.00
Antagonistic	Cc+ Nt	Methanol + Hexane	231.70	84.00
Antagonistic	Cc+ Nt	Methanol + Hexane	231.65	84.00
Antagonistic	Cc+ Nt	Ethylacetate + Hexane	241.45	78.00
Antagonistic	Cc+ Nt	Ethylacetate + Hexane	241.45	78.00
Antagonistic	Cc+ Nt	Ethylacetate + Hexane	241.42	78.00
Antagonistic	Cc+ Nt	Ethylacetate + Hexane	241.10	78.00
Antagonistic	Ec +Nt	Ethanol + Aqueous	242.80	86.00
Antagonistic	Ec +Nt	Ethanol + Aqueous	242.80	86.00
Antagonistic	Ec +Nt	Ethanol + Aqueous	243.00	86.00
Antagonistic	Ec +Nt	Ethanol + Aqueous	243.50	86.00
Antagonistic	Ec +Nt	Methanol +Ethylacetate	219.45	91.00
Antagonistic	Ec +Nt	Methanol +Ethylacetate	220.10	91.00
Antagonistic	Ec +Nt	Methanol +Ethylacetate	219.45	91.00

Antagonistic	Ec +Nt	Methanol +Ethylacetate	220.00	91.00
Antagonistic	Ec +Nt	DCM + Hexane	135.55	89.00
Antagonistic	Ec +Nt	DCM + Hexane	135.55	89.00
Antagonistic	Ec +Nt	DCM + Hexane	135.60	89.00
Antagonistic	Ec +Nt	DCM + Hexane	135.58	89.00
Antagonistic	Ec +Nt	DCM +Methanol	149.35	90.00
Antagonistic	Ec +Nt	DCM +Methanol	149.30	90.00
Antagonistic	Ec +Nt	DCM +Methanol	149.35	90.00
Antagonistic	Ec +Nt	DCM +Methanol	149.38	90.00
Antagonistic	Ec +Nt	DCM +Ethylacetate	211.55	89.00
Antagonistic	Ec +Nt	DCM +Ethylacetate	211.58	89.00
Antagonistic	Ec +Nt	DCM +Ethylacetate	211.55	89.00
Antagonistic	Ec +Nt	DCM +Ethylacetate	211.70	89.00
Antagonistic	Ec +Nt	DCM + Aqueous	158.43	72.00
Antagonistic	Ec +Nt	DCM + Aqueous	159.00	72.00
Antagonistic	Ec +Nt	DCM + Aqueous	158.55	72.00
Antagonistic	Ec +Nt	DCM + Aqueous	158.43	72.00
Antagonistic	Ec +Nt	Methanol +Aqueous	264.65	58.00
Antagonistic	Ec +Nt	Methanol +Aqueous	265.00	58.00
Antagonistic	Ec +Nt	Methanol +Aqueous	264.65	58.00
Antagonistic	Ec +Nt	Methanol +Aqueous	264.80	58.00
Antagonistic	Ec +Nt	Methanol + Hexane	220.75	74.00
Antagonistic	Ec +Nt	Methanol + Hexane	220.70	74.00
Antagonistic	Ec +Nt	Methanol + Hexane	220.73	74.00
Antagonistic	Ec +Nt	Methanol + Hexane	220.75	74.00
Antagonistic	Ec +Nt	Ethylacetate + Hexane	262.60	60.00
Antagonistic	Ec +Nt	Ethylacetate + Hexane	262.58	60.00
Antagonistic	Ec +Nt	Ethylacetate + Hexane	262.60	60.00
Antagonistic	Ec +Nt	Ethylacetate + Hexane	262.59	60.00
Antagonistic	Ec +Nt	Methanol + Ethanol	144.58	88.00
Antagonistic	Ec +Nt	Methanol + Ethanol	144.58	88.00
Antagonistic	Ec +Nt	Methanol + Ethanol	144.60	88.00
Antagonistic	Ec +Nt	Methanol + Ethanol	144.59	88.00

Table 4.19 Post Hoc Tests

Plant

Multiple Comparisons (Antagonistic)

Dependent Variable: Mortality_Ant

LSD

(I) Plant	(J) Plant	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Cc. + Ec	Cc+ Nt	11.6333*	2.42520	.000	6.8286	16.4381
	Ec +Nt	10.6333*	2.42520	.000	5.8286	15.4381
Cc+ Nt	Cc. + Ec	-11.6333*	2.42520	.000	-16.4381	-6.8286
	Ec +Nt	-1.0000	2.36052	.673	-5.6766	3.6766
Ec +Nt	Cc. + Ec	-10.6333*	2.42520	.000	-15.4381	-5.8286
	Cc+ Nt	1.0000	2.36052	.673	-3.6766	5.6766

Based on observed means.

The error term is Mean Square(Error) = 111.441.

*. The mean difference is significant at the 0.05 level.

```
UNIANOVA Mortality_Ant BY Plant Extract
  /RANDOM=Extract
  /METHOD=SSTYPE(3)
  /INTERCEPT=INCLUDE
  /POSTHOC=Plant(LSD)
  /CRITERIA=ALPHA(0.05)
  /DESIGN=Plant Extract Plant*Extract.
```

Note: Could not run a post Hoc because there were too many extract interactions.

4.11 Resistance ratio (RR) of the crude leaf extracts upon *Anopheles gambiae* third instar larvae

Tests for RR involved two strains of *A. gambiae s.s.***Giles** mosquito. One was the laboratory reared strain (susceptible strain) and the other was field strain (larvae collected from stagnating water pools – wetlands in Langas area of Eldoret). The laboratory and field strains were tested separately following WHO (2013) procedures as described in the methodology and their results were compared in a ratio (ratio has no units).

The results indicated that the laboratory reared larvae did not develop any resistance to five extracts namely C. cinerariifolium ethanol,methanol and DCM; E. camldulensis

methanol and DCM; and N. tabaccum ethanol. Other than C. cinerariifolium extract exhibiting 98% larval mortality the rest of these extracts achieved 100% mortality. On the other hand the laboratory strain developed resistance of 11 extracts: C. cinerariifoliumhexane, ethyl acetate and aqueous; E. camaldulensis ethanol, hexane, ethyl acetate and aqueous; N. tabaccum methanol, DCM, hexane, ethyl acetate and aqueous.

The field strain of the larvae did not develop any resistance to five extracts i.e. C. cinerariifolium ethanol and DCM; E. camaldulensis methanol and DCM; and N. tabaccum ethanol but developed resistance to 13 extracts which were C. cinerariifolium methanol, hexane, ethyl acetate and aqueous; E. camaldulensis ethanol, hexane, ethyl acetate and aqueous; N. tabaccum methanol, DCM, hexane, ethyl acetate and aqueous.

The laboratory reared larvae LC50 resistance varied from 0.993 (E.camaldulensis, lowest) to 1.066 (C. cinerariifolium, highest), which is 0.993 – 1.066 folds. The field strain larvae LC50 resistance varied from 1.081 (C. cinerariifolium, lowest) to 1.891(N. tabaccum, highest), translating to 1.081 – 1.891 folds. In both cases this gave ratios of 1:1.77 (lowest) and 1:1.75 (highest) indicating that the field strain larvae developed resistance to the extracts twice as much as the susceptible strain that is 2-fold resistance. It may be stated that the resistance of the field strain larvae could have been caused by the synthetic agicultural insecticides and pesticides applied to protect crops.

It was noted from Table 4.22 that the development of resistance in larvae from the 11 extracts of susceptible larvae and 13 extracts of the field strain larvae was inconsistent and gradual suggesting of presence of heterozygous population in response to these extracts. There was no marked difference in the resistance pattern to those resistance producing extracts in LC_{50} values where the two strains only produced resistance at a lower rate. However, the field strain larvae showed a significant reduction in resistance towards LC_{50} values particularly in the months of March, April, May and June 2010. This

was possibly because of heavy rains in these months which contributed to temephos dilution by runoff and thus lowering larvae resistance to extracts. While in the dry seasons when the agriculturally used chemicals were highly concentrated in waters and becoming magnified in the mosquito larvae or their parents this increased larvae resistance to the extracts. Increase in resistance possibly occurred in the dry months of January and February 2010.

The results in Table 4.20 revealed that the field strain larvae exhibited varying resistance ratio of extracts. The highest larvae resistance was recorded for *N. tabaccum* aqueous extract (5.560) in the month of April, followed by same extract at RR of 4.989 in the month of March. The lowest RR was observed in the Cc. ethanol (0.003) in the month of January 2010 followed by the same extract (RR) (0.015) in February, 2010. High RRs in most larvae were seen to occur between March and June 2010.

It can be argued that in the months of March, April and May there was heavy rains. The runoff flow which collected agricultural chemicals earlier applied to protect crops infiltrated into mosquito habitats. These chemicals (pesticides, fertilizers etc.), when applied persistently year after year, adult mosquitoes and the larvae are most likely to develop resistance of these chemicals. As some of the weaker insecticides such as the flower and leaf extracts of the plants under study were applied later to the larvae, the anticipated mortality could not be realized because of the already established resistance from prior applied superior pesticides. Thus, appearing as if the extracts were deprived the opportunity to effectively work. Comparatively, January and February were dry months in which agricultural chemicals were not in application and therefore the mosquitoes experienced no burden of chemical contact. Their exposure to agricultural chemicals to develop early resistance in them was nil and therefore this rendered them

susceptible to any applied insecticide and could be killed with applicaton of much less dose of an insecticide.

Table 4.20 shows variations of resistance ratio of extracts to the larvae. RR ratio seemed to be low in the first two months of the year and increased in March, April and May 2010. However, larvae also appeared to increase to increase their resistance ratio to the month of June 2010.

Table 4.20 Resistance Ratio (RR) of the Field strain larvae in the months of January – June 2010.

	Jan	**Feb**	**Mar**	**Apr**	**May**	**June**
Pyrethrum						
Ethanol	1.003	1.015	1.160	1.175	1.180	1.195
Methanol	1.018	1.045	1.145	1.185	1.194	1.198
DCM	1.092	1.084	1.144	1.165	1.172	1.175
Hexane	1.050	1.115	1.310	1.340	1.290	1.298
Ethylacetate	1.140	1.175	1.195	1.290	1.296	1.255
Aqueous	1.155	1.185	1.418	1.435	1.385	1.295
Eucalyptus						
Ethanol	1.080	1.145	1.278	1.165	3.200	3.155
Methanol	1.090	1.165	1.280	1.530	2.240	2.130
DCM	1.060	1.085	1.165	1.190	3.240	2.240
Hexane	1.085	1.080	1.288	1.462	4.122	1.320
Ethylacetate	1.069	1.162	1.380	1.395	2.285	2.265
Aqueous	1.108	1.110	1.318	1.395	3.226	2.460
Tobacco						
Ethanol	1.094	1.078	1.800	1.920	2.720	2.660
Methanol	1.096	1.140	1.298	2.295	2.345	3.136
DCM	1.055	1.075	1.295	2.220	2.270	2.255
Hexane	1.085	1.354	2.390	2.415	2.432	2.425
Ethylacetate	1.075	1.085	1.216	2.240	2.292	2.265
Aqueous	1.130	1.225	4.989	5.560	4.915	4.975

Table 4.21 shows RRs for the laboratory reared larval. Larvae succumbed to very high concentrations of the extracts in all months. Notably, the larvae were exterminated by the highest concentration of Ec Hexane (RR 0.140) in the month of February, 2010, followed by Cc DCM (RR 0.850) in the month of February 2010. The lowest recorded RRs were Ec Aqueous (RR 1.198 and 1.196), in the months of May and April, 2010.

Table 4.21 Resistance ratio (RR) of the laboratory reared larval in the months of January to June 2010.

	Jan	Feb	Mar	Apr	May	June
Pyrethrum						
Ethanol	0.885	0.888	0.896	0.890	0.945	0.942
Methanol	0.987	0.992	0.988	0.998	0.920	0.999
DCM	0.855	0.850	0.880	0.960	0.972	0.985
Hexane	1.144	1.070	1.043	1.046	1.020	1.049
Ethylacetate	0.966	0.968	0.978	0.980	0.988	0.985
Aqueous	1.153	1.160	1.160	1.250	1.146	1.145
Eucalyptus						
Ethanol	0.990	0.989	0.995	0.999	1.125	1.135
Methanol	0.892	0.898	0.856	0.905	0.984	0.998
DCM	0.886	0.868	0.894	0.920	0.955	0.986
Hexane	1.025	0.140	1.080	1.135	1.130	1.115
Ethylacetate	1.155	1.155	1.166	1.175	1.178	1.170
Aqueous	1.168	1.182	1.190	1.196	1.198	1.185
Tobacco						
Ethanol	0.955	0.960	0.982	0.992	0.998	0.900
Methanol	0.994	0.906	0.910	0.955	0.970	0.995
DCM	1.005	1.012	1.050	0.999	1.108	1.115
Hexane	1.085	1.035	1.149	1.151	1.162	1.155
Ethylacetate	1.168	1.055	1.165	1.175	1.175	1.171
Aqueous	1.188	1.105	1.085	1.175	1.156	1.140

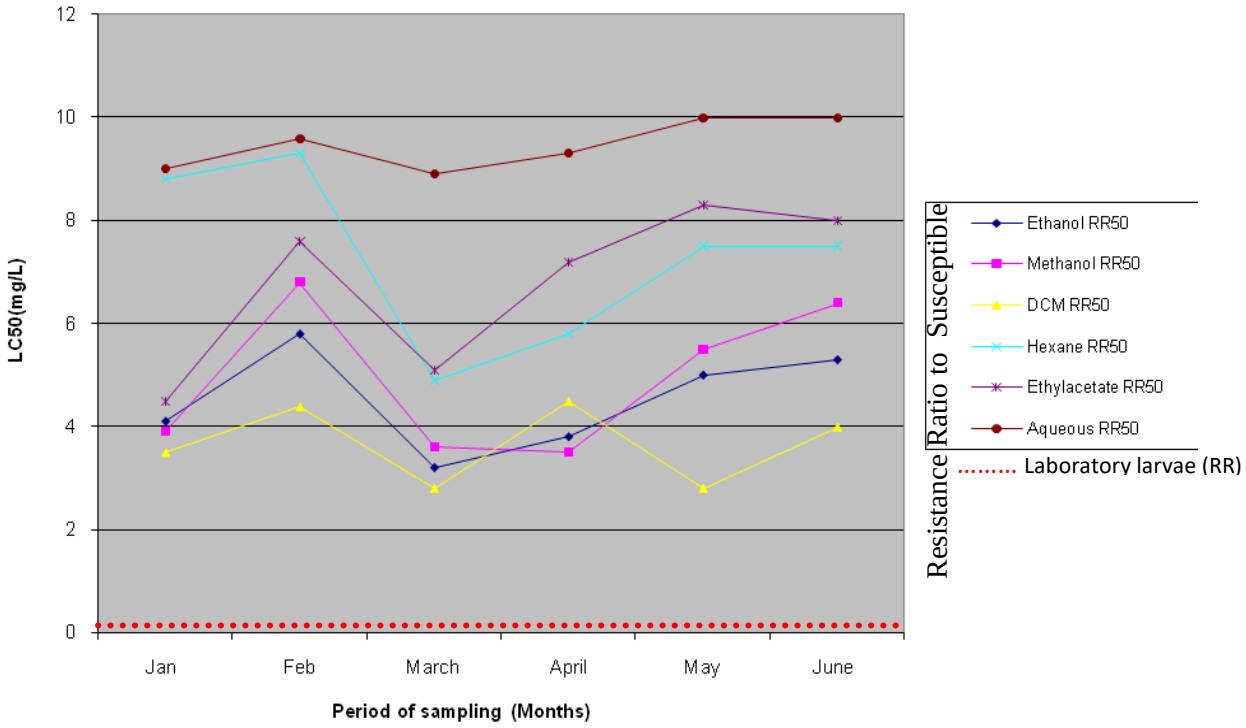

Fig 4.5 *C. cinerariifolium* crude leaf extracts resistance ratio – January to June 2010.

When the six extracts are traced up-down in Figure 4.5 their resistance ratio highest to lowest follow in the order of aqueous, ethyl acetate, ethanol, methanol, hexane and DCM. Aqueous extract showed the highest resistance ratio at 1.714 to the field strain larvae and achieving 60% larval mortality compared to 84% mortality for the individual extract test. DCM extract had the least RR or to say larvae were susceptible to it. It established a RR of 1.004 with larval mortality of 98.85% compared to 100% of individual extract test. Other extracts showed remarkable RRs: ethyl 1.066, mortality 68%; ethanol 1.008, mortality 98.5%; and methanol 1.180, mortality 80%. RRs appeared to be elevated in the months of March, April, May and in the month of June for methanol (1.160), hexane (1.200), and aqueous (1.550). The reasons for these high RRs in these months are described in paragraph 1 above.

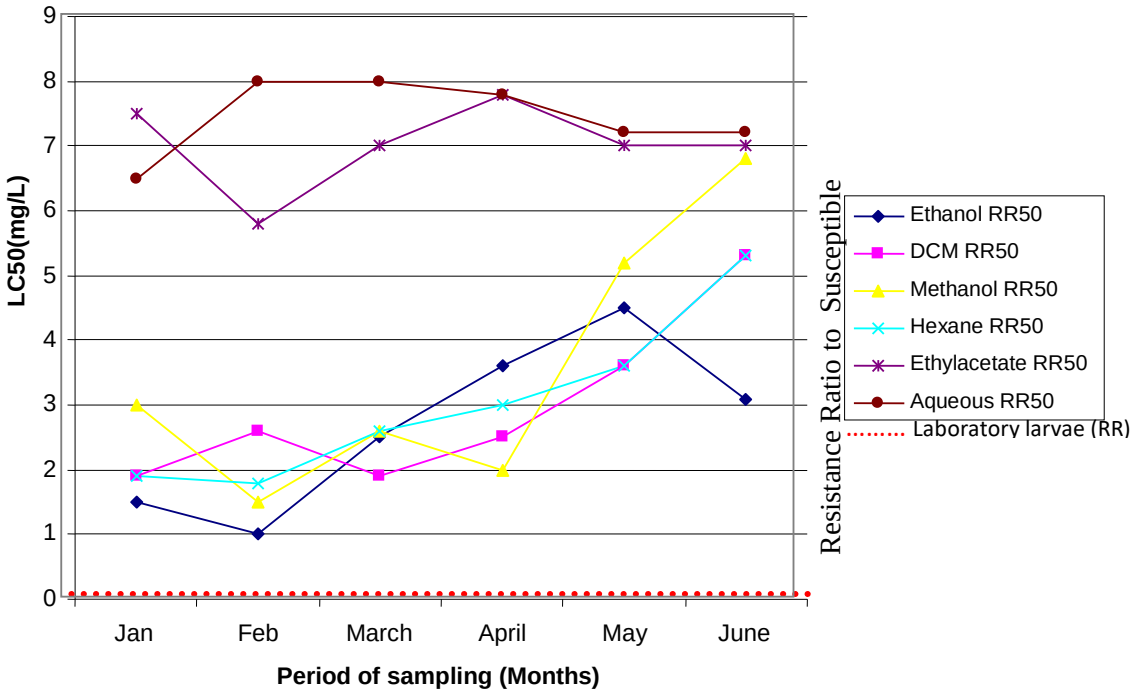

Fig 4.6. *E. camaldulensis* crude leaf extracts resistance ratio – January to June 2010

In the determination of RR of the larvae to *E. camaldulensis* extracts, aqueous and ethyl acetate extracts indicated remarkable effect as they resulted to high RRs of 1.823 and 1.195 respectively and achieving mortality of 64 and 62% respectively against mortality of 80% each when they were tested individually. The two extracts have a clear mark drawn between them and other four extracts (DCM , ethanol, hexane,and methanol) which claster together in view of their very low RR (0.993,0.998, 1.005 and 1.014 respectively). Their respective mortality were DCM 95% (compared to individual extract mortality of 100%), ethanol 90% (individual mortality 98%), hexane 72% (96%), and methanol 93% (100%). Apparently, of the six extracts methanol showed steady larval mortality and RR throughout the six months period. There were sharp drops for both mortality and RR in the months of January and February and March/mid-April. But from mid-April to June 2010 the larvae developed a higher RR of methanol reaching the peak of 1.014 in the month of June. Aqueous exract is the only one that constantly indicated high RR from the months of February 2010 thoughout to June 2010. Unlike other

extracts, aqueous showed high RR in February most likely for the reason that the larvae sampled that time had no prior exposure to any temephos.

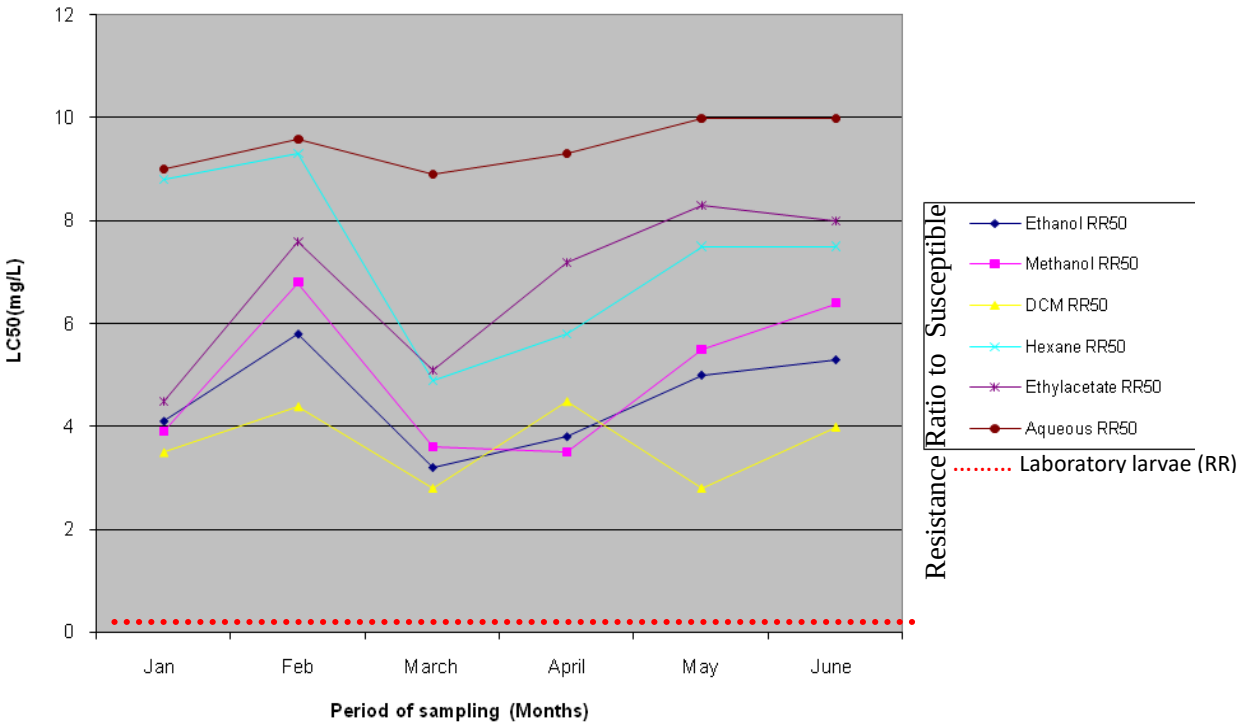

Fig 4.7 *N. tabaccum* crude leaf extracts resistance ratio (RR) – January to June 2010

In Figure 4.7 the aqueous extract showed the highest RR (1.891) followed by ethyl acetate (1.655), hexane (1.561), methanol (1.326), ethanol (1.288), and DCM (1.115). The aqueous extract maintained high RR to the larvae for the 6 months of the year without any sign of decline. Ethyl acetate and hexane induced a moderately high RR to the larvae although hexane which had very high RR to the larvae in January and February, 2010 seemed to drastically decline to exerting low RR to the larvae in March and April 2010. This sudden drop of the extract resistance to the field strain of larvae was possibly as a result of high rainfall that diluted the temephos applied in agriculture or insecticides applied in households for mosquito control. In May and June 2010 the larvae RR from ethyl acetate rose to a reasonably high level which may have been caused by

the larvae prior resistance adjustment to the temephos in the field. The larvae did not develop resistance against methanol, ethanol, and DCM as these extracts seemed to influence low RR especially between the months of March and June, 2010.

4.12 Crude leaf extracts persistence.

Persistence refers to biodegradation. The period of time the extracts will took to completely disappear from the solution.

In this study three plant extracts were tested for their persistence under light-dark regimes. For the light regimes, a bulb of 120W was provided to supply light during the study period. The need for the use of this bulb was to maintain uniform lighting of good intensity able to see throught the test periods as opposed to natural lighting which could vary at times. Dark regimes were prevailed by use of black canvass or black sheets sealing light from the windows. Experiments for the two regimes were performed separately ensuring 12:12 (dark:light) periods were maintained.

The highest individual extract concentration of each plant that exhibited 100% larval mortality was selected for use in these tests. These concentrations were: C. cinerariifolium DCM (164.86 ppm), *E.camaldulensis* DCM (168.65 ppm) and *N.tabaccum* ethanol (189.58 ppm). The essence of these tests was to test for the biodegradation of these concentrations with time from these levels to zero level. Zero level was translated as the point at which there was no trace of concentration in the solution. Intervals of samples taking for concentration decline determination by Gas Chromatography-Mass-Spectrometry (GC/MS)-(GC/MS-QP2020, of Perkin Elmer, UK) method. Results were recorded as shown in Table 4.22

Twenty five third instar larvae were emmersed separately in the three test concentrations each mixed with 300 ml distilled water in a holding bowl. Water/test concentrations holding larvae was sampled as described in the methodology.

Table 22: Percent recovered oils in the degradation of *C. cinerariifolium, E.camaldulensis and N.tabaccum*

Hrs	**Pyr**	**Euc**	**N.t**	**Pyr**	**Euc**	**N.t**	**Pyr**	**Euc**	**N.t**
0	164.6	144.45		151.50-182.70	132.4-157.2	132.4-155.3		y=10.64x+35.50	Y=13.88x+9.35
30	124.6			113.25-132.55				y=9.40x+17.80	
1	100.00			62.40-97.35				y=8.75x+20.75	
1.30	95.00			29.50-53.70				y=11.40x+12.15	
2.00	90.00	4.00	12.50	11.80-33.55				y=12.70x+21.70	
2.30	50.00	4.50	6.60	0-00					
3.0	25.00	5.00	1.50						
3.30	20.50	5.30	0.00						
4.00									
4.30									
5.00									
Days									
2		75.00	117.30		62.45-89.70	98.5-129.3		y=13.25x+9.70	y=7.85x+16.20
3		62.50	112.45		51.70-80.50	82.6-127.4		y=8.25x+9.40	y=6.35x+10.25
4		50.00	100.00		37.65-61.70	90.40-114.0		y=16.7x+9.25	y=10.70x+13.15
5		46.70	98.70		30.45-	81.7-113.7		y=7.45x+8.00	y=9.00x+11.55

					58.20				
6		25.45	96.40		16.28-34.70	79.8-111.5		y=11.85x+9.40	y=4.78x+8.15
7		25.45	85.25		13.60-32.80	69.6-100.4		y=6.40x+12.80	y=5.55x+10.20
8		25.00	76.80		13.45-32.50	60.2-88.7		y=7.70x+14.80	y=3.87x+9.10
9		25.00	75.00		13.45-32.50	69.87.9		y=8.80x+9.40	y=7.00x+10.85
10		12.50	62.50		3.55-19.70	51.3-76.5		y=16.25x+10.50	y=9.25x+3.98
11		6.25	54.00		1.12-4.80	36.7-63.8		y=5.20x+41.60	y=17.20x+10.16
12		1.00	52.00			32.8-60.7		y=11.70x+8.85	y=2.58x+6.25
13		0.00	50.00			31.7-59.2		y=27.50x+11.50	y=3.95x+4.80
14			48.00			37.5-56.8		y=21.50x+9.95	y=6.15x+7.15
15			46.30			35.3-54.6		y=10.75x+6.25	y=8.15x+5.55
16			43.40			33.8-52.9		y=9.85x+7.95	y=3.95x+5.25
17			25.00			12.7-32.6		y=17.80x+12.70	y=9.50x+16.50
18			24.00			12.3-32.1		y=14.75x+13.55	y=5.15x+21.85
19			19.40			10.5-28.7		y=18.25x+12.85	y=4.90x+8.65
20			10.27			1.75-17.5		y=5.45x+15.99	y=8.15x+13.25
21			0.00			0.00			
	Control		0.00						

The results of the three plants indicated that under light regime *C. Cinerariifolium* took 5 hours and 30 minutes to completely decompose and 27 hours to decompose under darkness. *E. camaldulensis* decomposition under light regime was 12 days and that of dark regime was 35 days. The dark-light degradation periods for *N. tabaccum* were 18 days of light and 28 days of darkness.

Figure 4.8 shows a comparison of the three plant extracts tested under light regime. *Chrysanthemum cinarerііfolium* had the shortest time of biodegradation at 5 hours 30 minutes. *Eucalyptus camaldulensis* got exhausted on a biodegradation period of 12 days while *Nicotiana tabaccum* showed the longest biodegration period of 21days. Consequently, *C. cinarirariifolium* flower extract was superior to the leaf extracts of E. *camaldulensis* and *N. tabaccum.* The period of the extracts biodegradation under light (solar energy) regimes is an important factor in the application of insecticide for the control of mosquitoes in the field.

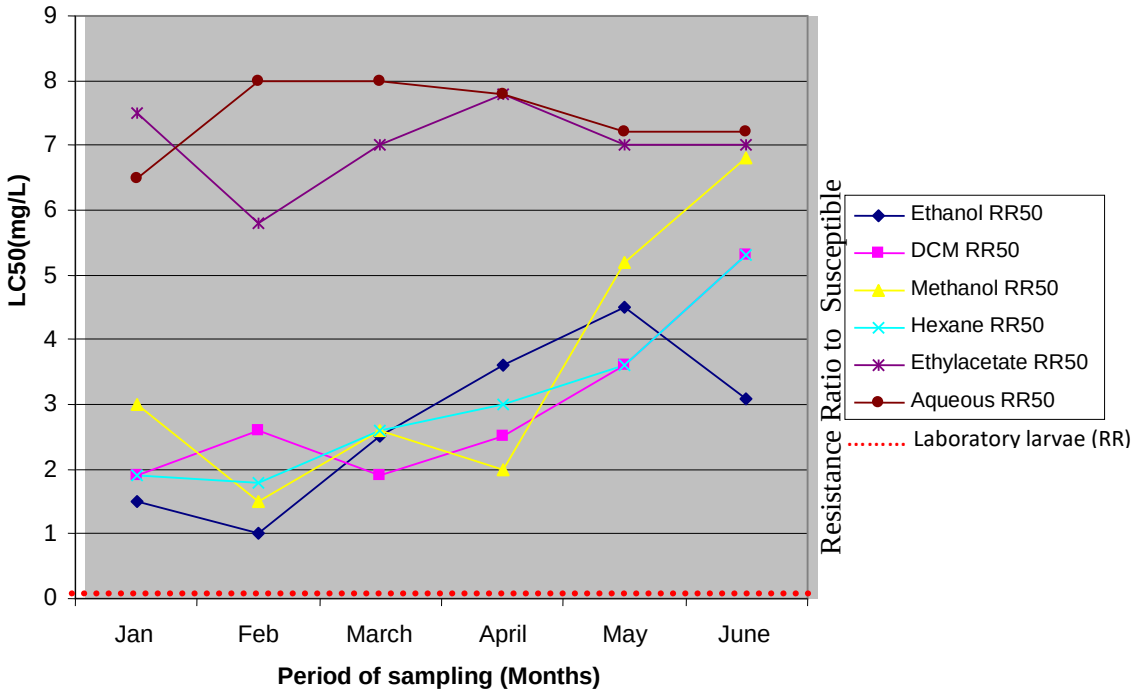

Fig 4.8. *E. camaldulensis* crude leaf extracts resistance ratio – January to June 2010

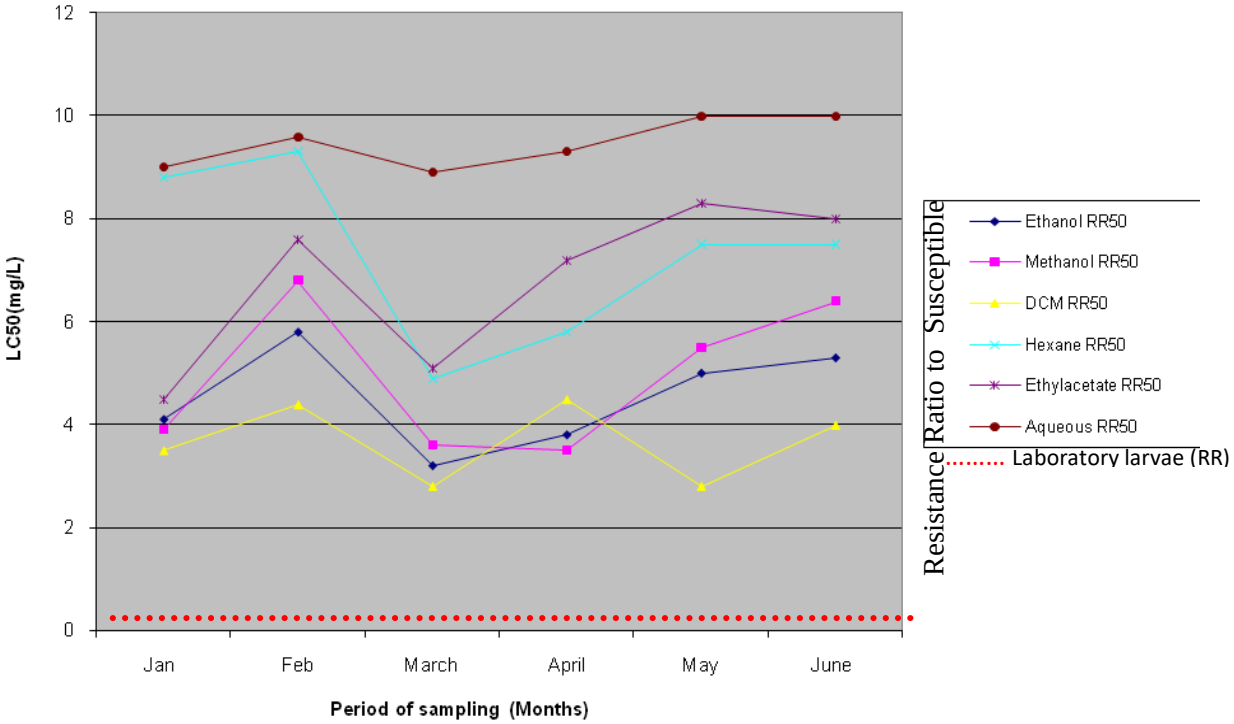

Fig 4.9. *N. tabaccum* crude leaf extracts resistance ratio – January to June 2010

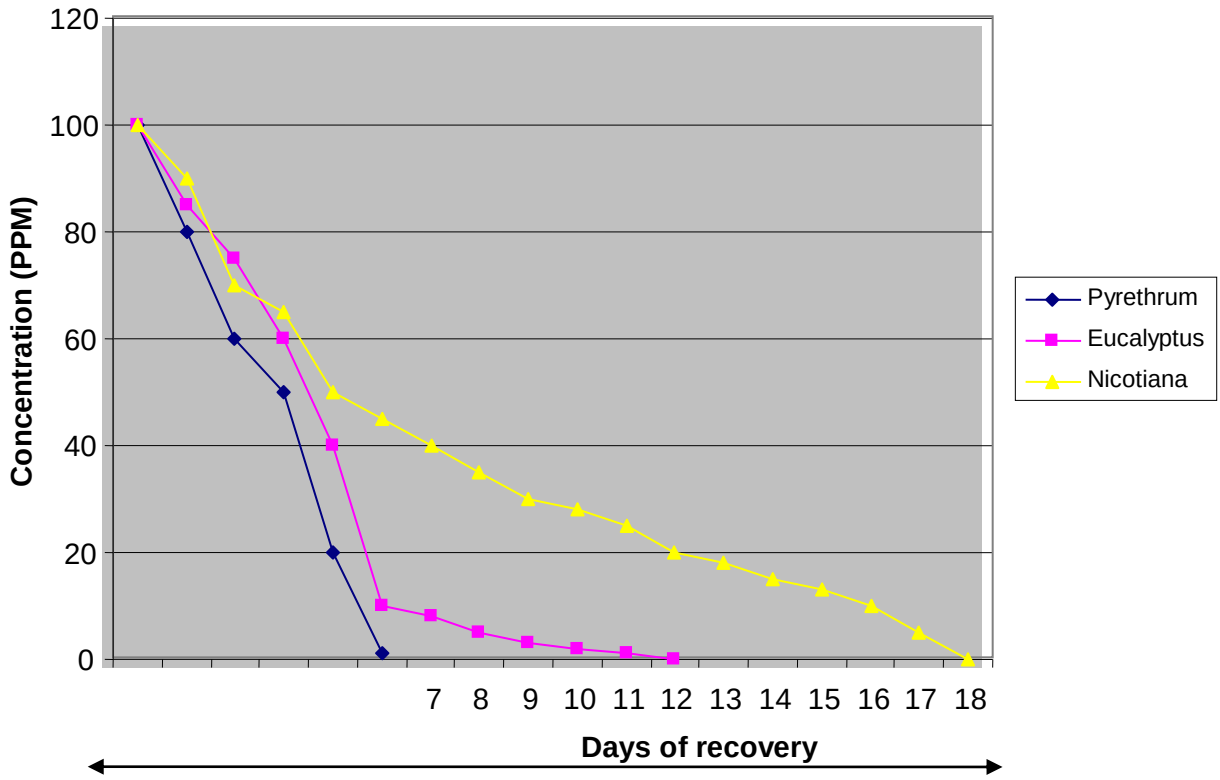

Fig 4.10 Persistance of *C. cinerariifolium*, *E. camaldulensis*, and *N. tabaccum* essential oils under light regimes.

In Figure 4.11 *Chrysanthemum cinerariifolium* indicated the shortest period of clearing from the environment (5hours 30minutes). *Nicotiana tabaccum* under light regime decomposed in 18 days and *Eucalyptus camaldulensis took* 12 days.

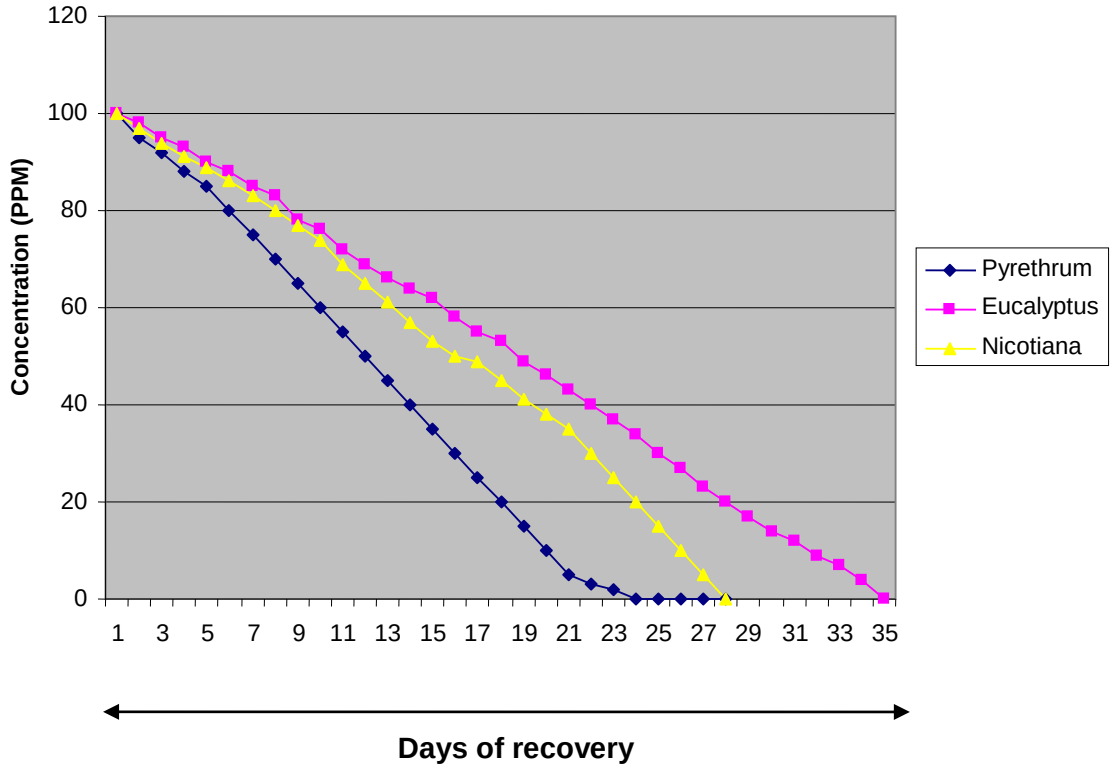

Fig 4.11 Persistance of *Chrysanthemum cinerariifolium*, *Eucalyptus camaldulensis*, and *Nicotiana tabaccum* essential oils under dark regimes.

All the extracts indicated a long period of decomposition under dark regimes.

However, among the three plants, *Chrysanthemum cinerariifolium* indicated the shortest period of clearing off from the environment in 28 days. *Nicotiana tabaccum* was at bar with *Chrysanthemum cinerariifolium* in biodegrading for 28 days, while *Eucalyptus camaldulensis* biodegraded in the longest time of 35 days.

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS.

5.1 Rearing of *Anopheles gambiae* Gilesmosquito in the laboratory.

The rearing of *Anopheles gambiae* **Giles** mosquito in the laboratory succeeded. Mating took place and eggs were oviposited and concequently larvae hatched. However, there was observed occurance of egg and larvae population decline. The eggs oviposited were below the expected number of eggs as it is cited that an adult female *A. gambiae s.s.* mosquito lays 50 – 200 eggs per oviposition (CDC 2015 in https://www.cdc.gov/malaria/about/biology/mosquitoes/ 2015). Other authorities state that adultlt *A. gambiae* mosquito lays 30 to 150 eggs every 2 to 3 days (www.malariasite.com/anopheles-mosquito/2008). In this syudy the egg oviposition ranged between 65 and 102 eggs per adult female *A. gambiae* mosquito. The shortfall could not be justified, however, in the past studies Hitchcock (1968) attributed mosquito egg laying shortfall due to the ability of female mosquitoes to retain some mature eggs and is influenced by some physical and chemical factors (Dhileepan 1997) including visual olfactory and ductile responces (Bentley and Day 1989). Further, is case of oviposition behaviour interrupted by oviposition repellents, or the immediate lack of suitable aquatic site (large medium) for egg laying. In this case the gravid female would be forced to retain mature eggs (Bentley and Day 1997) . The contribution by Zeev and Tamar (1968) supported that insect repellent DEET (N, N-diethyl-3-methylbenzamide) provided repellency to ovipositing Aedes *aegypti* for about 2 weeks. Xue *et al*. (2001a) fo und that DEET at 0.1% rate of application could deter oviposition of *Aedes albopictus* Skuse in containers for 2 weeks. It is important for a future investigation done to review the related insecticides to DEET used in Langas area either for agricultural purposes or for the control of mosquitoes that may cause repellence to egg oviposition to *A. gambiae s.s.***Giles.**

Other factors for the egg decline have been stated by the past workers and include missed mating (Downes, 1968); suitability of the habitat for egg laying, potential fitness of the

progeny i.e. progeny of mixed marriages in the mosquito population, site characteristics including olfactory cues and light-dark cycle (Agyapong *et al.* (2014) Sumba *et al.,* 2004); and canabalism to eggs and larvae (MR4, 2014).

According to these authors mosquitoes locate themselves in space and time to ensure they are available to mate. The interplay of time of initiation, mark,er type and height in swarm formation reduce the probability of intraspecific mating. Probably in the case of this study during adult culture the male mosquitoes did not locate themselves in totality in space to mate and time available (dusk) may have been short. In addition other factors would have contributed such as possible marker type.

Early authors have reported various common infections in mosquito colonies particularly larvae. Viruses (MIV, *Parvoviridae*, and Mosquito Densovirus (MDV) were singled out by Dodson and Rasgon (2017) and Benelli *et al.* (2016) while bacteria and intestinal infections (*E. coli*, *Pseudomonas fluorescens, Leptothrix buccalis Lankesteria culicis, Vavraia spp (Plistophora*), Ascarids; and *Amblyosporidae* family which included *Amblyospora* and *Parathelohania* were singled out, Parratt *et al.* (2016) Jenkins (1964) and Andreadis (1999) respectively. The Fungi (*Coelomomyces spp;* and *Leptolegmia spp, Entomophthora spp*) infections were reported by Saliba *et al.* (2016) Kramer (1964) and George *et al.* (2013) Scholte *et al.* (2004) respectively Patil *et al.* (2016) Larson (1967) and Patil *et al.* (2015) Schober (1967) observed *articella spp* in mosquitoes while *Vorticella spp;* and Nematodes (Mermithids) were a subject of Amstrong and Bransby-Williams (1961), Patil *et sal.* (2016) Hati and Ghosh (1961), and Kabyhrski et al. (2012) Kalucy (1972) respectively. It is possible that in this study the eggs oviposited and the larvae population hatched out could have encountered some of these predators and disease causing pathogens to reduce their numbers.

5.2 Independent crude extract activities to establish LC_{50} and LC_{90} of the extracts.

Each crude extract of *C. cinerariifolium*, *Eucalyptus camaldulensis* and *Nicotiana tabaccum*

was tested for its larvicidal activity for the determination of the median lethal concentration (LC_{50} and LC_{90}) to kill 50% and 90% of the exposed third instar larvae of *Anopheles gambiae s.s.* **Giles**. The results indicated that all the three crude plantflower and leaf extracts had the potential to kill the mosquito larvae since the extracts showed significant larvicidal activity at P = < 0.05 level significance.

However, DCM flower extract of *C. cinerariifolium (Cc)* and DCM leaf extract of *Eucalyptus camaldulensis (Ec)* exhibited the highest activity of LC_{50} 164.86 ppm, LC_{90} 255.17 ppm and LC_{50} 168.65 ppm, LC_{90} 315.85 ppm respectively thereby exbiting 100% larval mortality. Other extracts that achieved 100% larval mortality were ethanol of *Cc* (LC_{50} 187.78 ppm, LC_{90} 268.26 ppm), methanol of *Ec* (LC_{50} 197.46 ppm, LC_{90} 329.68 ppm) and ethanol of *Niciotiana tabaccum (Nt)* (LC_{50} 189.58 ppm, LC_{90} 320.75 ppmn).

The four extracts that as well showed good results were methanol of Cc (LC_{50} 225.45 ppm, 98% larval mortality; ethanol of Ec (LC_{50} 210.15 ppm, LC_{90} 335.58 ppm, 96%); hexane of Ec (LC_{50} 198.56 ppm, LC_{90} 338.35 ppm, 96%); methanol of Nt (LC_{50} 224.35 ppm, LC_{90} 332.75 ppm, 96%); and ethyl acetate of Nt (LC_{50} 201.52 ppm, LC_{90} 332.84 ppm, 96% larval mortality). The last extract in the nineties percent mortality was ethyl acetate of Cc (LC_{50} 227.56 ppm, LC_{90} 347.38 ppm, 92% larval mortality).

The last extracts that exhibited eighties percent in larval mortality were DCM of Nt (LC_{50} 229.72 ppm, LC_{90} 342.64 ppm, 88% larval mortality); hexane and aqueous of Nt LC_{50} 235.85 ppm, LC_{90} 314.70 ppm and LC_{50} 258.42 ppm LC_{50} LC_{90} 393.36 ppm respectively larval mortality of 84% each); and ethyl acetate of Ec (LC_{50} 260.46 ppm, LC_{90} 347.38 ppm, 80% larval mortality.

There is no past research work documented on toxicity of *C. cinerariifolium* on third instar larvae of *A. gambiae s.s.* **Giles** mosquito. But some work has been done on *E.camaldulensis* and other *Eucalyptus* species and are well documented although not specifically on *A. gambiae* larvae. It is believed that results of this study will serve good reference material for future researchers. The research work done by the following authors is appreciated: Elsiddig (2011) on *E. camaldulensis* crude leaf extract against *A. arabiensis* **Patton,** a member of *A. gambiae* complex. The author found that the mortality of the larvae upon the administration of hexane leaf extract of this plant ranged from 82% to 100% and he attributed this efficacy to cinoel (*eucalyptol*) oil contained in the plant as a major component. In this study, hexane of *Ec* achieved 96% larval mortality and therefore can be concluded that the results concur with those recorded by Elsiddig.

Further, Elsiddig results were in agreement with those of Corbet (1950) and Reekie (2005), however, with these two authors stating that the initial level of the cinoel oil was greater than the commercial larvicide.

Barbosa *et al.* (2016) Sen-Sung Cheng *et al.* (2009) studied on leaf extracts of *E. camaldulensis* and *E. urophylla* on *Aedes aegypti* larvae and both plants induced 100% mortality of the larvae at 200µg/ml after 24 hours exposure. They attributed this high mortality to the major compounds in these leaf extracts i.e. χ-terpinyl acetate (14.87%), p-cymene (21.69%) for *E. camaldulensis* and 1-8-cineole 58.34%, χ-terpinylacetate (14.87%) for *E. urophylla*. Similarly, Bilal *et al.* (2012) and Guerrero *et al.* (2015) carried out a study on *E.camaldulensis* on *Ae. Albopictus* larvae and a concentration of 78.00 ppm achieved 100% larval mortality while Wada and Singh (2014) searched on *E. camaldulensis* leaf extract against *Culex quinquefasciatus* mosquito larvae also achieving 100% larval mortality.

Many other *E. camaldulensis* sister species plants have also been studied for their efficacy in mosquito larvae and only a few can be cited here: *E. citriodora* against 3rd instar larvae of *Ae. Aegypti* larvae, Uthayarasa *et al.* (2010); *E. citriodora* leaf extracts upon *A. stephensi* Liston, Pankaj and Anita (2014); *Eucalyptus* species Karthikeyan *et al.* (2012); *E. globules* on *A. stephensi* and *Ae. Aegypti* larvae Nair *et al.* (2014); *E. citriodora* against *Culex quinquefasciatus* Navendhiran *et al.* (2014); *E. globules* leaf extract on *Culex pipiens* Ghoshi *et al.*(2012); and *E. citriodora* plant essential oil against *A. gambiaes.s.* **Giles** Bosou *et al*., (2013).

Both Navendhiran and Ghoshi observed that the essential oil of *E. citriodora* is rich in citronellal, citronellol and opulegol which were revealed to be toxic to larvae. As it can be observed from the cited researchers toxicity of a crude leaf extract depends on the major components which similarly in this study had influence in high larval mortality achieved. This is in confirmation to the high mortality of 100% induced by five extracts of this study, including those which larval mortality ranged from 96 to 98%. However, those extracts in this study that exhibited between 80 to 92% larval mortality can be said to have had no major compounds to cause 100% larval mortality or if the major comounds were there they were weak.

In this study aqueous extracts of all the plants indicated low larval mortality *(Cc* 84%, *Ec* 80% and *Nt* 84%. This low exhibition of larval mortality was an indication that water as a solvent did not dissolve all major acting compounds from the flower and leaf extracts of the plants. This compares well with Ileke *et al*. (2015) and Azokou *et al*. (2013) Rahuman *et al*. (2009) studies where aqueous extracts showed low larval mortality and the results of Ileke et al. (2015) Olofintoye *et al*. (2011) in the study of aqueous extracts of *Datura stramonium* – Jimson Weed and *N. tabaccum* on *Anopheles* and *Culex* mosquitoes showed low larval mortality and hence supports this present work.

5.3 Synergistic activities of the crude extracts when they acted in combination.

Synergism means toxic effect of two is much greater or worse than one alone. It is clear from the results that *Eucalyptus camaldulensis* possess larvicidal effect against *A. gambiae s.s.* **Giles.**

The combinations of DCM *C. cinerariifolium* with other crude extract solvents Simwas found to be the most effective with lethal values of 100% for larval exposure of 24 hours. Wada and Singh (2014) made a screening and the crude extract showed that *E. camaldulensis* contain large amount of 1,8- cineole, β-pinene, y-terpinene as the major component in leaves. In the present study synergism was tested and a total of fifteen synergistic activities and their synergists were identified (Table 4.21). Out of these top five highest synergistic activities were singled out. These were in their order: *C. cinerariifolium* DCM extract +*Eucalyptus camaldulensis* methanol extrac; *C. cinerariifolium* DCM extract + *Eucalyptuscamaldulensis* ethyl acetate extract; *C. cinerariifolium* DCM extract + *Eucalyptus camaldulensis* hexane extract; *C. cinerariifolium* DCM extract + *Nicotina tabaccum* hexane extract and *Eucalyptus camaldulensis* DCM extrat + *Nicotiana tabaccum* hexane extract.

Although these five concentrations appeared merging from low to high and exhibited 100% larval mortality, there was one possibility to explain this. This was by the fact that each extract had toxic compounds which when combined became more toxic and achieved total larvae mortality in their smaller concentrations. These concentrations were apparently reduced to much lower than the individual extracts concentrations and similarly to those of the combining extracts. From the same table it was observed that DCM extracts of the three plants dominated in synergistic activities by appearing as synergists ten times compared to other extracts such as methanol, hexane and aqueous extracts of which each appeared six times as synergists. While this was the case, ethanol

and ethyl acetate each appeared four times. Simply, then it can be concluded that DCM extact was the best synergist, followed by three extracts i.e. methanol, hexane and aqueous and finally two extracts, ethanol and ethyl acetate.

From the results obtained, four lessons were learnt:- (i) Two highest combining extracts e.g. Cc DCM (164.86 ppm) + Ec hexane (168.65 ppm) still yielded to the highest concentrations (127.85 ppm) for the mortality of the larvae. The resultant 127.85 ppm described as highest concentration was better than the two combining extracts; (ii) The synergistic result formed by two low concentrations e.g. Cc methanol (224.45 ppm) + Ec aqueous (259.58 ppm) still yielded to low concentrations (201.55 ppm), much closer to each of the two combining extracts. The resultant 201.55 ppm described as low concentration was just slightly better or no better than the concentrations of the two combining concentrations; (iii) When a high concentration was combined with a low concentration e.g. Cc DCM (164.86 ppm) + Ec ethyl acetate (260.56 ppm), a high concentration 121.45 ppm resulted for the mortality of the larvae. The resultant 121.45 ppm described as high was a creation of the low Ec ethyl acetate which improved on the high concentration of Cc DCM and (iv) Synergistic combinations yield to smaller concentrations and therefore this would be economical. Smaller concentrations when used appropriately will distergrate from the place of application faster than large doses which will tend to persist in the environment. In this view, persistence of chemical insecticides for example, will render the untargeted organisms likely to die.

The three pairs which were neither synergestic nor antagonistic and these were: methanol of *Chrysanthemum cinerariifolim*in combination with ethyl acetate of *Eucalpytus camaldulensi,* Ethanol of *Eucalyptus camaldulensis*in combination with Aqueous of *Nicotiana tabaccum* and Methanol of *Eucalyptus camaldulensis*in combination with

hexane of *Nicotiana tabaccum.* These extracts may not be useful when synergism is in demand for use in the control of mosquitoes.

Consider extracts combination of methanol of *C. cinerariifolim* and ethyl acetate of *N. tabaccum*in column 1. Methanol of *C. cinerariifolim* achieved 98% mortality of LC_{50} by 224.45ppm. When it combined with ethyl acetate of *N. tabaccum*, it exhibited 96% mortality of LC_{50} by 224.35ppm. In this combination, the two extracts exhibited 50% larval mortality at 232.66ppm. This translates that ethyl acetate at 224.35 ppm was higher than methanol at 224.45 ppm and the resultant of these two is 232.66ppm meaning that methanol of *C. cinerariifolim* lowered the effect of ethyl acetate of *N. tabaccum* by 3.7%. Again consider combination of DCM of *C. cinerariifolim* and methanol of *N. tabaccum* in column 4. DCM extract of *C. cinerariifolium* at 164.86 ppm exhibited 100% larval mortality and methanol of *Nicotiana tabaccum* caused 96% larval mortality at 224.35ppm. In their combination 85.7% larval mortality resulted from the application of 225.80 ppm. In this combination, DCM at 164.86 ppm was higher than methanol at 224.35 ppm and their resultant concentration of 225.80 ppm indicated that DCM of *C. cinerariifolim* was lowered from 164.86 ppm to 225.80 ppm and hence methanol of *N. tabaccum* was an antagonist.

Documented evidence on synergism tests based on the plants of this study is limited. Despite this the very few documented are mainly outside the plants of study and may not form good comparison.

5.4 Antagonistic activities of the crude extracts when they acted in combination.

Antagonistic is the opposite of synergistic i.e. opposing, when it is combined. The best that will be expected of antagonism is reduced performance. In this study there were twelve antagonistic activities. The highest to lowest crude leaf extract combination concentrations occurred between 0.10 ppm *C. cinerariifoliumC. cinerariifolium*

(methanol extract -224.45 ppm; *N. tabaccum* ethyl acetate extract – 224.35 ppm) and 59.70 ppm (*E. camaldulensis* DCM extract – 164.65 ppm; *N. tabaccum* ethyl acetate extract – 260.56 ppm).

The most important principle observed in antagonism was that a high concentration (indicated by less ppm) was converted to a weaker concentration (indicated by more ppm). The more the concentration applied the more it indicated the extract's weakness or it also indicated mosquito resistance to the extract. It was observed that every antagonistic extract rendered the corresponding combining extract weaker such that the concentration could not achieve 100% larval mortality. From the combinations, it was observed that crude flower and leaf extracts with low (more ppm) concentrations were easily antagonized (Table 4.21 serials No. 1, 3, 4, 5, 6, 7, 9, and 10). However, for reasons not known, two extracts with high concentrations (shown by less concentrations) namely both DCM and methanol of *E. camaldulensis* (164.65 ppm and 197.46 ppm, respectively) were lowered to 211.55 ppm and 219.45 ppm respectively. It was also observed that four of *N. tabaccum* extracts (aqueous , ethyl acetate, hexane, methanol), acted as antagonists in various combinations while two of *E. camaldulensis* (ethyl acetate and hexane) and one extract of *C. cinerariifolium* (methanol) as well acted as antagonists. Extracts of *N. tabaccum* therefore dominated in rendering other plants extracts inactive with an exception of ethanol and DCM.

As the activities of the antagonized extracts continued to be lowered, larval mortality percent also reduced (Table 4.21). Therefore a decrease in larval mortality became an indicator of antagonism. For example, individual extracts of E. camaldulensis methanol and DCM had achieved 100% larval mortality but when ethyl acetate of N. tabaccum combined with these two extracts larval mortality declined to 91% and 89% respectively. Methanol of N. tabaccum reduced larval mortality of DCM *C. cinerariifolium* from 100%

to 90%. Even in the lower larval mortality cases the individual extracts which attained average larval mortality were seen to tremendously running low in larvae mortality. This is in reference to extract hexane of E. camaldulensis which antagonized ethyl acetate of *E. camaldulensis* to have mortality decreased from 80% to 68% and ethyl acetate of *E. camaldulensis* acting against hexane of *N. tabaccum* pushind down larval mortality from 84% to 60%.

Two important issues were observed and recorded in the combinations: i) Crude leaf extract with low concentration in combination reduced the crude leaf extract with higher concentration. The resultant effect was that the concentration of the two combining extracts was lowered below the concentration of each of the combing extract. This trend would complicate the efforts for the malaria vector control particularly when the accompanying antagonism in biopesticides is not well understood ; ii) Amongst the combinations four different solvents of *N. tabaccum* i.e. ethyl acetate, methanol, aqueous and hexane appeared commonly and overwhelming other solvents which simply implies that the resultant solvent extracts had a major role in antagonism. Table4.19, 4.20, and 4.21 indicate those activities that were antagonistic, the actual antagonizing crude leaf extracts and a list of antagonists respectively.

Following this description, it was thus concluded that a majority of *N. tabaccum* extracts were antagonists with an exception of It was notable, therefore, that it will be important to avoid the use of *N. tabaccum* extracts in combination with other extracts in mosquito control in the field. There is much need for further investigation to identify which compounds in the antagonistic extracts that induce antagonism in other extracts.

5.5 Resistance ratio (RR) of the larvae to the extracts

Resistance is defined as the acquired ability of an insect population to tolerate doses of insecticide which can kill the majority of individuals in a normal population of the same

species (WHO, 1977). Under this subject two strains of *An. gambiae* **Giles** mosquito were tested. One was the laboratory reared strain (susceptible strain) and the other was field strain (larvae collected from stagnating water pools – wetlands in Langas area of Eldoret municipality). The results of the two larvae were then compared WHO (2013) (WHO, 2005) and as adapted by Rocha *et al*. (2015), Grisales *et al*. (2013) Mulyatno *et al*, (2012) and Johan and Shahid (2012).

Both strains were subjected to the diagnostic dose of the crude flower and leaf extracts of the three plants. The results indicated that all the laboratory susceptible strain showed complete larval mortality (100%) when subjected to test concentrations. The larvae showed no resistance and the RR ranged from 0.885 lowest (Cc ethanol January 2010) to 1.070 highest (Ec aqueous March 2010). Considering the field strain RR varied from 1.009 lowest (Cc ethanol January 2010) to 1.560 highest (Nt aqueous in March 2010). The field strain was susceptible to low concentrations of the crude leaf extracts. *N. tabaccum* DCM crude leaf extract showed RR varying from 1.003 to 1.891. Our results indicate a rather strong resistance to *N. tabaccum* aqueous extract (1.981), *E.camaldulensis* aqueous extract (1.823) and *N. tabaccum* hexane extract (1.561). A weak resistance was portrayed by *C. cinerariifolium* ethanol flower extract (1.003) , *E. camaldulensis* hexane leaf extract (1.005), *C. cinerariifolium* ethyl acetate flower extract (1.066), *N. tabaccum* DCM leaf extract (1.039) , and *N. tabaccum* methanol leaf extract (1.081). The crude leaf extracts which exhibited no resistance to the laboratory reared larvae strain and which susceptibility to the strain were *C. cinerariifolium*DCM (0.996), *E.camaldulensis* methanol (0.998), *E. camaldulensis* DCM (0.993) and *N. tabaccum* ethanol crude leaf extract (0.999).

Results indicated presence of cross-resistance among the field strain in 24 hours post-recovery period. Probably this was due to the selection of a certain insecticide of one or

more genes which would generally extend to other compounds that share either a metabolic pathway or a target site (Subbiah *et al.* (2009) Wirth *et al*., (2000). More so one obvious reason for this is that different groups of genes can be selected with one insecticide (Hitayati *et al*. 2011; Karunamoorthi & Sabesani, 2012). The field strain on the other hand has a high probability of previous exposure to temephos and may therefore be expected to exhibit higher tolerance for temephos. Variation in the resistance was seen to occur in crude leaf extracts administered and may be this could be contributed by heterozygous genes in the populationwhich caused quick dilution of resistant genotypes resulting in the decline of resistance level Low *et al.* (2013) (Selvi *et al.,* 2005). Among other common factors enhancing resistance are impacts from pyrethroids used in household insecticides, fogging for mosquito control and agricultural practices (Reid & MacKenzi, 2016). On the researcher's personal survey of the site of mosquito collection it was observed that other on-site factors could also contribute to this resistance such as smokers and tobacco leaf brokers and *eucalyptus* timber users (carpentry workshops) of which waste products is saw dust which pollutes wetlands through runoff. Both these factors could emmit nicotine and *eucalyptus* oil respectively to pollute the malara vector habitats and developing resistance to malaria vector mosquitoes

Indeed, some previous works demonstrate that oils containing mainly oxygenated compounds have a higher persistence and lose their activity more slowly than those with a high content of hydrogenated compounds (Rathore & Nollet, (2012); ISGlobal Barcelona Institute of Global Health (2017) WHO (1995); Liao *et al.* (2017) Shaaya & Kostyukovsky, 2006). Apparently, due to very short persistence time demonstrated by the oils of the three plants investigated in this study, may have a high content of hydrogenated compounds as opposed to oxygenated compounds.

Detection of resistance of biopesticides in malaria vectors will help public health personnel to formulate appropriate steps to counter reductions in effectiveness of control effort that may accompany with the emerging problems of insecticide resistance. Further, more cross-resistance or resistance as a result of agricultural uses of insecticides may evolve and adversely impact the options to switch an alternative method or insecticides for disease control and hence focus to plant extracts and other biological control agents.

5.6 Persistence of extracts in *Anopheles gambiae s.s.* **Giles** larvae habitat as a function of time.

In this study the three plant extracts were tested for their persistence under light-dark regimes. A bulb of 120W was provided to supply light during the study period. Darkness was provided by black canvass that sealed light during the daytime. Over the nights the natural darkness predominated and was used. Howevewr, bulbs of low voltage (60-80W) were applied during the nights as a balance between the the day use of canvass and dack sheets. Under these circumstances light – dark regime of 12:12 (light: dark) was allowed throughout the study period. In this experiment 25 third instar larvae were exposed to the concentrations for 24 hours however, mortality was not observed since the essence of this experiment was persistence. Water/test concentrations holding larvae were sampled hourly and daily to analyze using Gas Chromatography- Mass Spectroscopy (GC-MS) analysis Model No SQ8 GC/MS, England, to determine residuals of the essential oils in the mosquito bath after every one hour or one day until the oils complete biodegradation (Table 4.24).

The results of the three plants indicated that under light regime *C. cinerariifolium*took 5 hours and 30 minutes to completely decompose and 28 days to decompose under darkness. *E. camaldulensis* decomposition under dark regime was 35 days and that of light regime was 12 days. The dark-light degradation periods for *N. tabaccum* slightly

stretched higher than those of other plants and disappeared within 18 days of light and 28 days of darkness. Generally, these are good periods for mosquito control especially when mosquitoes will tend to prolong their breeding periods in stagnating waters. In mosquito control programme these extracts will keep mosquitoes in abeyance for a period of about 1 ½ months. However, it must be realized that application of insecticides for mosquito control in Kenya takes place during the day. For this reason, it could be considered whether it would be appropriate to apply insecticides for mosquito control at dusk other than applying insecticides in the early hours of the day.

The photodecomposition results for *C. cinerariifolium* concur with those of Chen and Casida (1969) whose their results of study indicated 5 hours and 30 hours light-dark periods respectively for pyrethrin 1, allethrin, phthalthrin and dimethrin. Other researchers including Gunasekera (2005) and Crosby (1995) also attempted tests on environmental fate of pyrethrins.

The breakdown (deactivation) of natural pyrethrins when exposed to daylight is perhaps their most prominent and best-recognized chemical characteristic (Chen and Casida, 1969). Pyrethrin photodegradation is rapid. In dilute solution in an organic solvent, but in the virtual absence of atmospheric oxygen, the principal reaction is isomirizationof the pyrethrolone side-chain, from a cis-(Z-) to a trans-(E-) configuration (Kawano *et al.*, 1980; Ramirez, 2013; Bullivant & Pattenden, 1976). Under aerobic conditions, photooxidation predominates. Other than pyrethrin 1 all of the other pyrethrins (Bioallethrin, Cinerin I, Jasmolin I, pyrethrin II, Cinerin II, and Jasmolin II) also undergo photodegradation. Pyrethrin I and Cinerin I are degraded more rapidly than pyrethrin II and Cinerin II (Brown *et al.*, 1957). The rapid and extensive decomposition of the pyrethrins very likely is due primarily to UV-energized autoxidation (direct reaction with

atmosphere triplet oxygen). Pyrethrins are stable in the dark (Chen and Casida, 1969; Dickinson, 1982).

These differences in degradation could have possibly been as a result of the chemical composition of the plants' oils. This is so because some compounds in an extract may resist decomposition while others may decompose in a very short time depending on the type of chemical in the extract. Components of *N. tabaccum* and *E.camaldulensis*oils may require further investigation to confirm which components of the oils have high resistance to decomposition. Since the trend of insecticides application for mosquito control in Kenya takes place during the day it is therefore important to take into account of the period of the extracts biodegradation under light (solar energy) regimes. The persistence of *E. camaldulensis* and *N. tabaccum* are not documented and hence no record available for comparison to this study.

5.7 Conclusions and Recommendations

5.7.1 Conclusions

The following conclusions were made in respect of this study:

(i)The extracts of the three plants as independed extracts demonstrated good results. They exhibited high larval mortality ranging from lowest 80% (Ec ethyl acetate) to highest 100% (*Cc* ethanol, *Cc* DCM, *Ec* methanol and *Nt* ethanol). These crude leaf extracts can be effectively used in the control of mosquitoes by replacing the chemical pesticides which generally pollute the environment and specifically have deleterious effects to human health.

(ii) A good number of synergistic extracts were identified in the three plants. In all they were 28 in number (12 DCM, 10 methanol, 4 ethanol and 2 ethyl acetate). This gives a wide range of synergistic extracts for choice in their use during mosquito control

programmes. These synergists acted positively in the improvement of the combining extracts for better results.

(iii) Seven antagonistic extracts were identified. These extracts tended to lower the activities of the combining extracts resulting to to poor results.

(iv) The highest RR of the field strain of the larvae was observed to occur during the heavy rain season (March, April, May and June 2010). This was most likely contributed by the agriculturally applied chemicals (temephos) swept into the larval habitats by runoff giving the larvae acquire prior exposure and therefore the larvae developing resistance of the weaker later applied insecticides.

(v) The three plants short periods of biodegradation in both light and dark regimes was a characteristic of decomposable insecticides. The plants, therefore when used for mosquito control will be environment friendly.

5.7.2 Recommendations

The following recommendations are made in view of the extracts of the three plants:

(i) It is important that the extracts that indicated best performance can be considered for use in the mosquito control programmes both in the Central and the county governments. These extracts are: Cc ethanol, and DCM, Ec methanol and Nt ethanol all which exhibited 100% larval mortality; Cc methane (98%), Ec ethanol, hexane and Nt methanol which gave 96% larval mortality. These extracts can be directly applied immediately extracted in the form of liquid or can be commercially reconstituted into powder for sale in shops.

(ii) The extracts with synergistic properties should be used in combination with other extracts to achieve good results in mosquito control. This is particularly in cases where the malaria vector mosquitoes tend to develop resistance to the applied insecticide(s). In the synergistic activities DCM of Cc acted 12 times as synergist, methane of the same extracts acted 10 times as synergist, ethanol 4 times and ethyl acetate of Cc and Ec 2

times. Hence, giving wide choice of the best synergist can be considered in the above order of synergism.

(iii) The use of antagonistic extracts during the mosquito control programmes should be avoided. These extracts are likely to weaken the anticipated well performing extracts. Ten antagonists were identified. These were methanol of Cc (acted x 1) and Nt (x1); Hexane of Ec (x1) and Nt (x2); Ethyl acetate of Ec (x1) and Nt (x2); Aqueous of Nt (x1).

(iv) The Larval Source Management (LCM) is the only method of malaria vector control (WHO,2013). In order to avoid *Anopheles gambiae s.s.* larvae resistance development to insecticides, it would be appropriate to apply the extracts during the low rain periods as opposed to during heavy rains. The latter is a factor for the temephos (agriculturally applied chemicals) accumulation in the larval habitats through runoff. The field srain of larvae therefore would have experienced previous (prior) exposure to temephos and are likely to resist the weaker extracts when later applied.

(v) The three plants indicated short periods of persistence both in dack and light regimes. However, it was observed that Cc had the shortest periods of persistence and therefore biodegraded within the shortest time of 5.5 hours light and 28 days darkness. This plant's extracts should be given priority in their use as mosquito control insecticide, however, notwithstanding the fact that other extracts showed good quality in toxicity.

5.7.3 To Researchers

Researchers to be encouraged to make further studies on the following areas:

i. Make use of other polar (e.g. Acetone, Dimethyl sulfoxide, Nitromethane, Acetic acid, Formic acid, Isopropanol, Acetonitrile etc) and non-polar solvents (e.g. Pentane, Cyclopentane, Cyclohexane, Benzene, Toluene, Chloroform, Diethyl ether, 1,4-Dioxane etc.) to make comparison with the ones used in this study.

ii. Carry out research on *Anopheles gambiae s.s.* Giles larvae to determine effects of Additive and Potentiation activities of the crude flower and leaf extracts.

iii. Control of mosquitoes at the eggs stage using these three plant extracts.

iv. Need to carry out a study using these crude leaf extracts against adult *Anopheles gambiae* s.s. Giles mosquitoes.

v. Large male mosquito population for swarming is a major pre-condition for mosquito mating. Can mosquitoes occasionally mate without swarming especially those very few in households?

REFERENCES

Adebiyo A.O and Uluwafemi, A. 92017). Assessment of tobacco control efforts in three sub-saharan African countries. *Nigerian postgraduate medical Journal*, volume 24 Issue 1, pp. 8-13.

Africa Business Start (2014). Industrial Africa Business idea with a flower: Pyrethrum. In:africajumpstart.com/industrial-africa-business-idea-flower-pyrethrum. Accessed 7th July, 2018.

Ageep, T.B., Damiens, D., Alsharif, B., Ahmed, A., Salih, E.H.O., Ahmed, F.T.A,, Diabate, A., Lees, R.S. Gilles, J.R..L and El Sayed, B.B. (2014). Participation of irradiated Anopheles Arabiensis males in swarms following field release in Sudan. *Malaria Journal* 13: 484.

Agriculture, farming, Tobacco production by country worldwide (2016). Leading tobacco producing countries worldwide in 2016 (in 1,000 metric tons). Statistica, the statistics portal. In: https://www.statistica.com/statistics/261173/leading-countries-in-tobacco-production/accessed 10/6/2018.

Agyapong, J., Chabi, J., Ablorde, A., Kartey, M.D., Osei, J.H.N., desouza, D.K., Dadzie, S., Boakye, D.A. Ohta, N., Hadi, M.P and Suzuki, T. (2014). Ovipositional Behaviour of Anopheles gambiae mosquitoes. *Tropical Medicine and Health*, 42(4): 187 – 190.

Agyapong, J., Chabi, J., Ablorde, A., Kartey, W.D., Osei, J.H.N., de Zouza, D. K., Dadzie, S. Boakye, D.A. Ohta, N., Hadi, M.P. and Suzuki, T. (2014). Ovipositional behaviour of Anopheles gambiae mosquito. Tropical Medicine and Health 42(4):187-190.

Akogbeto, M.C., Salako, A.S., Dagnon, F., Aikpon, R. Konletio, M. Sovi, A. and Sezonlin, M. (2018). Blood feeding behaviour comparisoin and contribution of Anopheles Coluzzi and Anopheles gambiae, two sibling species living in sympathy to Malaria transmission in Alibori and Donga region Northern Benin, West Africa. Malaria Journal 17:307.

Alam, M.F., Chopra, A.K., Safhi, M. M. and Dua, V. (2011). Toxicological properties of several medicinal plants from the Himalayas (India) against vectors of malaria, filariasis and dengue. Tropical Biomedicine 28(2): 343-350.

Alternative paper (Ref) Although not in Anopheles is: Danielle, H.B. (2017). Monitoring insecticide resistance mechanisms in Culex tarsalis from Sutter County, California. A thesis submitted to the office of the graduate school in partial fulfillment of the requirements for the degree of Master of Science, College of the Pacific Biological Sciences.

American Mosquito Control Association (AMCA) (2011). Manual for Mosquito Rearing and Experimental Techniques, Bulletin No. 5. *Journal of American Mosquito Control Association* 5:5.

American University of Beinit (AUB) (2017). AUB leads the way to a healthier tobacco-free future. In: https:website.aub.edu.16/news/2017/pages/aub-tobacco-free.aspx accessed 13.6.2018.

Anna Sengeeva (2017) Pyrethrum market: which countries produce the most. Market analyst indexbox marketing.

Anopheles species diversity and distribution of the malaria vectors of Thailand. Tainchum, K. Kongmee, M., Manguin, S., Bangs, M.J. and Chereonviriyaphap, T. (205). *Trends in Parasitology*, volume 31, Issue 3 pp. 109-119.

Arap Chepkwony, (2014). Pyrethrum farmers get new market 29th May, 2014, Bahati, Nakuru, Kenya.

Arshad, J., (2011). Tobacco production: Beating the average global yield. In *The Express Tribune*, December, 5th, 2011.

Artis, M. L., Lehmann, T. and Huestis, D. (2014). The effects of oviposition – site deprivation on longevity and blood feeding rate in Anopheles gambiae. *Parasites and Vectors* 7(1)

Ashauer, R., Connor, 1.0 and Escher, B.I. (2017). Toxic mixtures in time – The sequence makes the poison. Environmental Science and Technology pp. 1-9, ISSN 0013-936X https://doi.org/10.1021/acs.est.6606163.

Asmeng J. (2000). Larval stage of mosquito lasts 1-3 weeks, and hence a stable stage to handle. Pg. 88.

Australian Pesticides and Veterinary Medicines Authority (2016). Review of the mammalian toxicology and metabolism /toxicokinetics of Paraquat; Supplement II: Neurotoxicology. In: Metabolism and toxicokinetics, supplement II: Neurotoxicology prepared by the Agricultural and Veterinary chemical assessment team, Office of Chemical safety of the Department of Health, Canberra, Austraia, pp. 37-96.

Awuah, S.B. Annan, A.A. Ascofare, O.M., Diendonne, S.D., Kusi, P.a., Dabo, E.O., and Danso, K.O., (2016). Insecticide resistance in Malaria vectors in Kumasi, Ghana. Parasite and Vectors 9:633 http://doi.org/10.1186/S13071-016-1923-5.

Awuah, S.B. Annan, A.A., Ascofare, O.M. Dieudonne, S.D., Kusi, P.A., Dabo, E.O. and Danso K.O. (2016). Insecticide resistance in malaria vectors in Kumasi, Ghana. Parasites and Vectors 9:633.

Azab, M., Khabour, O.F. Alzoubi, K.H., Hawamdeh, H. and Nassar, L. (2017). Assessment of genotoxicity of Pyrethrum in cultured human hymphocytes. Drug and Chemical Toxicology 40(3): 251 – 255.

Azokou, A., Kone, M.W., Koudou, B.G. and Tra Bi, H.F. (2013). Larvicidal potential of some plants from West Africa against Culex quinguefasciatus (Say) and Anopheles gambiae Giles (Diptera: Culicidal). Journal of Vector Borne Diseases 50: pp. 103-110.

b) Dambach P., Traore, I. Becker, N., Kaiser, A. Sie, A. and Sanerborn (2014). EMIRA: Ecologic Malaria reduction in Africa – innovative tools for integrated malaria control. Global Health Action 7: 10.3402/gha.v7 25903.

Bahadar, K., Munir, A. and Asad Shahzad (2016). Biological Properties of Eucalyptus L. SPS. Extracts against microbial pathogens: An updated review. *Journal of Biodiversity and Environmental Sciences* vol. 9 No. 5, P. 72 – 85.

Baker, D.G. and Lipman, N.S (2015). Laboratory animal medicine (Third edition). Chapter 33 – Factors that can influence animal research. Elsevier 2015, pp 1441-1496.

Barbosa, C.L.A., Filomeno, C.A. and Teixeira, R.R. (2016). Chemical variability and biological activities of Eucalyptus spp. Essential oils. Molecules 21:1671;doi:103390/molecules21121671.

Barbosa, L.C.A., Filomeno, C.A., and Teixeira, R.R. (2016). Chemical Variability and Biological activities of Eucalyptus spp. Essential oils. Molecules 21, 1671, doi: 10.3390

Bargielowski, I.E., Lounibos, L. P., Shin, D., SMatt, C.T. Carrasquilla, M.C., Henry, A., Navarro, J.C., Panpy, C. and Dennett, J.A. (2015). Widespread evidence for interspecific mating between Aedes aegypti and Aedes allopictus (Diptera: Culicidae) in nature. Infection, Genetics and Evolution 36:456-461.

Baughman, T., Peterson, C., Ortega, C., Preston, S.R., Paton, C., Williams, J., Guy, A., Omodei, G., Johnson, E., Williams, H., O'Neill, S.L., Ritchie, S.A., Dobsoni, S.L., and Madan, D. (2017). A highly stable blood meal alternative for rearing Aedes and Anopheles mosquitoes. PLOS Neglected Tropical Diseaes 11(12):e0006142.https://doi:org/10.1371/journal.pntd.0006142.

Becnel, J. J. (2007). Current status of *Deltabaculoviruses, Cypoviruses* and *chloriridoviruses* pathogenic for mosquitoes. *Virol sin* 22:117-127.

Becuel, J. J. and Whtie, S. E (2007). Mosquito pathogenic viruses – The last 20 years. *American Mosquito Control Association Bulletin.*7 (23):36-49.

Behbahani. R.V., Sharififard, M., Djadid, N.D., Shamzi, J., and Fakoorziba, M.R. (2014). Laboratory evolution of the entomopathogenic fungus Beguveria bassiana against Anopheles Stephensi Larvae (Diptera: Culizida). The Asian Pacific *Journal of Tropical Medicine Disease* 4(supple. 2): 5799 – 5802.

Benedict, M. Q., Knols, B. G. J., Bossin, H. C., Howell, P. I., Mialhe, E., Careres, C. And Robinson, A. S. (2009). Colonization and mass rearing: Learning from others. *Malaria Journal*. 8: 2

Benelli G., Jeffries C.L and Walker T. (2016). Biological Control of Mosquito vectors. Past, present and Future. Insects 7(4): 52.

Benett, B.M. (2014). The origins of timber in India. *Agricultural History Review* 62(1) pp. 98-118(21).

Benito, J.L., Jaca., and Aragon, G. (2018). Biovision foundation. Pyrethrum: General information on pyrethrum, pyrethrins and pyrethroids. In: https: www.infonet-biovision.org/planthealth/plant-extract-pyrethrum.accessed June, 5, 2018.

Berg, R.F. (2015). Apparatus to measure the vapour pressure of slowly decomposing compounds from 1 pa to 10^5 pa. *Journal of Chemical and Engineering Data* 60(12): 3483 – 3495.

Berk-Johnson, L.M. Nelson, W.A., Paaijmans, K.P. Read, A.F., Thomas, M.B. and Bjorhstad, O.N. (2013.

Bilingual Teaching Courses (2009). Medical parasitology, Pre-clinical Medical College, Sun-Yat-Sen University 2009. In: jpkc. sysu.edu.cn/jscx/Textbook/senven-3.html. Retrieved 12.1.2014.

Birget, P.L.G. and Koella, J.C. (2015). An epidemiological model of the effects of insecticide-treated bednets on Malaria transmission. PLOSONE 10(12): e0144-173 https://doi.org/10.1371/journal.pone.0144173.

Blakely, W. F. (1965). A key to the Eucalypts: with descriptions of 522 species and 150 varieties. Third edition. Forest and Timber Bereau, Canberra.

Bohura, S.K., Sarro, J. Li, P., Mysore, K., Severson, D.W. Emrich, J.S. and Duman-Scheel, M., (2016). High-through cis-regulatory element discovery in the vector mosquito Aedes aegypt. Biomedical Cenral Genomics. 17: 341.

Bojanowska, A. and Brzesicka-Bak, M. (1967). Acute and subacute toxicity of diedrin, administered percutaneously (in Polish). *Rocz, Panstw, Zakl, Hig.*18:161-169

Borio, G. (2018), *The Tobacco timeline*. 2018/07/09

Bossom A.D., Mangelinckx, S., Yedomonhan, H., Boko, P.M., Akogbeto, M., Kimpe, N.D., Avlessi, F. and Sohonnhlone, D.C.K. 92013). Chemical Composition and insecticidal activity of plant essential oils from Benin against Anopheles gambiae (Giles0. *Parasites and Vectors* 6:337.

Bosson, A.D. Mangelinckx, S., Yedomonhan, H., Boko,. P.M., Akogbeto, M.C., Kimpe, N.D., Avlessi, F. and Sohounhlone D.C.K (2013). Chemical compositon and insecticidal activity of plant essential oils from Benin against Anopheles gambiae. Parasites and Vectors 6:337.

Bossou, A. D. Mangelinckx, S. and Love,S., D. C. K. (2013). Chemical composition and insecticidal activity of plant essential oils form Benin against *Anopheles gambiae*(Giles).*Parasites and Vectors*. 6:337.

Bossou, A. D., Akogbeto, M. C,. Kimpe, N. D. Avlessi, F. and Dominique, C. K. (2013). Chemical composition and insecticidal activity of plant essential oils from Benin against *Anopheles gambiae*(Giles).*Journal of Parasites and Vectors 6*: 337.

Bossou, A. D., Mangelinckx, S. Yedomonhan, H., Boko, P. M., Akogbeto, M., Kimpe, N., Arlessi, F. and Sohuonhloue D. C. K. (2013). Chemical composition and insecticidal activity of plant essential oils from Benin against *Anopheles gambiae* Giles. *Parasite and vectors.*6:337.

Bossou, A.D. Mangelinckx, S., Yedomonhan, H. Boko, P.M., Akogbeto, M.C., Kimpe, N., Avlessi, F. and Sohounhloue (2013). Chemical composition and insecticidal activity of plant essential oils from Benin against Anopheles gambiae (Giles). *Parasites and Vectors* 6:337.

Botanical Database (2015). *Australian Plant Name Index* (APNI); Australian National Botanic Gardens and Centre for Australian National Biodiversity Research, Canberra.

Brant, H.L. (2015). Impacts of deforestation on mosquito community dynamics: thesis submitted for the degree of Doctor of Philosphy, Imperial College, University of London, Park Campus, Buckhurst Road, Ascot, SL57 PY, UK.

Breman, J.G., Daily J. and Baron, E.L. (2018). *Malaria: Epidemiology, Prevention and control*. In https://www.update.com/contents/malaria-epidemiology-prevention-and-control/print. retrieved 2.6.2018.

Brodsky, J.B. (2018). Recent advances in anaesthesia of the obese patient version 1: referees: 2 approved). 06 Aug. 2018, 7(F1000 Faculty Rev): 1195 (doi: 10.12688/f1000research.15093.1).

Brown, D. (2010). Malaria – transmitting mosquito splitting into two species. Malaria journal 3:17-26.

Burke, A., Dandalo, L., Munhenga, G., Dahan-Moss Y., Mbokazi, F., Ngxongo, S., Coetzee, M., Koekemoer, L. and Brooke, B. (2017). A new malaria vector mosquito in South Africa. Science Reports 7:43779; doi: 1038/srep43779.

Burkett – Calena, N. D, McClure, C. J. W., Estep, L. K., and Eubanks, M. D. (2013). Hosts or habitats. What drives the spatial distribution of mosquitoes? *Ecosphere* ; 4 (2): 30

Burnett, D., Farrel, G. and Kiiru, M. (2002). Strategies for the development of a competitive pyrethrum – based pesticide sector in Kenya. Final Report (NRI report (NRI report No. 2695). *Technical Report*. Natural Resources Institute, Chatham, United Kingdom.

Burnett, D., Farrell, G. and Kiiru, M. (2002). Strategies for the development of a competitive pyrethrum – based pesticide sector in Kenya. Final Report (NRI report No. 2695). *Technical Report*. Natural Resources Institute, Chatham, United Kingdom.

Butail, S., Manoukis, N. C., Diallo, M., Ribeiro, J. M. and Paley, D. A., (2013). The dance of male *Anopheles gambiae* in wild mating swarms. *Journal of Medical Entomology*;50 (3): 552-9.

Caputo, B., Nwakanwa D., Jawara, M., Adiamoh, M., and Dia, I. (2008).*Anopheles gambiae* complex along the Gambia river, with particular reference to the molecular forms of *Anopheles gambiaes* s.s. *Malaria J*ournal; 7:182.

Caralca, P. A. M., Gomes de Assumpcao, L. M. I., Bruno, R.; and Bonato, C. M. (2010). Homeopathic and Larvicide effect of *Eucalptus cinevera* essential oil against *Aedes aegypti*. *Journal of Brazilian Archives of Biology and Technology*; 53:4

Carnevale, P., Toto, J.C. Besnard, P., Dos Santos, M.A. Fortes H., Allan, R. and Manguin, S. (2015). Spatio-Temporal variations of Anopheles coluzzii and An. Gambiae and their Plasmolium infectivity rates in Lobito, Angola. *Journal of Vector Ecology* 40(1): 172-179.

Carvalho, D. O., Nimno, D., Naish, N., McKemey, A. R., Gray, P., Wilke, A. B. B. Marrelli, M . T., Virginio, J. F., Alfey, L. and Capurro, M., (2014). Mass Production of genetically modified *Aedes aegypti* for field releases in Brazilian *Journal of Visualized Exp*eriments. (83): e3579.

Cassone, B.J. Kay, R.G.G., Daugherty, M.P. and White, B.J., (2017). Comparative transcriptomics of Malaria mosquito testes: Function, evolution and linkage. G3. Genes, Genomes, Genetics 7(4): 1127-1136.

CDC (2014), Health, United States (2014). Health Risk Factors: Current Cigarette Smoking. U.S. Department of Health and Human Services. CDC DHHS Publication No. 2015 – 1232 pp. 12 of 473. In:https://www.cdc.gov/richs/data/hus/hus/4.pdf.

CDC (2017). Smoking and Tobacco use. Health effects of Cigarette smoking. CDC 24/7: Saving Lives, Protectign People: In: https://www.cdc.gov/tobacco/data-statistics/fact-sheets/health-effects/effects-cig-smoking/index.html retrieved in 12.2.18.

CDC Office of smoking and health (2018), Smoking and tobacco use; efforts to reduce deaths and prevent chronic diseases that result from tobacco use. In: https://www.cdc.gov/tobacco/about/ash/index.html. accessed July, 10, 2018.

CDC(2014). LD50 of Nicotine: Immediately dangerous to life or health concentrations (IDCH). In: National Institute for Occupational Safety and Health (NIOSH), centres for Disease Control and Prevention (CDC) 24/7: Saving lives, Protecting people.

Centres for Disease Control and Preventio (CDC), (2010). Methods in *Anopheles* Research. In www.mr4.org/portals3/MR4-publications/Anopheles Protocol Manual second Edv. 2011/2011 Complete Manual pdf TOC. pdf (Retrieved 8/9/13).

Centres for Disease Control and Prevention (CDC) (2015). CDC 24/7: Saving lives, protecting people. In:https://www.cdc.gov/malaria/about/biology/mosquitoes

Centres for Disease Control and Prevention (CDC) (2017). Cigarette smoking is the leading preventable cause of death in the United States, CDC, 247/7. Saving Lives, Protecting People. In https://www.cdc.gov /tobacco/data-statistics/fact-sheets/health-effects/effects-cig-smoking/index.html retrieved 122.18.

Centres for Disease Control and Prevention (CDC) (2018). Burden of Tobacco use in the United States: Tips from former smokers:CDC 24/7: Saving lives Protecting people. In https://www.cdc.gov/tobacco/campaign/tips/resouces/data/cigarette-smoking-in-United States.html. retrieved 12.2.18.

Centres for Disease Control and Prevention (CDC) 2018. Malaria – about Malaria; biology CDC 247/7: Saving Lives, Protecting People. In: https://www.cdc.gov/malaria/about/biology/index.html-retrieved 12.2.18.

Centres for Disease Control and Prevention (CDC), (2008). Burden of Tobacco use in the United States: Tips from former smokers CDC24/7: Saving lives, Protecting People.

Centres for Disease Control and Prevention (CDC), (2008). Tobacco use is the leading preventable cause of death in the United States. Fact sheet: cigarette-smoking related mortality. (http://www.cdc.gov/Tobacco/data-statistics/fact-sheets/health - effects/cig-smoking-mort.html. Retrieved 1.12.2013.

Centres for Disease Control and Prevention (CDC), (2012). Malaria – About malaria. Centres of Disease Control and Prevention, CDC 24/7: "Saving Lives. Protecting People", In www.cdc.gov/malaria/about/faqs.html. Retrieved 6.6.2014.

Centres for Disease Control and Prevention (CDC), (2013), Quickstars: Number of deaths from 10 leading causes – National Vital Statistics System, United States, 2010. *Morbidity and Mortality weekly Report.* 62(08); 155.

Centres for Disease Control and Prevention (CDC), (2013). Centres for Disease Control and Prevention. CDC 24/7: "Saving Lives; Protecting People". Global Health – Division of Parasitic Diseases and Malaria, Atlanta, USA. 2012.

Centres for Disease Control and Prevention (CDC), (2014) Atlanta USA 8.2.12. CDC 24/7: "Saving Lives: Protecting people". Content source: Global Health – Division of Parasitic Diseases and Malaria.

Centres for Disease Control and Prevention (CDC), (2018). Economic Trends in Tobacco CDC 24/7: Saving Lives. Protecting people.

Centres for Diseases Control and Prevention (2018):Tobacco related mortality. CDC 24/7: Saving lives. *Protecting people*: In: https://www.cdc.gov/tobacc/data-statistics/fact-sheets/health-effects/tobacco-related-mortality/index.htm retrieved 1.2.2018.

Centres of Disease Contry (CDC) (2014) Methods in Anopheles Research (MR4), Fourth Edition, 2014. In: https://www/beiresources. org/portals/2/MR4 /MR4 Publications/methods in Anopheles Research / 2014/2014Mr. Accessed 20.7.2018.

Chabi, J., Eziefule, M.C., Pwalia, R., Joannitta, J., Obuobi, D., Amlalo, G., Addae, C.A., Alidu, I., Baidoo, D.A., Akporh, S., Gbagba, S., Frempong, K.K., Hadi, M.P., Jamet, H.P., and Dadzie, S.K. (2018). Impact of urban agriculture on the species distribution and insecticide resistance profile of Anopheles gambiae s.s. and Anopheles cohizzii in Accra metropolis, Ghana. Advances in Entomology 6, 198 – 211.

Chambers, E. W., Hapairai, L., Peel, A. B., Bossin, H., and Dobson, S. L. (2011). Male mating competitiveness of a Wolbachia – introgressed *Aedes Polynesiensis* strain under semi-field conditions. *PLoS ONE Neglected Tropical Diseases*. 5 (8); e1271

Chandler, J. A., Highton, R. B. and Hill, M. N. (1976). Mosquitoes of the Kano plain, Kenya: Results of indoor collectiosn in irrigated and nonirrigated areas usin human bait and light traps. *Journal of medical Entomology*. 12(5):504-10.

Charlwood, J.D. (2017). Some like it hot: a differential response to changing temperatures by the malaria vectors, Anopheles funestus and Anopheles gambiae Peer. *Journal* 5: e3099 https://doi.org/10.7717/peerj.3099.

Chen, Y.L., and Casida, J.E.(2008). Photodecomposition of pyrethrin I. allethrin, phthalthrin and dimethrin. Journal of Agriculture and Food Chemistry. 17:208-215. *Journal of Agriculture and Food Chemistry*. 17:2008-215.

Chiogna, G., Herrera, P.A., and Cirpka, O.A. (2016). Helical flow and transient solute dilution in porous media. Transport in Porous Media 111.59.603.

Citation (2014) Embo Conference on Molecular and population Biology of Mosquitoes and other disease vectors: From basic Vector Biology to Disease control. *Pathogens and Global Health*, 107(8): 400 – 457 (doi 10.1179 /2047772413Z.000000000160).

Clayton, A.M. Dong, Y. and Dirnopoulos, G. (2014). The Anopheles innate immune system in the defence against malaria infection. *Journal of innate immunity* 6:169-181.

Costa, A.A., Lucia, A., Naspi, C. and Masuli, H.M. (2017). Repellent and larvicidal activity of the essential oil from Eucalyptus nitens against Aedes aegypti and Aedes albopictus (Diptera: Culicidae). *Journal of Medical Entomology* 54(3) 1-7.

Crawford, J.E. Bischoff, E. Garnier, T., Ghenie, A., Eiglmeier, K., Holm, I., Riehle, M.M., Guelbeogo, W.M., Sagnon, N. Lazzaro, P. and Vemick, K.D. (2012). Evidence for population – specific positive selection of immune genes of Anopheles gambiae. G3: Genes / Genomes / Genetics volume 2 p. 1505 – 1519 (volume 2, pp. 1505 – 1519).

Crosby, D.G. (1995). Environmental fate of pyrethrins. In: Pyrethrum Flowers; Production, Chemistry, Toxicology and uses, J.E. Casida and G.B. Quistad (eds). *Oxford University Press, NewYork*, N.Y. pp. 194 – 213.

Dambach, P. (2018). New approaches for integrated and cost-effective malaria vector control. Journal of Rare diseases Research and Treatment 3(1):6-10.

Damdangdee, N., Khongtak, W., Kertmanee, Y., Evans B. P. and Schuster, A. L. (2013). Maintenance of mosquito vectors: effects of blood source on feeding, survival, fecundity and egg hatching rates. *Journal of Vector Ecology*. 38 (1): 38-45.

Danbach P., Schleicher, M., Korir, P., Quedraogo, S., Dambach, J., Nightly biting cycles of Anopheles species in Rural North Western Burkina Faso. *Journal of medical Eutomology* Vol. 55 Issue 4 pp. 1027 – 1034.

Davies, C., Coetzee, M. and Lyons, C.L., (2016). Effect of stable and fluctuating temperatures on the life history traits of Anopheles arabiensis and A. quadriannulatus under conditions of inter and intra-specific competition. Parasites and Vectors 9:342 doi:10.1186/S1307 – 016-1630-2.

Dawidar, AEM., Mortada, M.M., Raghib, H.M. and Abdel-Mogib, M. 2013. A method of computing the effectiveness of an insecticide on Molluscicidal activity of Balanites aegyptiaca against monacha cartusiana. *Pharmaceutical Biology volume*: 50 Issue 10 pages 1326 – 1329.

Diabate Abdoulage and Tripet Frederic (2015). Targeting male mosquito mating behaviour for malaria control. *Parasites and vectors*. 8;347.

Diabate, A. and Tripet, F. (2015). Targeting male mosquito mating behaviour for malaria control. *Parasites and Vectors* 8:347 doi:10.1186/s/13071-015-0961-8.

Diabate, A. and Tripet, F. (2015). Targeting male mosquito mating behaviour for Malaria control. Parasites and Vectors 8:347.

Diabate, A., Ullastres, A. and Gonzalez, J. (2014). Adaptation through chromosomal inversions in *Anopheles. Frontiers in Genetics*. 5:129.

Djegbe, I., Dramane, G., Zeykeng, F., Akgbeto, M. and Djouaka, R. (2017). Relationship between Kur L1014F genotypes and phenotypic – resistance to pyrethroids and DDT insecticides in Anopheles gambiae s.l *International Journal of Innovation and Applied Studies* vol. 20 No. 3 pp. 983 – 993.

Djouaka, R. F., Bakare, A. A., Coulibaly, O. N., Akogbeto, M. C,. Ranson, H., Heminigway, J. and Strode, C (2008). Expression of the cytochrome P4SOS, CYP6P3 and CYP6M2 are significantly elevated in multiple pyrethroid resistant

populations of *Anopheles gambiae s.s.* from Southern Benin and Nigeria. *Biomedical Central Genomics*. 9:538.

Djouaka, R.J., Atoyebi, S.M., Tchigossou, G.N., Riveron, J.M., Irving, H., Akoton, R., Kusimo, M.O. Bakare, A.A. and Wondji C.S., (2016). Evidence of a multiple insecticide resistance in the Malaria vector Anopheles funestus in South West Nigeria. *Malaria Journal* 15: 565.

Dlamini, S.N. and Franke, J. (2015). Assessing the relationship between environmental factors and malaria vector breeding sites in Swaziland using multi-scale remotely sensed data. Geospatial Health 10(1): 302.doi:10.408/gh.2015.302.

Doccola, J.J. and Wild P.M. (2012). Tree injection as an alternative method of insecticide application.www.intechopen.com. assessed 10.8.2018.

Dodson, B.L. and Rasgon, J.L. (2017). *Vector Competence of Anopheles and Culex mosquitoes for Zika virus*. Peer J. 5: e3096 https:

DOI – The USAID/Uganda Development Objective (DOI), Economic Growth (2015) Initial Environmental Examination Amendment: Uganda pesticide evaluation report and safer use action plan (PERSUAP), USAID/Uganda, 2015.

Downes, J. A (1968). The sworming and mating flight of Diptera: *Annual Review of Entomology*. 14: 271-298.

Duchon, S., Bonnet, J., Marcombe, S., Zairn, M. and Corbel V. (2009). Pyrethrum: A mixture of natural pyrethrins has potential for malaria vector control. *Journal of Medical Entomology*; 46:516-522.

Dumitrache, G. (2018). Malaria. Factors contributing to malaria transmission urban malaria in sub-sahara. In: Geography NCEA level 2: Malaria-contributing factors and processes, Linkedin, corporation pp 1-61.

Dwijendra, S., (2014). Role of Plant biopesticides in managing vectors of communicable Diseases; in Advances in Plant Biopesticides, Durijendra (Ed, Springer Nature, Switzerland AG. Pp. 243-270.

Eke, B.N. (2016). Toxicokinetics; Disposition of xenobiotics (Absorption, distribution and excretion of xenobiotics). Eke, B.N., Professor of Toxicology, Department of Toxicology, Faculty of Pharmacy, Ankara University, Zagrep, Crotia.

Eliningaya, K. Himeidan, Y.E.S. Munga, S. and Yan, G. (2015). Assessment of Mosquito larval productivity among different land use types of for targeted malaria vector control in the Western Kenya highlands. *Parasites and Vectors* 8(1): 356.

El-Maghraby, S., Nawwar, G., Bakr, R.F., Helmy, N., and Kamel, O.M.H.M (2012). Toxicological Studies of some agricultural waste extracts on mosquito larvae and experimental animals. *Journal of Egyptian Society of Parasitology*30(1): 101-115.

Elroby, S.A. K and Aziz, S.G. (2011). Understanding the decomposition reaction mechanisms of Chrysanthemic acid: a computational study. *Chemistry Control Journal* 5:66.

Elumadai, K., Dhanasekaran, S., Anandam, A., Krishnappa, K., Gokulakrishnan, J. and Elangovan, A. (2012). Mosquitocidal activities of Abrus precatonus L. (Fabaceal against Chikungunya vector, Aedes aegypti (L) and Japanese encephacitis vector, Culex tritaeniorhynchus (Giles) (Diptera:culicidae). International Journal of Current Life Sciences vol. 2, issue 6, pp 31-38, July, 2012. *International Journal of current life sciences*. 2(6): 31-38.

En.wikipedia.org/wiki/Pyrethrum 2014. The use of pyrethrum for flea control in dogs and cats.

English, M. (2011). "Mosquito" attacking malaria with spermless mosquitoes August 10, 2011, 02:49 PMET// Marianne

English/news.discovery.com/animals/tags/mosquito.htm. Retrieved 3.4.2013.

Environmental Pollution Agency (EPA) (2014). Ecological risk assessment. In: en.wikipedia. org/wiki/toxicology. Retrieved 3.7.2014.

Environmental Pollution Agency (EPA), (2014). Ecological risk assessment, 2014.

Environmental Pollution Agency (EPA), (2014). Major factors that influence toxicity.

Environmental Protection Agency (FPA, (2016) and Stockholm Convention on Persistent organic pollutants (2017). Update of inventories of persistent organic pollutants (POPs) in Ireland 2006 – 2015 – by Ricardo Energy and Environment, Clean Technology Centre SPCP 20-16 – 42; and the Stockholm Convention on Persistent Organic Pollutants. American Journal of International Law, volume 95, issue 3, pp. 692-708 https://doi.org/10.2307/26685/7.

Eriksen, M., Judith, M. and Hana, R. (2012). The Tobacco, Atlas, (4th ed). American Cancer society. In: Brenya Edward, "Struggling to weaken the giant. Litigation as a measure to compel the adaptation of tobacco control instrument in Malawi". *African Journal of Political Science and Internation Relations*.6 (7) : 155-166.

Escher, R.L. (2008). Sex ratios of mosquitoes from long term consensus of Florida tree holes. Journal of the American Mosquito Control Assoication (24(1): 11-15 2008. *Journal of American Mosquito control Association*. 24(1):11-15.

Essential Oils In: www.bojensen.net/Essential Oil Eng/Essential Oils/29.htm. Retrived 14.1.15.

Ethiopian Public Health Institute (EPHI) (2017). Anopheles mosquito rearing and insectary handling guidelines: Bacterial parasitic and zoonotic diseases research directorate, Addis Ababa, Ethiopia.

Eucalyptus Caltivation – In: en.wikipedia.org/wiki/Eucalyptus.

Eucalyptus Cultivation Practices: In www/tnpl.com/cultivation-Eucalyptus.pdf.

Eucalyptus oil is rich in cineole approximately 35-90% by volume, (http://www.patentstorm.us/patents/62514410/description.html. Retrieved Oct. 10, 2010.

European Commission Directorate General, Environment (2014). Guidance efficacy Evaluation of Insecticides part 18 and 19. 49th meeting of representatives of members states competent Authorities for the Implementation of Directive 98/8/EC concerning the placing of Biocidal Products on the Market; Document CA-Dec 12-Doc.6.2.1

Everett, S. (2013). Eucalyptus tree HTMT, Dartmouth, England, United Kingdom, May 18, 2013.

Experience – Essential – oils.com (2010-13) in www.experience essential-oils.com/users-of-eucalyptus.html. Retrieved 12.11.2013

Eynde J.J.V. (2014). Prodrug Strategies for enhancing the percutaneous absoption of drugs. Molecules 19: 20780 – 20807; doi: 10.3390/molecules 191220780.

Facchinelli, L., Valerio, L., Lees, R.S. Oliva, C.F., Persampieri, T., Collins, C.M., Crisanti, A., Spaccapelo, R. and Benedict M. (2015). Stimulating Anopheles gambiae swarms in the laboratory: applicaton for behavioural and fitness studies. Malaria Journal 14:271.

Fadjo, B.K., Kondou, B.G., Tia, E., Saric, J., N'dri, P.B., Zoh, M.G., Gba, C.S., Kropf, A., Lesse, N.B. and Chouaibou, M.S. (2018). Insecticide resistance status of An. Gambiae in areas of varying agrochemical use in cote D'lvoire. BioMedical Research International volume 2018, Article ID 2874160, 9 pages.

Fahmy, N.T., Villinski, J.T. Bolay, F., Stoops, C.A. Tageldin, R.A., Fakoli, L., Okasha, O., Obenauer, P.J and Diclaro II, J.W. (2015). The seasonality and ecology of the Anopheles gambiae complex (Diptera: culicidae) in Medical Entomology 52(3): 475 – 482.

FAO, IFAQ, UNICEF, WFP and WHO (2018). The State of Food Security and nutrition in the World. Building Climate resilience for Food security and nutrition, Rome FAO.

FAO/WHO (2006). Joint FAO/WHO Food standards programme. Codex Committee on Pesticides residues. Thirty –eight session, Hotel Vila Gale, Fortaleza, Brazil, 3-8 April, 2006. Draft and propoed draft maximum residue limits in foods and feeds at steps 7 and 4, including dried chili peppers at step 7.

FAOSTAT (Food and Agriculture Org. Statistics) Factfish research made simple. In:www.factsfish.com/statistic/pyretherum%2cproduction quantity. Africa:Pyrethrum-market report-Analysis and Forecast to 2025.

Farida, A., Ahmad, A.H., Hadi, P.K., Hambal M. Falirimal, Y and Shatitri, R. (2011). Ovitras use in epidemiology study of Aedes aegypti and Aedes albopictus in Kuta Alam sub-district Banda Aceh, Indonesia. *Proceeding of The Animal International Conference* Syiah Kuala University 2011, Banda Aceh, Indonesia, November, 29-30, 2011.

Fensham, R.J. and Fairfax, R.J. (2009). Drought related tree death of Savanna eucalyptus: species susceptibility, soil conditions and root architecture. Journal of Vegetation Science volume 8 issue 1 pp. 71-80.

Fettene, M., Olana, D., Christian, R.N., Koekemoer, L.L. and Coetzee M. (2013). Insecticide Resistance in Anopheles arabiensis from Ethiopia African Entomology 21(1): 89 – 94.

Finnley, D. J. (1971). Probit analysis, 3rd ed. London: Cambridge University Press; 1971, p. 338.

Firestorm: The story of a catastrophic fire that struck Tasmanian township of Dunalley January, 4, 2013. *Guardian*, Thursday, 23 May, 2013.

Food Agricultural Organization and International Atomic Energy (FAO/IAEA) (2017). Guidelines for standardized mass rearing of Anopheles mosquitoes. Joint FAO/IAEA programme, version 1.0. edited by Human Disease Vectors (HDV) group of the insect Pest Control Laboratory Insect Pest Control section, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture.

Gachie, T (2018). Pyrethrum farming in Kenya: Price, byproducts, fertilizer, pests, clones, varieties, diseases, climate, rainfall, counties, harvesting, picking, commercial companies, researchers, nursery. Kenyanlife 2018.

Gachie, T. (2018). Pyrethrum Farming, harvesting, drying and marketing. Kenyanlife 2018.

Galizi, R., Doyle, L.A., Menichelli, M., Bernardin, F., Deredec, A., Burt A., Stoadard, B.L., Windbichler, N. and Crisanti, A. (2014). A synthetic sex ratio distortion system for the control of the human malaria mosquito. *Nature communications* 5, Article number: 3977 doi: https: //doi/org/10.1038/ncomms 4977.

Ganasekara, A.S. (2005). Environmental fate of pyrethrum. Environmental Monitoring Branch, Department of Pesticide Regulation 10011 Street, Sacramento, CA95812.

Gentile, J.E. Rund, S.S.C. and Madey, G.R. (2015). Modellign Sterile insect technique to control the population of Anopheles gambiae. *Malaria Journal*, 14:92.

George, J., Jenkins, N.E., Blanford, S., Thomas, M.B. and Baker, T.C. (2013). Malaria mosquitoes attracked by Fatal fungus. PLOSONE 8(5): e62632. https://doi.org/10/1371/journal.pone.0062632.

Georgiou, T., Mavrikiou, G., Alexandrou, A., Aristidou, E.S Sarva, I., Christodoulides, T. Krasia, M., Anastasiadou, V.C. Sismani, C., Drousiotou, A. and Tanteles, G.A (2016). Novel GLA Deletion in a Cypriot female presenting with cornea verticillata. Case reports in Genetics Volume 2016, Article ID 5208312, 6 pages.

Ghalem, B. R. and Mohamed B. (2014). Antibacterial activity of essential oil of North-West Algeria *Eucalyptus camaldulensis* against *E. Coli* and staphylococcus aureus. *Journal of Coastal Life Medicine*. 2 (10): 799 – 804.

Ghosh, A., Chowdhury, N. and Chandra, G. (2012). Plant extracts as potential mosquito larvicides. *Indian Journal of Medical Res*ources. 135(5): 581-593.

Ghoshi, A., Nandika, C., and Chandra, G., (2012). Plant extracts as potential mosquito larvicides. *Indian Journal of Medical Research*. 135: 581-598.

Giamba, N. Choi, K. S. and Thomson, H. (2006). Malaria vectors in Angola: distribution of species and molecular forms of the *Anopheles gambiae* complex, their pyrethroid insecticide knockdown resistance (kdr) status and *Plasmodium falciparum* sporozoites rates. *Malaria Journal*. 5:2

Gilles, M., Zhao, J., A. Min. And Agboola, S. (2010). Chemical composition and antimicrobial properties of essential oils of three Australian Eucalyptus species. *Food Chemistry*. 119 (2) 731 – 737

Gillies, M.T and Coetzee, M. (1987). Mosquito Taxonomic Inventory: A supplement to the Anophelinae of Africa South of the Sahara (Afrotropical Region). Publications of the South African Institute for Medical Research, vol 55 pp. 1-143.

Gnanguenon, V., Govoetchan, R., Agoesa, F.R., Osse, R., Agbo, F.O., Azondekon, R. Sovi, A., Attolon, R., Badirou K., Tokponnon, F., Padonou, G.G. and Akogbeto, M.C. (2014). Transmission pattern of Plasmodium Falciparum by Anopheles gambiae in Benin. *Malaria Journal* 13:444 https://doi.org/10/1186/1475-13-444.

Gneme, A., Guelbeogo, W.M., Riehle, M.M., Sanou, A., Traore, A., Zongo, S., Eighneir, K., Kabre, G.B., Sagnon, N.F. and Vernick, K. D., (2013). Equivalent Susceptibility of Anopheles gambiae M and S. molecular forms and Anopheles arabiensis to Plasmodium Falciparaum infection in Burkina Faso. Malaria Journal 12:204.

Golding, N. (2…). Mapping Malaria with Gaussion Processes: Malaria atlas project, Part Malaria; Spatial ecology and epidemiology group. In: ml.dcs.shef.ac.uk/gpss/gpst14/gpgpst14 NGMAP.pdf. accessed July 10 2018.

Gomenou, M. (2016). Introduction to toxicology: The dose-response and basic concepts. European Food Safety Authority; Scope and ethical Principles of toxicology and 3rd Eurotox course of basic toxicology, Zagreb, Croatia.

Gonzales, K.K. and Hansen, I.A (2016). Artificial diets for mosquitoes. *International Journal of Environmental Research and Public Health*, 13, 1267; doi 10.3390/ijerph 13121267

Goodman, L.S., Gitman A. and Brunton, L.L. (2015). The pharmacological basis of Therapeutoties: Neostigmine (Professional Patient Advice); In http://www.drugs.com/ppa/neostigmins.html. accessed 12Aug. 2015.

Gotifrid G. M. Urasa, F. M., Katunzi, G., Yaro, J. G., Munga, S. and Kweka, E. J. (2014). Reduced hatchability of *Anopheles gambiae s.s.* eggs in presence of third instar larvae. *Biomedical Centre Research Notes*. 7:231

Goutam, C. Anupam, G., Indranil, B. and Ghosh, S.K. (2013). Use of larviporous fish in biological and environmental control of disease vectors. In: Biological and Environmental control of Disease Vectors (ed. By Gameron M. and Lorenz L.M.), Wallingford, UK, CABI.

Govindarajan, M. and Karappannan, P. (2011). Mosquito Larvicidal and Ovicidal properties of Edipta alba (L). Hassk (Asteraceae) against Chikungunya vector, Aedes aegypti (Linn.) (Diptera: Culicidae). Assian Pacific Journal of Tropical Medicine Vol. 4, issue 1, January 2011, Pages 24-28. 4(1): 24-28.

Govoetchan, R., Sovi, A., Aikpon, R. and Salako A., (2013). The impact of oviposition – site deprivation in gravid females of Anopheles gambiae (Diptera: Culicidae) on fecundity, trophic behaviour and life expectance. International *Journal of Tropical Insect – Science volume* 33, Issue 4, pp. 207-215.

Griffin, J. T. Ferguson, N.M. and Ghani, A.C. (2014). Estimates of the Falciparum Malaria disease in Sub – Sahan Africa. Nature Communications 5, Article number; 3136.

Grisales, N., Poupardian, R., Gomez S. Gonzalez, I. F. Ranson, H. and Lenhart, A. (2013). Temephos resistance in Aedes aegyph in Columbia compromises Dengue vector control. *Journal of PLoS ONE Neglected Tropical Disease*. 7 (9): e 2438

Guerrero – Ruiz, R., Perez-Rodriguez, M.A., and Campos-Norzagaray, M. (2015). Toxicity of native plant extracts against larvae of Aedes aegypti (Diptera: Culicidae). *Asian Pacific Journal of Tropical Biomedicine volume* 5, Issue 4, pp. 287 – 291.

Gunasekara A.S. (2005). Environmental fate of Pyrethrins. Environmental monitoring Branch, Department of Pesticide Regulation, 10011 Street Sacramento, CA95812.

Gurusamy, R. and Natarjan, S. (2013). Current status on Biochemistry and Molecular Biology of microbial degradation of Nicotine. The Scientific World Journal volume 2013 Article ID 125385, 15 pages http://dx.doi.org/10.1155/2013/125385.

Hancock, P.A., Wiebe, A., Gleave, K.A., Bhatt, S., Cameron, E., Trett, A., Weetman, D., Smith, D.L., Hemingway, J., Coleman, M., Gething, P.W. and Moyes, C.L. (2018). Associated Patterns of insecticide resistance in field populations of malaria vectors across Africa. Proceedings of the National Academy of Sciences of the United States of America (PNAS) 115 (23): 5938-5943.

Harshmi, L.S.A, Hossain, M.A. Well., A.M., Al-Riyami Q, Al-Sabahi, J.N. (2013). Gas Chromatography – Mass spectrometry analysis of different organic crude extracts from the local medicinal plant of Thymus vulgaris L. Asian Pacific Journal of Tropical Biomedical 2013 Jan., 3(1): 69-73. doi:10:1016/52221-1691 (13) 60026-x. *Journal of Tropical Biomedical Research*. 3(1) 69-73.

Hartung, A. and Commission, M.A.K. 92016). Pyrethrum (MAK, Value Documentation) 2008. Wiley Online Library. In: https://doi.org/ 10/1002/3527600418 .mb800334e4516.

Hashmi, L.S.A, Hossain, M.A., Weli, A.M., Al-Riyami, Q and Al-Sabahi, J.N. (2013). Gas Chromatography – Mass spectrometry analysis of different organic crude extracts from the local medicinal plant of Thymus vulgaris L. Asian Pacific *Journal of Tropical Biomedicine* (APJTB), 3(1): 69 – 73..

Hazrat B., Waseem, A.; Hafiz, A.A.K. soaib, A.H.; and Intinan, A.K. (2012). Toxicity of selected indegenoous plant extracts against *Aedes allopictus* (Diptera: Culicidae): A potential Dengue Vector in Dengue positive areas. *Pakistan Journal of Zoology*. 44 (2), pp. 371 – 375.

Heuler, H. (2013). Missing the forest for the trees. In: Uganda believes tree plantations are a perfect solution to the nations deforestation crisis. Critics say planting non-native trees causes more harm than good. *Earth Island Journal*. 27:4 in: www.earthisland.org/journal/index.php/issues/toc/winter-2013/

Hikal, W.M., Baechen, R.S. and Said – A- A. (2017). Botanical Insecticides as simple extractives for pest control. *Cogent Biology*, 3(1), 1404274. http://doi.org/10.1080/23312025.2017.1404274.

Hitayati, H., Nazni, W.A. Hanlim, L. and Azirum, M.S. (2011). Insecticide resistance development in Aedes aegypt upon selection pressure with malathion. *Journal of Tropical Biomedicine*28(2): 425-437).

Holland –Letz, T. and Kopp-Schneider, A. (2015) Optimal experimental designs for dose-response studies with continous endpornts. Archives of Toxicology 89 (II): 2059-2068.

Horton, M. K., Rundle, A., Whyatt, R. M., Rauh, V. A., Camann, D. E., and Barr, D. B, (2011). Impact of Prenatal Exposure to Piperonyl Butoxide and Permethrin on 36 – month Neurodevelopment. *Journal of Paediatrics*. 127 (3): e699-e706.

Hoy, J., Swanson, N. and Seneff, S. (2015). The high cost of pesticides: Human and animal diseases. Poultry, Fisheries and Wildlife Sciences 3:132. doi.10.4172/2375-446x.1000132.

Hu, T. and A.H. (2015). Tobacco Control and Tobacco Farming in African countries. *Journal of public Health Policy* 36(1): 41-51.

Ileke, K. D., Oyeniyi, E. A, Ogungbite, O. C. and Adesina, J. M. (2015). *Nicotiana tabaccum* a prospective mosquitocide .

Ileke, K.D., Ogungbite, O.C., Oyeniyi, E. and Adesina, J.M., (2015). Nicotiana tabaccum a prospective mosquitocide in the management of Anopheles gambiae (Giles) *International Journal of Mosquito Research* 2(4): 19-23.

Ileke, K.D., Oyeniyi, A.E., Ogungbite O.C. and Adesina, J.M. (2015). Nicotiana tabaccum a prospective mosquitocide in the management of Anopheles gambiae (Giles). *International Journal of Mosquito Research*2(4): 19-23.

Imam, H. Sofi, Z.G. and Seikh, A. (2014). The basic rules and methods of mosquito rearing (Aedes aegypt). Trop Parasitol 2014 Jan – Jun, 4(1): 53-55. *Journal of Tropical Parasitology*. 4(1) 53-55.

Imbahale, S. S., Mukabana, W. R., Orindi, B., Githeko, A. K. and Takken, W. (2012). Variation in Malaria transmission dynamics in three different sites in Western Kenya. *Journal of Tropical Medicine*. 201(2012), Article ID 912408, 8 pages http://dx.doi.org/10.1155/2012/912408.

Imbahale, S. S., Paaijmans, K. P. Mukabana, W. R., Lammeren, R., Githeko, A. K. and Takken, W. (2011). A longitudinal study on Anopheles mosquito larval abundance in distinct geographical and environmental settings in Western Kenya. *Malaria Journal*. 10:81.

Improved Ref: http://dx.org/10.5772/54853. or intechopen, doi:10.5772/54853. available from: https://www.intechopen.com/books, anopheles-mosquitoes-new-insights-into-malaria-vectors/systematic-techniques-for-the-recognition-of-anopheles species-complexes. Or

Intellectual Ventures Lab (2014). Mosquito rearing. In: IV labarchive.com.70-32-104-217. intrenpreview.com/?page-id=3771, Retrieved 8.11.2013.

Intellectual Ventures Laboratory (2014). IV Lab is developing an artificial mosquito diet to help scientists and health programmes raise mosquitoes effectively. In:www.intellectualventureslab.com/work/artificial-diet). Retrieved 8.11.13.

Interactive Learning Paradigms Incorporated (ICPI) (2016). The MSDS hyper glossary: "Acute toxicity" safety emporium: Methods for determination of acute toxicity. In: www.ilpi.com/msds/ref/acute toxicity.html (accessed June, 18, 2018.

Irving, H. and Wondji, C. (2017). Investigating knockdown (Ledr) mechanisms against pyrethroids/DDT in malaria vector, Anopheles funestus across Africa. *BioMedical Central* 18:76.

ISGlobal Barcelona Institute for Global Health (2017). Innovative tools to tackle malaria and other vector-borne diseases urgently needed. Universitat Barcelona Institute for Global Health C/Rossello, 132, 5th. 2nd 08036.

israelensis WDG in Dengue vector from Lahore, Pakistan. *Pakistan Journal of*

Iwuanyanwa, K. C. P., Amadi, U., Charles, I.A. and Ayalogu, E. O. (2012). Evaluation of acute and sub-chronic oral toxicity study of Baker cleansers Bitters – A poly-herbal drug on experimental rats. *Journalof Experimental and Clinical Sciences*. 11:632

Janko, M.M. Irish, S.R., Reich, B.J., Peterson, M., Doctor, S.M. and Mwandagalirwa, M.K. (2018). The links between agriculture, Anopheles mosquitoes and malaria risk in children younger than 5 years in the Democratic Republic of Congo: a population –based, cross sectional, special study. *The Lancet* volume 2, Issue 2 PE 74 – E82.

Jayakumar, J., Elumalai, K., Dalmin, G. and Krishnamurthy, R. (2014). Larvicidal and synergistic activity of *Anisomeles malabarica* and *Phyllanthus emblica* against the larvae of common malaria vector, *Anopheles stephansi* Liston. *Journal ofDiscovery*.20: 62.

Jew, E.K.K., Dongill A.J. and Sallu, S.M. (2017). Tobacco Cultivation as a driver of Land use change and degradation in the Miombo woodlands of South-West. *Tanzania Land Degradation Development* 28.2636-2645.

Johan, N and Shahid, A. (2012). Evaluation of resistance against *Bacillus thuringiensis*

Juan, L.W., Lucia, A., Zerba, E.N., Harrand, L., Marco, M. and Masuh, H. M. (2011). Chemical Composition and fumigant toxicity of the essential oils from 16 species of *eucalyptus* against *Haemotobia irritans* (Diptera:muscidae) adults. *Journal of Economic Entomology*. 104 (3) 1087-1092.

Jucht, C.C, Erguler, K., Shek, C.Y. Basanez, M.G. and Parham, P.E. (2015). Modelling Anopheles gambiae s.s. population dynamics with temperature and age dependent survival. International Journal of Environmental Research and Public Health 12.5975-6005, doi: 10.3390/ijerph120605975.

Jucht, C.C. Parham, P., Saddler, A., Koella, J.C. and Basanez, M.G. (2014). Temperature during larval development and adult maintenance influences the survival of Anopheles gambiae s.s. Parasites and Vectors 7:489.

Jucht, C.C. Parham, P.E. Saddler, A., Koella, J.C. and Basanez, M.G. (2014). Temperative during larval development and adult maintenance influences the survival of Anopheles gambiae s.s. *Parasites and Vectors* 7:489.

Kabbale, F.G., Ako, A.M., Kaddu, J.B., Matovu, E., Kazibwe, A., Yadouleton, A. and Onapa, A.W. (2016). Molecular identification of Anopheles gambiae sensu stricto Giles (Formerly Anopheles gambiae Savannah Firm) in Kamuli District Uganda. African Journal of Biotechnology, vol. 15(39), pp. 2124-2131.

Kajeguka, D.C., Kaaya, R.D., Mwakalinga, S., Ndossi, R., Ndaro, A. Chilongola, J.O., Mosha, F.W., Schloler, K.L., Kavishe, R.A. and Alifrangis, M. (2016). Prevalence of dengue and Chikunguya virus infections in North-Eastern Tanzania. *Biomedical Central (BMC) Diseases Journal* 11(1);, (183) doi: 10.1186/S12879-016-1511-5.

Kakkilaya, B. S. (2013). Source reduction in mosquito control. Dr. B. S. Kakkilaya's malaria website 2011-2013. In: malariasite.com2011-2013;www.malariasite.com/ malaria/source.htm and efficacy of source reduction in mosquito control. In:www.appropedia.org/efficacy-of-source-reduction-in-mosquito control. Retrieved 3.10.2013.

Kamali, M., Sharakhova, M. V., Banicheva, E., Dmitrii, K., Zhijian, T., Sharakhov, I. V. (2011). An integrated chromosome map of microsatellite markers and inversion breakpoints for an Asian malaria mosquito, *Anopheles stephensi. Journal of Heredity*. 102(6):119-726.

Kamau, ., Koekemoer, L.L. Hunt, R.H. and Coetzee M. (2003). Anopheles Parensis: The main member of the Anopheles funestus specis group found resting inside human dwellings in Mwea area of Central Kenya toward the end of the rainy season. *Journal of the American Mosquito Control Association*, 19(2): 130-133.

Kamdem C. Fossog, B.T. Simard, F., Etouna, J., Ndo, C., Kengne, P., Bousses, P., Etoa, F.X. Ambene, P.A., Fontenille, D., Nkondjio, C.A., Besansky, N.J. and Costantin, C. (2012). Anthropogenic habitat disturbance and ecological divergence between incipient species of the malaria mosquito Anopheles gambiae. PLOS ONE https://doi.org/10.137/journal, pone. 0039453.

Kansas State University (2018), Problem: Tree cavities: Kansas State University Research and Extension, 2021 Throckmorton Plant Sciences Center, Kansas State University, Manhattan, KS 66506: 785.532.6173 In:https:// hnr. kstate.edu/extension/infor-center/commonpest-problem/common-pest-problem-new/Tree-cavities.

Kansas State University (2018). Problem: Tree Cavities. Kansas State University Research and Extension, 2021 Throckmorton, Plant Sciences center, Manhattan, KS 66506:785.532.6173 In: https://hnr.kstate.edu/extension/infor-center/common-pest-problems/common-pest-problem-new/Tree cavities Accessed 5.7.2018.

Kant, R., Hay, S., Srivastava, H.C. and Sharma V.P. (2013). Review of the bioenvironmental methods for malaria control with special reference the use of larvivorous fishes and composite fish culture in central Gujarat, India. *Journal of Vector Borne Diseases* 50:1-12.

Karoki, S.M (2013). The role of agricultural chemicals on Anopheles arabiensis vector 'fitness' in malaria transmission in a rise agro-ecosystem in Kenya. A thesis master of Science in Epidemiology and Laboratory management submitted in particle fulfillment of the requirements for the degree of the Jomo Kenyatta university of Agriculture and Technology.

Karthikeyan, R. Venkatesh, P. and Chandrasekhar, N. (2012). Morpho anatomical studies of Leaves (leaves) of Abutilon indicum (linn) sweet. *Asian PacificJournal of Tropical Biomedicine*.1691(12) 5464-5469

Karthikeyan, V., Sivakumar, K., Gokuldass, A. and Mohanasundaram, S. (2012). Studies on larvicidal activity of *Leucas aspera, Vitex negundo and Eucalyptus thailam* against *culex quinquefastiatus* collected from Coovum river of Chennai, India. *Asianjournal of Pharmaceutical and clinical research*. 5: 3.

Karthkeyan, V. , Sivakumar, K., Gokuldass, A. and Mohanasundaram, S. (2012). Studies in larvicidal activity of Leucas aspera, Vitex negundo and Eucalyptus against Culex quinquefasciatus collected from Coovum River of Chennai, India. *Asian Journal of Pharmaceutical and Clinical Research*. 5: 3

Karunamoorthi, K., Girmay, A. and Hayleeyesus, S. F., (2014). Mosquito repellent activity of essential oil of Ethiopian ethonomedicinal plant against Afro-tropical malarial *Anopheles Stephensi*, *Journal of King Sand University. Science*. 26 (4) : 305-310

Karunamourth, K. and Sabesan, S. (2012). Insecticide resistance in insect vectors of disease with special reference to mosquitoes: a potential threat to global public health. *Health Scope* 2(1): 4-18.

Katzung, B.G., Masters S.B. and Trevor, A.J. (2012). Basic and Clinical Pharmacology, 12e. Section ix Toxicology. Chapter 56 Introduction to Toxicology: Occupational and Environmental. McGraw-Hill Global Education Holdings, LLC.

Kawada, H. (2017). Possible now controlling measures for the pyrethroid-resistant malaria vectors? *Annals of Community Medicine and Practice*, 3(1): 1019.

Kellison, R.C., Lea, R. and Marsh, P. (2013). Introducton of Eucalyptus spp. Into the United States with special emphasis on the Southern United States. *International Journal of Forestry Research*, volume 2013, Article ID 189393, 9 pages, http://dx.doi.org/10/1155/2013/189393.

Kenya Tobacco Control Country Profile. Government of Kenya (GoK), 2013). "Tobacco Industry Interference in Kenya: Exposing the tacticis": A joint report by the Ministry of Public Health and Sanitation (MoPH&S), International Institute for Legislative Affairs (ILA) and Centre for Tobacco Control in Africa (CTCA). In: Uakenya.org/wp-content/uploads/2015/10/ Ti-Interference-in-Kenya. Pdf Accessed 21-6.2018.

Kesteman, T., Randrianarivelojosia, M. and Rogier, C. (2017). The protective effectiveness of control interventions for Malaria prevention. A systematic review of the literature (Version 1, referees: 1 approved, 2 aprpoved with reservation) Fl000 REsarch 6: 1932(doi:10.12688/f1000research.12952.1).

Khan, I. (2010). Rearing of *Anopheles arabiensis* mosquitoes for use in sterile insect technique programme. A study report submitted to insert pest control laboratory, Food and Agricultural Organization/International Atomic Energy Agency (FAO/IAEA) Agricultural and Biotechnology Laboratories Seibersdorf, Austria.

Khan, I., Damiens, D., Solibani, S. M. and Gilles, J. R. L. (2013). Effects of drying eggs and egg storage on hatchability and development of *Anopheles arabiensis*. *Malaria Journal*. 12:318.

Khan, I., Farad, A. and Zeb, A. (2013). Development of inexpensive and globally available larval diet for rearing *Anopheles stephensi* (Diptera. Culicidae) mosquitoes. *Parasites and Vectors*. 6:90

Khan, M.M. Shah, S.W.H, Akhter, I. and Malik, H. (2017). Integrated Pest Management of fruit flies in guava orchids. *Journal of Entomology and Zoology Studies*, 5(2): 135 – 138.

Khater, H. F. (2011). Ecosmart Biorational Insecticides: Alternative insect control strategies, In: "Insecticides" – advances in integrated pest management, Dr. Farzana Perveen (Ed.). ISBN: 978-953-307-780.

Khater, H. F. (2012). Prospects of botanical biopesticides in insect pest management *Journal of Applied Pharmaceutical Science* ; 02(05): 244- 259.

Khater, H.F. (2012). Ecosmart Biorational insecticides: Alternative insect control strategies. In: "Insecticides", Perveen, F. (Ed).

Khubeiz, M.J., Mansour, G. and Bahira, Z. (2016). Chemical composition and antimicrobial activity of leaves Eucalyptus camaldulensis essential oils from four Syrian samples. International Journal of current Pharmaceutical Review and Research; 7(5); 251-257.

Kipyab, C. P., Khaemba, B. M., Mwangangi, J. M., and Mbogo, C. M. (2013). The bionomics of *Anopheles merus* (Diptera:culicidae) along the Kenyan Coast. *Parasites and Vectors*. 6:37.

Kissel, J.C. (2011). The Mismeasure of dermal absorption. *Journal of Exposure Science and Environmental Epidemiology*, 21(3): 302-309.

Kitau, J., Oxborough, R.M., Tungu, P.K, Matowo, J., Malima R.C., Magesa S.M. Bruce, J., Mosha, F.W. and Rowland M.M. (2012). Species shifts in the Anopheles gambiae complex: DOLLINs successfully control Anopheles arabiencies? PLoSONE 7(3): e31481.

Kivuyo, H.S. Mbazi, P.H., Kisika, D.S., Munga, S., Rumisha, S.F., Urasa, F.M. and Kweka, E.J. (2014). Performance of five food regimes on Anopheles gambiae

senso stricto larval rearing to adult emergence in insectany. *PLOSONE* 9(10): e11067/.https://doi.org/10.137/journal.pone.0110671.

Kobylinski K.C. Sylla, M., Black, W. and Foy, D.D (2012). Mermithid nematodes found in adult Anopheles from South-Eastern Senegal. *Parasites and Vectors*, 5: 131.

Koininki, H., Hance, T. Djossou, J., Nondjou, F., Lognay, G., Malaisse, F., Nagassoum, M.B., Mapongmetsem, P.M, Ngamo, T.L, and Hanbruge, E. (2010). Persistent effect of a preparation of essential oil from *Xylopia aethiopia* against *Callosobrunchus machulatus* (Coleoptera, Bruchidae). African Journal of Agricultural Research; 5:1881-1887.

Koininki, H., Hance, T., Nandjou, F.A., Lognay, G., Malaisse, F. ,Nagassoum, M.B., Mapongmetsem, P.M., Ngamo, T.L and Haubruge, E. (2007). Toxicity of some terpenoids of essential oils of *Xylopia aethiopica* from Cameroon against *Sitophillus zeamais* Motschulsky. *Journal of Appied Entomology*. 131:269-274.

Koitenya, (2012). World Tobacco Atlas rings alarm bells for India. TNN/Mar 29, 2012, 03.05am 1st In: Times of India. Indiatimes.com/india/world-Tobacco-Atlas-rings-alarm-bells-for-India/articleshow/12448418.cms?src=fbw. Retrieved 10.3.2013.

Kshetrimayum, B. (2017). Medicinal plants and its therapeutic uses. DOI: http://doc.doi.org/10.4172/978-1-63278-074-4-075. Published by OMICS Group eBooks. In: https://www.researchgate.net/profile/kshetrimayum_Burka-Singh/publication/39643644-medical-plants-anc.

Kudom, A. A., Mensah, B. A. and Agyemang, T. K. (2012). Characterization of mosquito larval habitats and assessment of insecticide – resistance status of *Anopheles gambiaesenso lato* in urban areas in South-Western Ghana. *Journal of Vector Ecology*. 37(1): 77-82.

Kulma, K., Saddler, D., and Koella, J. C. (2013). Effects of age and larval nutrition on phenotype expression of insecticide – resistance in *Anopheles* mosquitoes. *Journal of PLoS ONE*. 8 (3)e58322.

Kumar, S., Cruz, M. A. Gupta, L., Rodrigues, J., Mury, B. C. (2010). A peroxidase-dual oxidase system modulates midgnt epithelial immunity in *Anopheles gambiae*; *Journal of Science*.327 : 1644 – 1648.

Kumar, S., Wahab, N., Mishra , M. and Warikoo, R. (2012). Evaluation of 15 local plant species as larvicidal agents against an Indian strain of Dengue fever mosquito, Aedes aegypti. *Journal of Front Physiology*. 3:104.

Kweka, E. J. (2012). Effects of co-habitation between *Anopheles gambiae* s.s. and *Culex quinquefasciatus* aquatic stages on life history traits. *Parasites and Vectors*. 5(1) : 33

Kweka, E. J., Owino, E.A., Mwang'onde, B.J. Mahande, A.M, Nyindo, M. and Mosha, F. (2011). The role of cow urine in the oviposition site prevalence of culicine and Anopheles Mosquitoes. *Parasites and Vectors* 4:184.

Kweka, E., Kimaro, E.E. and Munga, S. 9206). Effect of deforestation and land use changes on mosquito productivity and development in Western Kenya highlands: implication to malaria risk. *Frontiers in Public Health* 4:238, doi: 10.3389/fpubh. 2016.00238.

Kweka, E.J., Munga, S., Himeidan, Y., Githeko, A.K. and Yan, G., (2015). Assessment of mosquito larval productivity among different land use types for targeted malaria vector control in the Western Kenya Highlands. *Parasites and Vectors* 8:356 https://doi.org/10.1186/S1307-015-0968-1.

Lacey, A. A. (2012). Manual of Techniques in invertebrate pathology, 2nd ed. Elsevier, London 2012. Chapter IV Bioassay of Bacterial endomopathogens against insect larvae; Bioassay of Baccillus thuringiensis against Diptera larvae pp. 113.

Langerhans, R.B. and Riesch R. (2013). Speciation by selection: A framework for understanding ecology's role in speciation. Current Zoology 59(1): 31-52.

Lawal, H. O., Adewuyi, G. O., Fawehinmi, A. B., Adeogun, A. O. and Etaturie, S. O. (2012). Bioaasay of herbal mosquito repellent formulated from the essential oil of plants. *Journal of Natural Products*. 5 : 109 – 115.

Lee, H. (2018). Register now for free live web event on epidermics. The New English Journal of Medicine (NEJM): In:https://blogs.nejm.org/now/index.php/epidermics-going---/20/

Lee, Y., Marsden, C.D., Norris, L.C., Collier, T.C., Main, B.J., Fotana, A., Corne, A.J. and Lanzaro, G.C. (2013). Spatiotemporal dynamics of gene flow and hybrid fitness between the M and S forms of the malaria mosquito Anopheles gambiae. Proceeding of the National Academy of Sciences (PNAS) 110(49):19854-19859.

Liao, M., Xiao, J.J., Zhou, L.J. Yao, X., Tang, F., Hua, R.M., Wu, X.W. and Cao, H.Q. (2017). Chemical composition, insecticidal and biochemical effects of Melalenca alternifolia essential oil on the Helicoverpa armigera. Journal of Applied Entomology volume 141 Issue 9 pages 721 – 728.

Lili. L.B, Laith, Y., Bian, L. and Yan, G. (2009). Temporal and Spatial stability of Anopheles gambiae larval habitat distribution in Western Kenya. International *Journal of Health Geographics* 8(1)70

Linah, J. N. Okal, M.N., Varela, M.H., Karlson, A.K.B, Torto, B., Lindsay, S.W. and Fillinger, U. (2015). Discovery of an oviposition attractant for gravid malaria vectors of the Anopheles gambiae species complex. *Malaria Journal* 14:119.

Loose-leaf chewing tobacco in India and Bangladesh. In:www.medical news today. com/articles/249233.php. Retrieved 2.4.2014

Loughrin, J. Anderson, R.A., Hamilton – Kemp, T.R., Hildebrand, D.F., and Andersen, R.A. (1990). Headspace compounds from flowers of Nicotiana tabaccum and related species. *Journal of Agriculture and Food Chemistry* 38(2).

Low, V.L., Chen, C.D., Lee, H.L., Tan, T.K., Chen, C.F., Leong, C.S., Lim, Y.A.L., Lim. P.E., Rashid Y.N. and Azirun, M.S. (2013). Enzymatic characterization of insecticide resistance mechanisms in field populations of Malaysian Culex quinguefasciatus Say (Diptera: Culicidae). PLOSONE 8(11): e79928.doi: 10.1371/journal.pone.0079928.

Lu, G. Traore, C., Meissner, P., Konyate, B., Wolf, G.K., Beiersmann, C., Safety of insecticide-treated mosquito nets for infants and their mothers randomized controlled community trial in Burkina Faso . *Malaria Journal* 14: 527.

Lufele, E., Umbers, A., Ordi, J. and Ome M. (2017). Risk factors and pregnancy outcomes associated with placental malaria in a prospective cohort of Papua New Guinean Women. *Malaria Journal* 16.427.

Lugo, A., Bosetti, C., Peven, G., Rota, M., Bagnardi, V. and Gallus, S. (2017). Dose-response relationships between cigarette smoking and site specific caner risk: Protocal for a systematic review with an original design combining umbrella and traditional reviews. British Medical Journal (BMT) vol. 7 issue 10.

Lusk, C.H. Kelly, J.W.K and Gleason, S.M (2013). Light requirements of Australian tropical is cool-temperate rain forest tree species show different relationship with seedling growth and Functional traits. *Analysis of Botany*, vol. III (3): 479 – 488.

Lutomiah, J., Bast, J., Clark, J., Richardson, J., Yahwala, S., Oullo, D., Mutisya, J. Mulwa, F., Musila, L., Khamadi, S., Schnabel, D., Wurapa, E. and Sang. R. (2013. Abundance, diversity and distribution of mosquito vectors in selected ecological regions of Kenya: Public Health implications. *Journal of Vector Ecology*. 38(1): 134 – 142.

Lynne, M., Brendan, G., and Hean, R. (2010).*Eucalyptus* dunnii. Prime facts for profitable, adaptive and sustainable primary industries. New South Wales (NSW) Government, Industry and Investment 2010,

Maciel, M. V. Morais, S. M., Bevilaqua, C. M. L, Silva, R. A., Barros, R. S., Sousa, R. N., Sousa, L. C., Brito, E. S. and Souza-Neto, M. A. (2010). Chemical composition of *Eucalyptus spp*. essential oils and their insecticidal effects on *lutzomyia longipalpis*. *Journal ofVeterinary Parasitology*. 167:1-7.

Mackay, E. M. and Ross, H. (2012). The Tobacco Atlas, Fourth Ed. Atlanta, GA: American Cancer Society; NewYork, N.Y; *World Lung Foundation*.

Madakacherry O. Lees, R.S. Gilles, J.R.C (2013). Aedes albopictus (Skuse) males in Laboraory and semi-field cages. Release ratios and mating competitiveness. Acta Tropica xxx(2013). Journal of Acta Tropica (2013).

Magesa, S. Kisinza. W. and David, J. P. (2014). Insecticide resistance mechanisms associated with different environments in the malaria vector *Anopheles gambiae*: a case study in Tanzania. *Malaria Journal*. 13:28.

Mahande, A. M., Dusfour, I., Matias, J. R. and Kweka, E. J. (2012). Knockdown resistance Roll alleles, and the annual entomological inoculation rate of wild mosquitoes populations from Lower Moshi, Northern Tanzania. *Journal of Global infectious Diseases*. 4 (2): 114 – 119.

Maia, M.F. and Moore, S.J (2011). Plant-Based insect repellents: A Review of their efficacy, development and testing. *Malaria Journal* 10(Suppl 1): S11.

Maia, M.F. Robinson, A., John, A., Mgando, J., Simfukwe, E. and Moore, S.J (2011). Comparison of CDC backpack aspirator and the Prokodack aspirator for sampling indoor and outdoor – resting mosquitoes in Southern Tanzania. Parasites and Vectors 4:124.

Maia, M.F., Robinson, A., John A., Mgando J., Simfukwe, E. and Moore S.J. (2011). Comparison of the CDC backpack aspirator and the Prokopack aspirator for sampling indoor – and outdoor- resting mosquitores in Southern Tanzania. Parasites and Vectors 4:124.

Maiga H., Damiens D. Diabate, A. Dabire, R.K., Ouedraogo, G.A., Lees, R.S. Gilles, J.R.L (2016). Large scale Anopheles arabiensis egg quantification methods for mass rearing operations. Malaria Journal 15:72. *Malaria Journal*. 15:72.

Maiga, H., Bimbile – Sonda, N.S., Yamada, H., Wood, H. and Gilles, J.R.L. (2017). Enhancements to the mass-rearing cage for the malaria vector, Anopheles arabiensis for improved adult longevity and egg production. *Entomologia Experimentalis et Applicata*. 2017/05/04.

Maiga, H., Damiens, D., Diabate, A., Dabire, R.K., Onedraogo, G.a., Lees R.S. and Gilles, J.R.L. (2016). Large scale Anopheles arabiensis egg quantification methods for mass ranking operations. Malar. J. 2016: 15:72. Malaria Journal 15:72.

Mains J.W. Brelsfoard, C.L. and Dobson S.L. (2015). Male mosquitoes as vehicles for insecticide: PLos Neglected Tropical Diseases 9(1): eooo3406

Malaria Research and Reference Reagent Resource Centre (MR4), (2014). Methods in Anopheles Research. Fourth Edition, CDC ATCC chapter 2: Anopheles Laboratory Biology and Culture pp 1-10.

Mamai, W., Sonda, N.S.B., Maiga, H., Juarez, J.G., Muosa, Z.A.I, Ali, A.B., Lees, R.S., and Gilles, J.R.L., (2017). Optimization of mosquito egg production under mass rearing setting: Effects of cage volume blood meal source and adult population density for the malaria vector, Anopheles arabiensis. *BIOMedical Central* 16:41.

Mancini E. Spanaci, M.I. Gordicho, V., Caputo, B., Pombi, M., Vicente, J.L., Dinis, J., Rodrigues, A., Petraria, V., Weetman, D., and Pinto, J. and Torre, A. (2015). Adaptive potential of hybridization among malaria vectors: Introgression at the immune locus TEP1 between Anopheles coluzzii and A., gambiae in 'Far-West' Africa. *PLOSONE* 10(6): e0127804.https://doi.org/10/1371/journal.pone.0127804.

Mann, R. S. and Kanfman (2012).Natural Product Pesticides: Their Development, Delivery and use against insect vectors. *Journal ofMini-Reviewsin Organic Chemistry*. 9: 185 – 202.

Mann, R.S. and Kaufman, P.E. (2012). Natural product pesticides: Their development, delivery and use against insect vectors. Mini-Reviews in Organic Chemistry Volume 9, Issue 2.

Manoukis, N. C. Diabate, A. Abdoulaye, A., Diallo, M. Dao, A., Yaro, A. S., Ribeiro, J. M. C and Lehmann, T. (2009). Structure and dynamics of male swarms *of Anopheles gambiae*. In press, *Journal of Medical Entomology pdf*.46(2) 227-235.

Manoukis, N. C., Diabate, A., Abdoulaye, A., Diallo, M. Dao, A., Yaro, A. S. Rebeiro, J. M. C. and Lehmann, T. (2010). Structure and dynamics of male swarms of *Anopheles gambiae. Journal of Medical Entomol*ogy. 46(2): 227 – 235.

Manoukis, N.C. Butail, S., Diallo, M., Ribeiro, J.M.C, and Paley, D.A. (2014). Stereoscopic Video Analysis of Anopheles gambiae behaviour in the field: Changes and opportunities. Acta Tropica, volume 132, pp. 580 – 585.

Marcombe, S., Farajollahi, A., Healy, S., Clark, G.G. and Fonseca, D.M. (2014). Insecticide resistance status of United States populations of Aedes albopictus and mechanisms involved. PLOSONE 9(7): e101992.

Massebo, F., Balkew, M., Teshome, G. M. and Lindtorn B. (2013). Blood meal origins and insecticide susceptibility of *Anopheles arabiensis* from Chano in South-West Ethiopia. *Journal ofParasites and Vectors*. 6:44.

Matowo, J., Jones, C.M., Kabula, B., Ranson, H., Steen, K., Mosha, F., Rowland, M., and Weetman, D. (2014). Genetic basis of Pyrethroid resistance in a population of Anophelesis Arabiensis, the primary malaria vector in Lower Moshi North-Eastern Tanzania. Parasites and Vectors 7: 274.

Mattah, P.A.D, Futagbi, G., Amekudzi, L.K., Muttah, M.M., DeSouza, D.K. Kartey-Attipoe, W.D., Bimi, L. and Wilson, M.D. (2017). Diversity in breeding sites and distribution of Anopheles Mosquitoes in selected urban areas of Southern Ghana. Parasites and Vectors 10:25.

McAthur, C.C. Meredith, J.M. and Eggleston, P. (2014). Transgenic Anopheles gambiae. Expressing an antimalarial peptide suffer no significant fitness cost. PLOSONe, https://doi.org/10.1371/journal. pone.0088625.

Meador, J.P., Yeh, A. and Gallagher, E.P. (2018). Adverse metabolic effects in fish exposed to contaminants of emerging concern in the field and laboratory. *Environmental Pollution* 236:850-861.

Medhi, S.M., Peza, S.A., Mahnaz, K., Reza, A.M., Abbas, H. Fatemer, M. and Hassan, V. (2010). Phytochemistry and larvicidal activity of Eucalyptus camaldulensis against malaria vector, Anopheles stephensi. *Asian Pacific Journal of Tropical Medicine*. Volume 3, Issue II pp. 841 – 845.

Medical Dictionary for Health Professions and Nursing, Farlex 2012. In: medical-dictionary the freedictionary.com/toxicology. Retrieved 6.6.2014.

Medical Entomology Centre for Disease Control Department of Health and Families. Northern Territory Government. Revised edition 2010. In: www.health. nt.gov.an /library/scripts/objectifymedia. aspox? file=pdf/51/36/pdfdsite ID=1&str-title. Retrieved 14.1.2014.

MedlinePlus (2018), Smoking. Quit Smoking. Effects of smoking. United States National Library of Medicine. In: https://medlineplus.gov/smoking.html accessed July, 1, 2018.

Merchant M. (2016). Insects in the city: Elimination of tree holes for mosquito breeding. In: insectis in the city. Blogspot. Com/2016/05/elimination-of-tree-holes-for-mosquito.html. accessed 5.7.2018.

Merchant, M. (2016). Insects in the City: Elimination of tree holes for mosquito breeding. In: insectism the city.Blogspot.com/2016/05/elimination-of-tree-holes-for-mosquito.html. Accessed 5.7.2018.

MicroHealth (2014). Smoking is single most preventable cause of disease, disability and dealth in the United States. In:https://www.microhealth//c.corn/2014/02/number-one-cause-of-preventable-disease-in-the-US. Accessed July, 10, 2018.

Ministry of Health (2016). The epidemiology and Control profile of malaria in Kenya: Reviewing the evidence to guide the future vector control. National Malaria control programme, Ministry of Health. Technical support provided by the LINK project (London School of Hygiene and Tropical Medicine and the information for Malaria (INFORM) project, KEMRI-Welcome Trust Research Programme), Nairobi, Kenya, April, 2016.

Mokdad, A. H., Marks, J. S., Stroup, D. F., and Gerberding, J. L. (2014). Actual causes of death in the United States. *JAMA: Journal of the American Medical Association*. 291(10): 1238-45

Moore, E.L. Scott, M.A., Rodriguez, S.D., Mitra, S., Vulean, J., Gordova, K.K. and Harisen, I.A (2018). An online survey of personal mosquito repellent strategies. Peer J. doi: 10.7717/peerj.5151.

Moreta, S. T., Yewhalaw, D., Boets, P., Shamed, A., Duchatean, L., Speybroeck, N., Vanwambeke, S.O., Legesse, W., DeMcester, L. and Goethals, P.L.M. (2013). Physio-chemical and biological characterization of *Anopheles mosquito* larval habitats (Diptera: culicidae): Implications for malaria control. *Journal ofParasites and Vectors*. 6:320

Morphological differentiation In: www.majordifference.com/2013/10/difference between Anopheles- and - vs.html/#. U8VL90B9vIx. Retrieved 5.1.2014.

Morphological differentiation of male and female *Anopheles mosquitoes*. In: (profwaqarhussain) blogspot.com/12/2/10/diffentiate-culexAnopheles-and-aedes.html; www.majordifferences.com/2013/10/difference between-Anopheles and vs. html# U8VL90BvIX. Retrieved 15.3.2013.

Mosby's Medical Dictionary, (2012). Definition of potentiation. Mosby's Medical Dictionary, 9th edition 2012 (Mosby ed.) Elsevier, US and UK, 2012 In: Store Elsevier.com/Mosbys-Medical-Dictionary/-Mosby/isbn-9780323085410/.Retrieved 4.9.2012.

MR4 Chapter 2:*Anopheles* Laboratory Biology and Culture 2.4.2 Hatching *Anopheles* eggs. In: https/www/mr4.org/portals/3/pdfs/Anopheles/2.4.2%20Anopheles%20eggs%201.pdf ,Retrieved 8.11.2013.

MR4: Methods in Anopheles Research (2015). Male and female Anopheles corpulation. In MR4: Recently Revised training manual "*Methods in Anopheles Research*, Malaria World

Mulagoli, I., (2011). Pyrethrum farmers look to liberalization to reverse. www.irinnews.org/report/94172/Kenya-pyrethrum farmers-look-liberalization-reverse_fortanes.accessed 3.7.2018.

Mulyatno, K. C., Yamanaka, A., Ngatino and Konishi, E. (2012). Resistance of *Aedes aegypti* (L). larvae to temephos in Surabaya, Indonesia. *Southeast Asian Journal of Tropical Medicine and Public Health*. 43:1

Mulyatno. K.C., Yamanaka, A., Ngatino, and Kinishi, E. (2012). Resistance of Aedes aegypti (L.). Larvae to temephos in Surabaya Indonesia. *Southern Asian Journal of Tropical Medicne and Public Health* 43:1.

Munga, S., Vulule, J., and Kweka, E. (2013). Response of Anopheles gambiae s.l. (Diptera: Culicidae) to larval habitat age in Western Kenya highlands. *Journal ofParasites and Vectors*. 6:13 (http://www.parasitesandvectors.com.content/6/1/13.

Munhenga, G. Brooke, B.D. Gilles, J.R.L., Slabbert, K., Kemp, A., Dandalo, L.C., Wood, O.R. Lobb, L.N., Govender, D., Renke, M. and Koekemoer L.L. (2016). Mating competitiveness of sterile genetic sexing strain males (GAMA) under laboratory and semi-field conditions:Steps towards the use of the sterile insect technique to control the major malaria vector Anopheles arabiensis, in South Africa. Parasites and Vectors 9:122.

Munhenga, G., Brooke, B.D., Gilles, J.R.L., Slabbert, K., Kemp, A., Dandalo, L.C., Wood, O. R., Lobb, L.N., Govender, D., Renke, M. and Koekemwer, L.L. (2016). Mating competitiveness of stelile genetic sexing strain males (GAMA) under laboratory and semi-field conditions: steps towards the use of the sterile insect technique to control the major malaria vector Anopheles arabiensis in South Africa. *Parasites and Vectors* 9:122.

Murad, A. K., (2013). Effects of humic acid and water hardness on acute cadmium toxicity by using Simocephalus exspinosus as a bioindicator. *Euphrates Journal of Agriculture Science*.5 (4): 17-25.

Murmu. A., Kundu, S.B. Pahari, A., De. A., Adhikari, D. Pal. S., Bhattacharya, A. and Battacharyya, C.(2015). Effect of Ondansetron on the analgesic efficacy of tramadol used for postoperative analgesia: a randomized controlled study. Southern African. Journal of Anaesthesia and Analgesia volume 21: issue 5 pp. 135-139.

Murray, C. J., Rosenfeld, L. C and Lim, S. S. (2012). Global malaria mortality between 1980 and 2010: a systematic analysis. *Lancet*.379:413.

Mwangangi, J. M., Mbogo, C. M., Orindi, B. O., Muturi, E. J. Midega, J. T., Nzovu, J., Gatakaa, H., Githure, J., Borgemeister, C., Keating, J. and Beier J. (2013). Shifts in malaria vector species composition and transmission dynamics along the Kenyan Coast over the past 20 years. *Malaria Journal*. 12: 13.

Naiga, H., Damiens, D., Niang, A. Sawadogo, S.P., Fatherhaman, O., Tripet, F., Diabate, A. and Gilles, J.R.L. (2014). Mating competitiveness of sterile male Anopheles coluzzil in large cages. *Malaria Journal* 13:460.

Nair, S. S. Shetty, V. and Shetty, N. J. (2014). Relative toxicity of leaf extracts of *Eucalpytus globulus* and *Centella asiatica* against mosquito vectors *Aedes aegypti* and *Anopheles stephensi*. *Journal of Insects*; *Volume* 2014(2014), Article ID 985463, 7 pages, http://dx/doi.org/10.1155/2014/985463.

Nair, S.S. Shetty, V. and Shetty, N.J (2014). Relative toxicity of leaf extracts of *Eucalyptus globules* and *Centeka asiatica* against mosquito vectors *Aedes aegypti* and *Anopheles stephensi. Journal of Insects*. Volume 2014, Article ID 985463, 7 pages.

Nair, S.S. Shetty, V. and Shetty, N.J., (2014) citation. World Health Organization, "Instructions for determining the susceptibility or resistance of mosquito larval to insecticides'. Technical Report WHO/VBC/81.807. World Health Organization, Geneva, Switzerland, 1981. Journal of Insects dor: 10.1155/2014/985463

Narendhiran, S., Mohanasundaram, J., Arun, J., Saravanan, L., Catherine, M., Subathra, M. Rannjith, R.V., Prassana, Alagesan, Buvaneshuru (2014). Comparative Study in larvicidal efficacy of medicinal plant extracts against Culex quinquefasciatus. *International Journal of Research in Plant Science*. 4(1); 22-25.

Nasrabadi, M. H. Ahmadi, F. and Batooli, H. (2012). Chemical composition of essential oil and in vitro antioxidant activities of the essential oil and methanol extracts of *Eucalyptus loxophleba*. *Natural product Research*: Formerly *Natural Product Letters*. 26 :7

Nasrabadi, M. H., Pourmortazavi, S. M., Nazarian, S., Ahmadi, F. and Batooli, H. (2013) Chemical composition, antioxidane and antibacterial activities of the essential oil and methanol extracts of *Eucalyptus obosa* leaves. *International Journal of Food Properties*. 17 :5

Nasuhoglu, D., Westlund, P., Isazadeh, S., Neamatallah, S., Yargeau, V. (2016), Development of a facile and high throughout bioluminescence essay using Vibro fischeri to determine the chronic toxicity of contaminated samples. Bulletin of Environmental Contamination and Toxicology (December, 2016).

National Resource Defence Council (NRDC) (2010). Proposed conditional registration of a pesticide product. In: www.urde.org/living/chemicalindex/pyrethrum. Retrieved 3.4.2011.

Natural Standard Professioanl Monograph, (2013).*Eucalyptus* oil (*E. globulusLabikardiere*, *E. fructicetorum*, *F. Von Mueller*, *E. Smithe R.T. Baker*), www.naturalstandard.com. Retrieved August, 10. 2013.

Ndiaye et al (2016). Chemical Composition of 8 eucalyptus species essential oils and the evaluation of their antibacterial, antifungal and antivirual activities. Journal of Biomedical Centre Complementary and Alternative Medicine. 12:81.

Ndiaye, E.H.B., Gueye, M.T., Ndiaye, I., Diop, S.M., Diop, M.B., Fauconnier, M.L. and Lognay, G. (2016). Chemical composition of essential oils and hydrosols of three Eucalyptus species from Senegal: Eucalyptus alba Renu, Eucalyptus camaldulensis, Dehnh and Eucalpytus tereticornis Hook. American *Journal of Essential Oils and Natural products* 5(1) 01-07.

Nganjiwa, J. I, Pukuma, M.S., Philemon, J. and Ekundayo, T.M., (2015). Evaluation of larvicidal properties of leaves and root ethanolic extracts of some plants herbs against fourth instar mosquito larval. *International Journal of Scientific and Research Publications*5:3.

Nganjiwa, J. I., Pukuma, M. S., Philimon J. and Ekuudayo T. M., Evaluation of larvicidal properties of leaves and root ethanolic extracts of some plants herbs against fourth instar mosquito larvae. *International Journal of Scientific and Reseach Publications*. 5 : 3.

Nicotiana Rustica (wild Tobacco) contains about 10 times the nicotine of N. tabacum: http.en.wikipedia.org/wiki/tobacco#cite-ref-I. Retrieved 4.3.2013.

Nkya, T.E. Akhouayri I, Kisinza, and W., David, J.P. (2013). Impact of environment on mosquito response to pyrethroid insecticides: Facts, evidences and prospects. *Insect Biochemistry and Molecular Biology*, volume 43, Issue 4, pp 407 – 416.

Nkya, T.E., Alkhouayri, I., Poupardin, R., Batengana, B., Mosha, F., Magesa, S., Kisinza, W. and David, J.P. (2014). Insecticide resistance mechanisms associated with different environments in the Malaria vector *Anopheles gambiae*: a case study in Tanzania. *Malaria Journal*. 13:13-28

Noor, A.M. Alegana, V.A Kamwi, R.N. Hansford C.F., and Ntomwa B. (2013). Malaria Control and the intensity of Plasmodium Falciparum Transmission in Namibia. PLos ONE 8(5): e6335.doi:10,137//journal.pone.0063350.

Norksis, M. (2008). Statistical Procedures companion (2nd edition).

Northern Territory Government, (2013). Morphological differentiation will be used to identify male and female Anopheles mosquitoes. In (profwaqarhussain. blogspot.com/1212/10/differentiate – culexAnopheles – and aedes. Html; and www.majordifferences.com/2013/10/difference-between-Anopheles-and-Vs.html# U8VL90BvIX. Retrieved March, 15, 2013.

Norusis, M. (2008), Statistical Procedures companion (2nd edition)

Nowell, L.H. Norman, J.E., Moran, P.W, Martin, J.D., and Stone, W.W. (2014). Pesticide toxicity Index – a tool for assessing potential toxicity of pesticide mixtures to fresh water aquatic organisms. 476-477 (2014), 144-157.

Nwane, P., Etang, J., Chouaibou, M., Toto, J. C., Koffi, A., Mimpfoundi, R. and Simard F. (2013). Multiple insecticide resistance mechanisms in *Anopheles gambiae* S.I. populations from Cameroon, Central Africa. *Journal ofParasites and Vectors*. 6:41

O'Loughlin, S.M. Magesa, S.M, Mbogo, C., Mosha, F., Midega, J. and Burt, A., (2016). Genomic signatures of population decline in the malaria mosquito Anopheles gambiae. *Malaria Journal* 15:182 https://doi:org/10.1186/S12936-016-1214-9

Obala, A. A., Kutima, H. L. Nyamagoba, D. N., Mwangi, A. W., Simiyu, C. J. Magak, G. N. Khwa-Otsyula, B.O., and Ouma J. H. (2012).*Anopheles gambiae and Anopheles arabiensis* population densities and malactivity in Kopele village, Western Kenya. *Journal of Iinf ection in Developing Countries*.6(8):637-643

Obino, V. O., Kamau, L., and Gicheru, M. (2013). Infections of *Anopheles gambiae* mosquitoes with *Plasmodium* oocysts and its effects on highland malaria in Kisii town and its surroundings. *American Journal of Epidemiology and Infectious Diseases*.2 (1): 13-18.

Ochomo, E. Bayoh, N.B., Kamau, L., Atieli, F., Vulule, J., Ouma, C., Ombok, M., Njagi, K., Soti, D., Mathenge, E., Muthami, L., Kinyari, T., Subramanian, K., Kleinschmidt, I., Donnelly, J. and Mbogo C. (2014). Pyrethroid susceptibility of malaria vectors in four districts of Western Kenya. *Parasites and Vectors* 7: 310.

Ochomo, E., Chahilu, M., Cook, J., Kinyari, T., Bayoh, N.M., West, P., Kamau, L., Osangale, A., Ombok, M., Njagi, K., Mathenge, E., Muthami, L., Subramanian, K. Knox, T., Mnavaza, A. Donnelly, M.J., Kleinschmidt, I. and Mbogo, C. (2017). Insecticide – treated nets and protection against insecticide – resistant malaria vector in Western Kenya. *Emerging Infections Diseases Journal*, volume 23, Number 5.

Odinga, W. A. and Angendu, C. A. (2003). The Relationship between pyrethrins and the yellow pigmentation in pyrethrum flowers. *African Journal of Science and Technology(AJST). Science and Engineering Series.* 4 (2):116 – 123.

Okal, M.N., Linah J.M. Torr S.J., Masinde E., Orindi, B., Lindsay, S.W. and Fillinger, U. (2015). Analyzing the Oviposition behaviour of Malaria mosquitoes: Design considerations for improving two- choice egg count experiments. *Malaria Journal* 14:250.

Olayemi, I.K., Ukubuiwe, A.C. and Wiman, A.O., (2014). Mosquito Species occurrence and diversity in conventional larval breeding sites in Minna. Metropolis, Nigeria. *International Journal of Innovation and Scientific Research* vol. 9 pp. 86-93.

Olayeni, I. K., Ande, A. T., Danlami, G. And Abdulahi, V. (2011). Used Anopheles gambiae and anopheles arabiensis sex rato of 1:1 p. 90.

Ole Sangba, M.L. Sidick, A. Govoetchan, R., Dide-Agossou, C., Osse, R.A. Akogbeto, M. Ndiath, M.O (2017). Evidence of Insecticide resistance mechanisms in Anopheles gambiae populations in Bangki, Central African Republic. *Parasites and Vectors* 10:23.

Oliva, C. F., Benedict, M. Q, Lemperiere, G. and Gilles, J. (2011). Laboratory selection for an accelerated mosquito sexual development rate. *Malaria J*ournal. 10:135

Olofintoye, L.K., Oke, S.I.A., and Omoregie O.B. (2011), Larvicidal properties of *Datura stramonium* (Jinson weed) and *Nicotiana tabaccum* (Tobacco) extracts against the larvae of (Anopheles and culex) mosquitoes. *An International Multidisciplinary Journal Ethiopia.5*(2):337-344.

Olofintoye, L.K., Simon – Oke, I.A, Omoregie O.B. (2011). Larvicidal properties of Datura Stramonium (Jimson Weed) and Nicotiana tabaccum (Tobacco extracts against the larvae of (Anopheles and culex) mosquitoes. *An International multi-Disciplinary Journal, Ethiopia*. 5(2).

Olyayemi, I.K., Danlami, G., Isah, B., Odeyemi, D.M., Ukubuiwe, A.C. and Mustapha, O.M. (2011). Indoor behaviour responses of the Principal Malaria vector, Anopheles gambiae (Diptera: Culicidae), In relation to macro-climate conditions in Minna, North central Nigeria. *Research Journal of Parasitology*, 6: 109-115.

Omukunda, E. Githeko, A., Ndonga, M.F., Mushinzimana, E. and Yan, G. (2012b). Effects of swamp cultivation on distribution of anopheline larval habitats in Western Kenya. (Marani, Kisii). *Journal of Vector Borne Dis*eases. 49(2) 61-71.

Omukunda, E. Githeleo A., Ndonga, M.F. Mushinzimana, E. and Yan G. (2012a). Effects of swamp cultivation on distribution of anophelinelarvae habitats in Western Kenya (Kombewa, Kisumu County) *Journal of Vector Borne Diseases*.49 : 1-71.

Omukunda, E., Githeko, A., Ndonga, M.F., Muskinzimana, E. and Yan Guiyan, (2012). Effect of swamp cultivation on distribution of anopheles larval habitats in Western Kenya. *Journal of Vector – Borne Diseases* 49:pp 61-71.

Onchuru, T.O., Ajamma, Y.U., Burugu, M. Kaltenpoth M. (2016). Chemical parameters and bacterial communities associated with larval habitats of Anopheles, Culex and Aedes mosquitoes (Diptera: Culicidae) in Western Kenya. *International Journal of Tropical Insect Science*. Volume 36 issue 3 pp 146-160.

Ondeto, B.M, C., Kamau, L., Muria, S.M. Mwangangi, J.M., Njagi, K., Mathenge, E.M., Ochanda, H. and Mbogo, C.M. (2017). Current status of insecticide resistance among malaria vectors in Kenya. Parasites and Vectors 10:429.

Open Chemistry Data Base (2018). Pyrethrins: Decomposition. National Centre for Biotechnology Information. Pubcherm Compound Database; CID = 71310221, http://pubchem.ncbi.nlm.nih.gov/compound/71310221(accessed July, 20, 2018).

Organization for economic cooperation and development (OECd) (2018). Guidance document on inhalation toxicity studies. Series on testing and assessment No. 39. (second edition), ENVI/JM/MONO (2009). 28/REVI, Paris 2018.

Otarigho, B. and Falade, M.O. (2013). Molecular evolution and Phylogenomics of the Anopheles gambiae complex. *Journal of Mosquito Research*, volume 3, No. 9 pp. 65-70.

Other major tobacco producers (www.statista.com/topics/1593/tobacco/. Retrieved July 3, 2018.

Owiti, Y.J. and Misire C. (2017). Effect of temperature and pH on egg viability and pupation of Anopheles arabiensis Patton (Diptera: Culicidae): Prospect for optimizing colony reproduction procedures. *Jordan Journal of Biological Sciences.* Volume, 10, No. 1.

Panigrahi, S.K., Barik, T.K., Mohanty S. and Kanti N. (2014). Laboratory evaluation of oviposition behaviour of field collected Aedes mosquitoes. *Journal of insects*. volume 2014(2014), Article ID207489, 8 pages.

Pankaj, T. and Anita S. (2014). An endevour to control *Anopheles stephensi* (Liston) larval by plant extracts. *Trends in Biosciences*; 7: 23.

Parratt, S.R and Laine, A.L. (2016). The role of hyperparasition in Microbial pathogen ecology and evolution. *International Society for Microbial Ecology* (ISME) 10: 1815 – 1822.

Patil, C.D. Narkhede, C.P., Suryawanshi, R.K. and Patil, S.V. (2016). Vorticella sp: Prospective mosquito biocontrol agent. *Journal of Arthropod Borne Diseases* 10(4): 602-607.

Patil, C.D., Narkhede, C.P., Suryawanshi, R.K., and Patil, S.V (2016). Vorticella SP: Prospective Mosquito Biocontrol agent. *Journal of Arthropod – Borne Diseases* vol. 10(4): 602 – 607.

Patil, C.D., Suryawanshi, R.K., Narkhede, C., and Patil, S. (2015). Vorticella sp: Prospective Mosquito biocontrol agent. Journal of Arthropod – Borne Diseases 10(4): 602-607.

Period of exposure in www.hyperdictionary.com/dictionary/subacute + toxicity. Retrieved May 5, 2012.

Pethybridge, S.J., Hay, F.S. Esker P. Gent, D.H., Wilson, C.R. Groon, T., and Nutter Jr. F.W. (2008). Diseases of Pyrethrum in Tasmania: Challenges and Prospects for management – Lowa state University, Plant pathology and Microbiology Publications. Plant Disease 92(2008): 1260, doi: 10.1094/PDIS-92-9-1260.

Phasomkusolsil, S., herdthusnee, K., Khuntirat, B., Kongtak, W., Pantuwatana K. and Murphy, J.R. (2011). Effect of temperature on laboratory reared *Anopheles. Dirus* Peyton and Harrisoni and *Anophelessawadnongporni*, Rattanarithikul and Green. *Southern Asian Journal of Tropical Medicine ana Public Health*. 42: 1

Phasomkusolsil, S., Lerdthusnee, K., Khuntirat, B. Kongtak, W., Pantuwatana, K. and Murphy, J.R. (2011). Effect of temperature on Laboratory reared Anopheles dirus Payton and Harrison and Anopheles salvadwongporni Rattanarithikul and Green. *Southern Asian Journal of Tropical Medicine and Public Health* 42(1): 63-70.

Phasomkusolsil, S., Tawong, J., Monkanna, N., Pantuwatana, K., Damdangdee, N., Khongtak, W., Kertmanee, Y., Evans, B.P. and Schuster, A.L. (2013). Maintenance of mosquitoes vectors: effects of blood source on feeding, survival, fecundinty and egg hatching rates. *Journal of vector Ecology*. 38 (1); 38-45.

Philips, R.S. (2014). Current status of malaria and potential for control. American society for microbiology, 2014.

Philpott, T., (2015). Bees Love Nicotine, even through its killing them. In: Mother Jones https://www.motherjones.com/food/205/04/bees-love-nicotine-even-though-its-killing-them/(accessed July, 10, 2018.

Pinto Jr, Airton R. (2010). Bioactivity of essential oils of Brazilian sassafras and *eucalyptus* against lesser mealworm and *Anopheles diaperinus* larvae. *Cienc. Rural (online)*. 40 (3) :637-643.

Primid, M., Ahmed, H., Hassan, A.A. and Kumara, T.K. (2015). Interspecific cross-mating between Aedes aegypti and Aedes albopictus laboratory strains: implications of population density on mating behaviours. Journal of the American Mosquito Control Association 31(4): 313 – 320.

Protopopoff, N., Matowo, J., Malima, R., Kaaya, R., Wright, A., West, P.A., Kleinschmidt, I., Kisinza, W., Mosha, F.W. and Rowland, M. (2013). High level of resistance in mosquito *Anopheles gambiae* to pyrethroids insecticides and reduced susceptibility to bendicarb in North – Western Tanzania. *Malaria Journal*.12:149.

Public Health England (2013). Background information on Malaria. In: www.hpa. org.uk/Topics/Infectious Diseases/Infectious Az/Malaria/General Information/ Mala10 background. Retrieved 13.12.2013.

Pugzhvendan, S.R. and Elumali, K. (2013). Larvicidal activity of selected plant essential oil against important vector mosquitoes: Dengue vector, *Aedes aegypti* (L.) Malaria vector, *Anopheles stephensi* (Liston) and Filaria vector, *Culex quinguefasciatus* (Say) Diptera:Culicidae). *Middle-East. Journal of Scientific Research*18 (1): 91-95.

Rajasekar, P., Jebanesan, A., Shamsudin, M. Jerald, I.J.A, Meeramaideen, M., Salahudeen, M. and Thirupathi, R. (2016). Larvicidal effects of essential oils from three medicinal plants against Aedes aegypti L. *International Journal of Modern Research and Reviews*, Volume 4, Issue 6, pp 1181-1183.

Ramirez, A. (2013). Pyrethrum Secondary Metabolism: Biosynthesis, localization and ecology of defence compounds. A submitted in fulfillment of the requirement for the degree of doctor at Wageningen University, Holland.

Ramirez, A., (2013). Pyrethrum secondary Metabolism: Biosynthesis, localization and Ecology of defence compounds. A thesis submitted in fulfillment f the requirements for the degree of Doctor of Philosophy at Wageningen University. Chapter 3: A Single cytochrome P450 Enzyme catalyzes the formation of chrysanthemic acid from chrysanthemolin pyrethrin Biosynthesis.

Ramphul, U., Boase, T., Bass, C., Okedi, L.M. Donnelly, M. J. and Muller, P. (2009). Insecticide resistance and its association with target – site mutations in natural populations of *Anopheles gambiae* from Eastern Uganda. *Journal of Transnational Research and Sociological Tropical Medicine* and *Hygiene.* 103(11) :1121-1126.

Rathore, H.S. and Nollet, L.M.L (2012). Pesticides. Evaluation of Environmental polluton. CRC Press. Taylor and Francis Group. Boca Raton London New York. In www.acaemia.edu/8239683/pesticides and environmental - pollution. Retrieved 3/7/2009.

Rawani, A., Chowdhury, N., and Ghoshi, A. (2013). Mosquito Larvacidal activity of Solanum nigrum berry extracts. Indian Journal of Medical Research volume 137 pages 972-976.

Reid, M.C. and McKenzie, F.E. (2016). The contribution of agricultural insecticide use to increasing insecticide resistance in African malaria vectors. *Malaria Journal*15:107.

Reidenbach, K. R. Neafsey, D. E., Costantini, C., Sagnon, N. F., Simard, F., Ragland, G. J., Egan, S. P., Feder, J. L., Marc, A., Muskaritch, T. and Besansky, N. J. (2012). Patterns of genomic differentiation between ecologically differentiated M and S forms of *Anopheles gambiae* in West and Central Africa. *Genome Biological Evol*ution. 4:1202 – 1212.

Reiss, J. and Sprenger, J. (2017). Scientific Objectivity, The Standard Encyclopedia of Philosophy (Winter 2017 Edition), Edward N. Salta (ed), URL-https://plato.stanford.edu/archives/win2017/entries/scientific-objectivity.

Revathi, J. (2014). Factors affecting absorption. Maua Reddy College of Pharmacy. In: https://www.slideshare.net/bharathpharmacist/factors-affecting-absorption-39685897.

Rios, N., Stashenko, E.E. and Deque, E. (2017). Evaluation of the insecticidal activity of essential oils and their mixtures against Aedes aegypti (Diptera: Culicidal). Revista Brasileira de Entomologia volume 61, Issue 4, pages 307 – 311.

Rocha, H.D.R., Paiva, M.H.S., Silva, N.M. Aranjo, A.P., Gamacho, D.R.R.A., Moura, A.J.F. Gomez, L.F., Ayres, C.F.J and Santos, M. A.V.M. (2015). Susceptibility profile of Aedes aegypti from Santiago, Island, Cabo Verde, to insecticides. Acta Tropica. 152:66-73.

Rodriguez, M., Perez, L., Pujol, M. and Borroto, C. (2018). Transgenic plants of Nicotiana tabaccum L. express aglycosylated monoclonal antibody with antitumour activity. Biotechnologia Aplicada 30: 157-161.

Rose, G., Krzywinska, E., Kim, J., Revuelta, L., Ferretti, L. and Krzywinski, J. (2016). Dosage compensation in the African Malaria Mosquito Anopheles gambiae. *Genome Biology and Evolution*, volume 8, Issue 2, pp. 411 – 425.

Roth, K. and Vaupel, E. (2018). Pyrethrum: History of a Bio-insecticide – Part 1. In German: Chem. Unserer Zeit 51:162-184. htt://doi.org/10.1002/ciuz.201700786. translated to English by Russey, W.E. (2018).

Rowe, V.K. (2004). Profiles in Toxicology: V.K. Rowe (1914 – 2004). *Toxicological Sciences*, volume 79, Issue 2, pp. 209 – 210.

Royston, P. and Parmar, M.K.B (2013). Resistricted mean survival time: an alternative to the hazard ratio for the design and analysis of randomized trials with time – to-event outcome. Minimal Bactericidal concentration (BMC). *Medical Research Methodology*, 13:152

Rudmann, D.G. (2013). On target and off target – based toxicological effects. *Toxicological Patholology*. 41(2) 310-314.

Rund, S.S., Gentile, J.E. and Duffield, G.E. (2013). Extensive circadian and light regulation of the transcriptome in the malaria mosquito *Anopheles gambiae*. Journal of Biomedical Centre *Genomics.*14: 214-218.

Ruschoni, M., Bettinetti, R., Polesello, S. and Stefani, F. (2017). Evolutionary Toxicology as a tool to assess the ecotoxicological risk in freshwater ecosystems. Water 10,490; doi: 10.3390/w10040490 www.mdpi.com/journal/water.

Saganuwan, S.A. (2015). Arithmethic – Geometric Harmonic (AGH) method of rough estimation of median lethal dose (LD50) up and down procedure. Journal of Drug Metabolism and Toxicity 6:3 doi: 1041721257-7609.1000180.

Salgueiro, P., Moreno, M., Simard F., O'Brochta, D. and Pinto, J. (2013). New insights into the population structure of Anopheles gambaie s.s. in the Gulf of Guinea Islands revealed by herves transposable elements. PLOSONE 8(4): e62964.https://doi:org/10.13071/journal.pone.0062964.

Saliba, K.S., Clarke, D., Santos, J.M., Menezes, M.J. Lim, C., Mascarenhas, A., Chery, L., Gomes, E., March, S., Bhatia, S.N, Rathod, P.K., Ferreira, M.U., Catteruccia, F. and Duraisingh, M.T. (2016). Infection of laboratory colonies of Anopheles mosquitoes with Plasmodium Vivax from cyropreserved clinical isolates. *International Journal of Parasitology* 46:679-683,.

Sanden, M. M., Monssiliou, A., Moiroux, N., Padonou, G. G., Massougbodji, A., Corbel, V., and Ndam, N. T. (2012). Optimized pan-species and speciation duplex real-time PCR assays for *Plasmodium* parasites detection in malaria vectors. *Journal of PLoS ONE*; December, 28, 2012. doi:10.1371/journals pone.00527

Sawadogo, P.S. Namountongou, M., Toe, K.H., Royamba, J., Maiga, H. (2018). Swarming behaviour in natural populations of Anopheles gambiae and Anopheles cduzzil: Review of 4 years survey in rural areas of sympatry, Burkana Faso (West Africa). *Acta Tropica, Elsevier 132 Suppl*. Pp.542-552.

Sawadogo, S. P., Diabate, A., Toe, H. K. Sanon, A., Leferre, T. and Baldet, T. (2013b). Effects of age and size on *Anopheles Gambiae S.S*. male mosquito mating success. *Journal of Medical Entomol*ogy. 50: 285 – 293.

Sawadogo, S.P., Niang, A., Bilgo, E., Millogo, A., Maiga, H., Dabire, R.K. Tripet, F. and Diabate, A. (2017). Targeting male mosquito swarms to control malaria vector density. *PLOSONE* 12(3): e0173273.https://doi:org/10.1371/journal.pone.0173273.

Sayal, S., and Gosh, S. (2014). Prey Selectivity and efficient biocontrol of dengue by guoppies: Effects of alternative prey and habitat complexity. *International Journal of Piere and Applied Zoology* volume 2 Issue, 4, pp. 339-347.

Scholte, E.J. Knols, B.G, Samson, R.A and Takken, W. (2004). Entomopathogenic fungi for mosquito control: a review *Journal of Insect Science*. 4:19.

School of Pharmacy, Biotechnology and Sport Science (2017). Analytical Chemistry of Pollutants. Analysis of atmospheric pollutants, University of Bologna.

Scott, T. W. and Takken, W. (2012). Feeding Strategies of anthropophilic mosquitoes result in increased risk of pathogen transmission. Journal of *Trendsin parasitology*. 28 (3): 114 – 121.

Sebei, K., Sakouhi, F., Herchi, W., Khonja, M.L., and Boukhchina, S. (2015). Chemical Composition and antibacterial activities of seven Eucalyptus species, *Biological Research* 48(1):7.

Sewe, M.,O., Ahlon, C. and Rocklov J. (2016). Remotely sensed environmental conditions and malaria mortality in three malaria endemic regions in Western Kenya. PLOSOne 11(4). E0154204. https://doi.org /10.1371/ journal.pone.0154204.

Sharokhov, I.V., (2013). Chromosome phylogenies of Malaria mosquito. Tsitologiia. 55(4): 238-40.

Shashibhushan, J., Venugopal, K., Lingaraja, M., Patanjali, C.P., Suresh, C. and Hugi, V. (2015). Paraquat: A fatal poison, a case report. Medical Journal volume 8 Issue 3 pp. 370 – 374.

Shaw, W.R., Teodori, E., Mitchell, S.N., Baldini, F., Gabrieli, P., Rogers, D.W. and Catteruccia, F. (2014). Mating activates the heme peroxidase HPX15 in the sperm storage organ to ensure fertility in *Anopheles gambiae*. *Procedure of Naitional Academy of SciencesUSA*.111(16) :5854 – 5859.

Shelf life of essential oils – and how to make them last longer. Using essential oils safely. In:https://www.usingeosafely.com/shelf-life-essential-oils-and how-to-make-them-last-longer/accessed10.6.2018.

Shelf Life of Essential Oils – and how to make them last longer. Using Essential Oils safely, In:https://www.usingeossafely.com/shelf-life-essential-oils-and-how-to-make-them-last-longer/assessed 28.6.2018.

Shishika et al., (2014). Male motion coordination in Anopheles Mating swarms. Scientific Reports 4, Article number 6318. doi:https://doi.org/10.1038/srepo6318 retrieved 22nd March 2015.

Shishika, D., Manonkis, N.C., Butail, S. and Paley D.A (2014). Male motion coordination in anopheline mating swarms. Scientific Reports 4, Article number: 6318(2014).

Shishika, D., Manonkis, N.C., Butail, S. and Paley, D.A. (2014). Male motion coordination in anopheline mating swarms. Scientific Reports 4, Article number: 6318(2014).

Shooshtari, M.B. Kashani, H. H., Heidari, S. and Ghalandari, R. (2013). Comparative mosquitor repellent efficacy of alcohol extracts and essential oils of different plants against *Anopheles Stephens*. *African Journal of Pharmacy and Pharmacology*. 7(5) : 310-314.

Siginaw County Mosquito Abatement Commission (SCMAC) (2013). Congress Av, Saginaw, MI 48602 (989) 755- 5751 www.scmac.org 2013. In: www.scmac. org/ pdf/webmosqkey2013.pdf. Retrieved 14.1.2014.

Silva, R.P., Rosario, V., Couto, J. and Domingos, A. (2016). The Anopheles gambiae transcriptome a turning point for malaria control. *Insect Molecular Biology* 26(2). With 37 reads doi:10.1111/imb.12289.

Singh, R. (2014). Studies on Mosquito larvacidal properties of Eucalyptus citnodora Hook (Family – Myrtaceae). Journal of communicable diseases 39(4): 233-236.

Singh, R. K., Mittal, P. K., Kumar, G. and Dhiman, R. C. (2014). Insecticide susceptibility status of *Aedes aegypti* and *Anopheles stephensi* larvae against temephos in Delhi, India. *International Journal of Mosquito Research*. 1(3): 69-73.

Sinha, K. (2012). World Tobacco Atlas rings alarm bells for India. TNN/March 29, 2012, 03.05 AM 1ST. In: times of India. Indiatimes.com/India/world-Tobacco-Atlas-rings-alarm-bells for India/articleshow/12448418.cms?8rc=flow. Retrieved 28.6.2014.

Siramon, P., Olitan, Y. and Ichiura H. (2013). Chemical composition and antifungal property of Eucalyptus camaldulensis leaf oil from Thailand. Journal of Records of Natural Products 7(1): 49-53.

Siteti, M. C., Injete, S.D., and Wanyonyi, W.A. (2016). Malaria parasite species prevalence and transmission dynamics at selected sites in the Western highlands of Kenya. *Christmed Journal of Health and Research* volume 3 Issue 1, pp. 45-50.

Siteti, M.C., Injete, S.D., and Wanyonyi, W.A. (2016). Malaria Parasite species prevalence and transmission dynamics at selected sites in the Western Highlands of Kenya. *Chrismed Journal of Health and Research* volume 3, Issue 1, pp. 45-50.

Soderlund, D.M (2012). Molecular mechanisms of pyrethroid insecticide neurotoxicity: recent advances. Journal ofv *Archives Toxicol*ogy; 86(2): 165-181.

Soni, V. and Anjikar, A. (2014). Use of Pyrethrin /Pyrethrum and its effects on Environment. PharmaTutor 2(6), 52-60.

Soultani, M. S. Papa-depoulos, N. T., Milonas, P. and Moyal, P. (2012). Abiotic factors and insect abundance. *Psyche: A Journal of Entomology*; Volume 2012(2012), Article ID 167420, 2 pages. http://doc.doi.org/10/1155/2012/167420.

Spatial Ecology and epidemiology Group (1913). Species of *Anopheles* mosquito. Spatial Ecology and Epidemiology group, Tinbergen Building Department of Zoology, University of Oxford, South Parks, Oxford UK, 1913. By Marriane E. Shirka, DOI: 10.5722/54163.

Stadman's Medical Dictionary (2002). The American Heritage stadman's medical dictionary. Houghton Mifflin company. In: www.dictionary.com/browse/threshold-limit-value. Retrieved on 11.9.2009.

Stanuszek, W.W. (2013). Saginaw County Mosquito Abatement Commission 2013. In: www.scmac.org/pdf/webmosqkey2013.pdf. Retrieved 14.1.2014.

Staples, D.P., Shelly, T.E and Yuval, B. (2012). Female mating failure and the failure of 'mating' in sterile insect programmes. Entomologia Experimentalis et Applicata 146: 66-78.

Statista Dosier (2013). Tobacco. Statistics and facts about the Tobacco industry.

Statistical Abstract (2003), Kenya. Central bureau of Statistics, MP and ND, Government of Kenya.

Statistical abstract, 2013 – 2016.

Stedman's Medical Dictionary for Health Professions and Nursing 2011

Stephen, M., Vulule J. And Eliningaya, J. K. (2013), Response of *Anopheles Gambiae* 8.1(Diptera: Culicidae) to larval habitat age in Western Kenya Highlands. *Journal ofParasites and Vectors*. 6:13

Stine, K.E. and Brown, T.M. (2015). The Relationship between the dose of a chemical (dependent variable) and the response produced (independent variable). In Principles of Toxicology, Third Edition, CRC, Press Taylor and Francis Group pp. 122.

Stone, C. M. (2013). Transient population dynamics of mosquitoes during sterile male releases: Modelling mating behaviour and pertubations of life history parameters. *Journal ofPLoS ONE.*(9)*:* e76228.

Sub-acute (www.hyperdictionary.com/dictionary/subacute + toxicity, Retrieved 5 May, 2012).

Subbiah, P., Ramesh, N., Sundaravadivelu, K., Samuel, P., and Tyagi, B.K. (2009). Larvicidal efficacy of various formulations of Bacillus sphaericus against the resistant strain of Culex Quinquefasciatus (Diptera: Culicidae) from Southern India. Tropical Biomedical 26(1): 23-29.

Sub-chronic, (www.businessdictionary.com/definition/toxicity.html, Retrieved 5 May, 2012).

Sugamar, S., Clarke, S.K., Nirmala, M.J., Tyaqi, B.K., Mukherjee, A. and Chandrasekaran, N. (2012). Nanoelmusion of Eucalyptus oil and its larvicidal activity against culex and quinguefasciatus. *Bulletin Entomological Research* 104(3): 403.

Sugumar, S., Nimala, J., Clarke, S.K. and Chandrasekaran, N. (2014). Nanoemulson of eucalyptus oil and its larvicidal activity against Culex quinque fasciatus. Bulleting of Entomological Research 104(3): 1-10.

Tabachnick, W.J. (2016). *Mosquito Oviposition behaviour and vector control*. Insects 7.65.

Taher, E., Mahmoud, N. and Mahmoud M. (20120. Laboratory evaluation of the effect of Egyptian native plants against some parasitic vectors. *Journal of Turkiye parazitoloji Dergisi* 36:160-165.

Taher, E., Malimoud, N and Mahmoud, M. (2012). Laboratory evaluation of the effect of Egyptian native plants against some parasitic vectors. *Turkiye Parazitoloji, Dergisi*. (36):160-165.

Tatiane, F., Trindade, T., Stabeli, R.G., Pereira, A.A., Facundo, V.A. and Silva, A.A. (2013). Copaifera multijuga ethanolic extracts, oil-resin, and its derivatives display larvicidal activity against *Anopheles darlingi* and *Aedes aegypti* (Diptera: Culicidae) *Rev. Bras.Farmacogn*. 23:3.

Thailayil, J., Gabrieli, P., Caputo, B., Bascunar South, A., Diabate, A., Dabire, Torre, A., and Catternccia, F. (2018). Analysis of natural female post-mating responses of Anopheles gambiae and Anophele coluzzii whravels similarities and differences in their reproductive ecology. Scientific Reports and Article number: 6594 (2018). Doi:https://doi.010.1038/541598-018-24923-W.

The American Heritage Stedman's Dictionary; 5th ed. 2013 publisher: Houghton, Mifflin Harcourt, Boston.
The Biology of Mosquitoes: In: https://www.sms/co/nz/site/ southernmonitoring/images/ NZB/mossieAwareness/The Biology of Mosquitoes.pdf.
The International Planet Names Index (IPNI) (2015). Database of the names and associated basic Bibliographical details of seed plant, ferns and lycophytes. *International Plants* Names Index 2015.
The Merck Veterinary Manual (2013). Factors affecting the activity of toxicants. October, 2013, by Steve M. Ensley. In: www.merek mamma is.com/vet/toxicology-introduction/factors-affecting-the activity-of-toxicants.html.
Thomas, R.J. (2013), Particle size and pathogenicity in the respiratory tract. Virulence 4(9): 847 – 858.
Threshold dose (TD). The minimum or the smallest dose of a substance which causes adverse change in an organism (www.preservearticles.com/2012021423360 / what-are-the-indices-of-toxicity.html. Retrieved March, 23, 2013.
Torris, G.J. and Hoback, W.W. (2013). Colour and Container size affect Mosquito oviposition. North Eastern Naturalist 20(2): 363-371.
Tovar, J. (2014). Tobacco cultivation in Latin America. Documents CEDE NO. 2014 – 12 SSRN: https://ssrn.com/abstract=2439417 or htt://dx.doi.org/10.2139/ ssrn. 2439417.
Toxtutor: United States National Library of Medicine. Factors influencing toxicity. In: https://toxtutor.n/in.gov/03-002 html.
Trindade, F.T.T., Stabeli, R.G., Peveira, A.A., Facundo, V.A. and Silva, A.A (2013). Copafera multijuga ethanolic extracts, oil-resin, and its derivatives display larvicidal activity against *Anopheles* darling and Aedes, aegypti (Diptera: cucicidae). Journal of Revista Brasileira de Farmacognosia 23:3.
Tulve, N., Olsen, M. Broder, M., Firestne, M., Nulford, E., Sheldon, L., Stahl, C. and Zartarian, V. (2016). USEPA guidelines for Human Exposure Assessment Forum U.S. *Environmental Protection Agency*.
Turek and Stintzing (2013). Stability of essential oils: A review Research Gatel comprehensive Reviews in Food and Science and Food Safety, 12(1). DOI:10.1111/154-4337.12006.
In:https://ww/researchgate.net/publication/260727283 – stability-of-Essential Oils-A-Review Assessed 28.6.2018.
U.S. Food and Drug Administration (FDA) (2015). Tobacco products: Women's Health and Smoking. In: https://www.fda.gov/tobaccoproducts/ publichealtheducation/ healthinforamtion/UCM445713.html. accessed July, 10, 2018.
United Nations (2009). Part 4: Environmental Hazards; 4.1 Hazardou to aquatic environment.
United Nations (2013). World Economic and Social Survey, Sustainable Development Challenges Department of Economic and Social Affairs, United Nations, NewYork E/2013/50/Rev1st/ESA/344.
United Nations (2017). Anex 9 Guidance on hazards to the aquatic environment. In: https://www.unece.org/fileadmin/DAM/trans/danger/publi/ghs/ghs-revo/English/12e-annex9.pdf accessed 21/6/2018.
United States Department of Health and Human Services (2007). Women and Smoking: A report of the Surgeon General Rockville (M.D): US. Department of Health and Human Services, Public Health Service, Office of the Surgeon General 2007.

United States Environmental pollution Agencr (USEPA) Guidelines for Exposure Assessment. http://cfpub.epa. gov/ucea/cfm/ recordplay.cfm? Deid=15263. Retrieved 4.5.2014.

United States Environmental Pollution Agency (USEPA) (2017). Pyrethrins and Pyrethroids., *Ingredients used in pesticide products*. https://www.epa.gov/ ingredients-used-pesticide-products/pyrethrins-and-pyrethroids. retrieved April. 2017.

United States Environmental Protection Agency (USEPA) (2014). Guidelines for exposure assessment and disease (Constant rate as a function of time (http://cfpub.gov/neca/cm/recordingplay.cfm?deid.

University of Bath (2018). Kenya Tobacco Control Country Profile. In: www.Tobacco Tactics.org Accessed 25 Sept. 2018.

USEPA (2006). Registration Eligibility Decision for Pyrethrins. List Case No. 2580, Special Review and Registration Division.

Uthayarasa, K., Surandra, S.N. Pathmanathan, M.K. and Jeyadevan, J.P. (2010).*Eucalyptus* efficacy of crude extracts of *Emblica officinalis* and *Eucalyptus citriodora* against *Aedes aegypti*. *International Journal of Phamaceutical and Biological Archives*. 1(4): 467-472.

Uthayarasa, K., Surendran, S. N., Pathmanathan, M. K; and Jeyadevan, J. P. (2010). Larvicidal efficacy of crude extracts of *Emblica officinalis* and *Eucalyptus citriodora* against *Aedes aegyptin* L. *International Journal of Pharmaceutical and Biological Archives*. 1(4); 467- 472.

Vannin, L., Reed, T.W. and Willis J. (2013). Temporal and Spatial expression of Cuticular Proteins of *Anopheles gambiae* implicated in insecticide resistance or differentiation of M/S incipient species. Parasites and Vectors 7:24.

Villaverde, J.J. Moran, B.S., Espana, P.S., and Prados, J.L.A (2016). Biopesticides from natural products: Current Development, Legislative Framework and Future trends. Bioresoni 11(2): 5618-5640.

Visciano, P., Schirone, M., Berti, M., Milandri A., Tofalo, R. and Suzzi, G. (2016). Marine biotoxins: occurrence, toxicity regulatory limits and reference methods. Frontiers in microbiology 7:1051.doi. 103389/fmcb.2016.01051.

Wada, A. and Singh K. (2014). Larvicidal effects of *Eucalyptus camaldulensis* plant leaf extract on *Culex guinquefasciatus* mosquito. *European Journal of Pharmaceutical and Medical Research*. 1(1): 192-203.

Wada, A. and Singh, K. (2014). Larvicidal effects of Eucalyptus camaldulensis plant leave extract on culex quinquefasciatus mosquito larvae. *European Journal of Pharmaceutical and medical Research* 1(1): 192-2003.

Wagner, J.L. Townsend, A.K., Velzis, A.E and Paul E.A (2017). Temperature and toxicity of the copper herbicide (Nautique) to freshwater fish in field and laboratory trials. Congent Environmental Science 3:1339386.

Walker, M., Winskill, P., Basanez, M. G., Mwangangi J.M., Mbogo, C., Beier, J. and Midega, J. T. (2013). Temporal and micro-spartial heterogeneity in the distribution of *Anopheles* vectors of malaria along Kenyan coast. *Parasites and vectors.* 6:311.

Walshe, D. P., Garner, P., Abdel-Hameed Adeel, A. A.., Pyke, G.H., and Burkot, T. (2013). Larvivorous fish for preventing malaria transmission: *Journal ofCochrane Database of Systematic Reviews*. (12):1-65.

Walter Reed Army institute Research (2013), Immunologic Receptors- Advances in Research and Application Q.A. Acton, 2013 ed.; Chapter 2, Complimentary receptors Walter Reed Army Institute of Research Silver Spring: *Plasmodium*

falciparum field isolates use compliment receptor 1 (CR1) as a receptor for invasion of erythrocytes, pp. 15.

Weiss L.M. and Bechel, J.J. (2014). Microsporidia in Insects. In: Microsporidia: Pathogens of Opportunity, First Edition, Chapter 21 pp. 521 – 570, Wiley online Library.

Wheat / CornFlour Mill (2017). Big Weed: Ten farms could supply all of America with marijuana. In:https://boingboing. Net/2017/12/31/amber-waves-of-green-html. Accessed July 7, 2018.

White, S.A. and Kaufman, P.E. (2014). African Malaria Mosquito: Featured Creatures, Entomology and Nematology/FDACS/DPI/EDIS Publication Number EENY – 601.

Whitnee, S., Haine A. and Beyrer, C. (2015). Safeguarding human health in the Anthropocene epoch: report of the Rockefeller Foundation – Lancet Commission on Planetary health. Lancet 2015; http://dx.doi.org/10.1016/S0140-6736(15) 60901-1.

WHO (2012), Global vector control Response. 2017 – 2030. World Health Organization 20, Avenue Appia, CH – 1211 Geneva 27, Switzerland.

WHO (2013). Guidelines for efficacy testing of spatial repellents: Control of neglected tropical diseases WHO pesticide evaluation scheme (WHOPES). WHO/Department of Control of neglected tropical diseases, Geneva, Switzerland.

WHO (2013). Guidelines for Laboratory and field testing of mosquito larvicides. WHO/CDS/WHOPES/GCDPP/2005.15.

WHO (2015). WHO commends the Roll back malaria partnership's contribution to global progress as governing board disbands secretariat: WHO remains committed to fuel filling its leading role in the fight against malaria. WHO, Geneva, Switzerland.

WHO (2018), Millenium Development Goals (MDGs): Key Facts. In: www.who/news-room/fact-sheets/detail/millenium-development-goals-(mdgs) 22/6/2018. accessed 23rd July, 2018.

WHO(2008). Pesticides. Children's health and the environment, WHO training package for the health sector, July 2008 version, www.who.int/ceh.

WHO, (2015) Malaria Core Vector control methods. WHO, Geneva, Switzerland.

WHO, (2018). *Overview of Malaria elimination*. Malaria Elimination Oversight Committee (MEDC) 2018.

WHO, M.W. Sengupta, D. and Chorodhury (2009). Impact of pesticides use in agriculture: Their benefits and hazards. *Journal of interdisciplinary Toxicology*.

Wijegunawardana. A.D. Gunathi lake H.N. Dassanayake, W. (2015). Establishment and Maintenance of laboratory colonies of Aedes albopictus mosquitoes. Proceeding of the peradeniya University International. Research sessions 2015;19:204

Wilke E. and Marrelli, M., (2012). Genetic Control of mosquitoes: Population suppression strategies. REvistar institute de medicina Tropical Sao Paolo 54(5): 287-92.

Williams, J. and Pinto, J. (2012). Training manual on malaria entomology for entomology and vector control technicians (Basic level: intergrated vector management of malaria and other infectious diseases Task order 2 contract GHA-1-02-04-00007-00; produced for United States Agency for International Development. In: https://www.researchgate.net/publicaton/262450744- training-manual-on-malaria-Entomology. accessed 13th July, 2018.

Wolf, K.M. and Ditomaso, J.M. (2015). Management of blue gum eucalyptus in California requires region-specific consideration. California Agriculture, 70(1): 39-47.

Wondji, C.S., Coleman, M., Kleinschidt, I., Mzilahowa, T., hving, H., Ndula, M., Rehman, A., Morgan, J., Barnes, K.G. and Hemingway, J. (2012). Impact of Pyrethroid resistance on operational malaria in Malawi. Procedure National Academy of Sciences USA. 109 (47): 19063-19070. (Proc Nati Acad Sci USA).

Wondwosen. B.W. Birgersson, G., Seyoum, E., Tekie, H., Torto, B., Fillinger, U., Hill, S.R. and Ignell, R. (2016). Rice volatiles lure gravid malaria mosquitoes. *Anopheles arabiensis*. Scientific Reports 6, 37930; doi:10.1038/srep37930.

Wong, A., and Glinski, J.A. (2017). Efficient, baseline separation of pyrethrins by centrifugal partition chromatography. *Journal of Chromatography* 8:362. doi.10.4172/2157-7064.1000362.

Wong, J., Bayoh, N., Olang, G., Killeen, G., Hamel, M.J., Vulule, J.M. and Gimming, J.E. (2013). Standardizing operational vector sampling techniques for measuring malaria transmission intensity: evaluation of six mosquito collection methods in Western Kenya. Malaria Journal 12:143 https://doi.org/10.1186/1475-2875-12-143.

World Health Organiation (WHO) (2015). In 2011, around 4.2million hectares of land were devoted to tobacco growing representing less than 1% total arable land. Bulletin of the WHO-past issues-volume 93, November, 12 – 817 – 892.

World Health Organization (2012). DDT in door residual spraying: Human health aspects. IPCS. Environmental Health Criteria No. 241. Copenhagen: World Health organization (WHO), collection Environmental Health Criteria 241, 2011, 295p, table fig, ISBN 978 – 9 – 4-157241-5, CHE, Geneva Switzerland.

World Health Organization (2012). WHO'S interim position statement on larviciding for malaria control in Africa. WHO, Geneva, Switzerland.

World Health Organization (2013). Guidelines for Laboratory and field testing of mosquito larvicides. Geneva: World Health Organization, Communicative Disease Control, Prevention and Eradication, WHO pesticide Evaluation Scheme 'WHO /CDS/WHOPES/GCDPP/2005.13.

World Health Organization (2014) NLM, Classification: WC 765. ISBN 978 – 92-4-1564830

World Health Organization (2014). World Malaria Report. Geneva: Switzerland.

World Health Organization (2015). Core Vector control methods: Doi in malaria vector control. WHO, Geneva, Switzerland.

World Health Organization (2015). Supplementary Vector Control Methods. Larval Source Management, WHO, Geneva, Switzerland.

World Health Organization (2016). Malaria World Malaria Report ISBN 9789241511711; pages 186, Geneva, Switzerland.

World Health Organization (2017). Malaria World Malaria Report. Geneva, Switzerland.

World Health Organization (2017). World Malaria Report. WHO, ISBN 978-92-4-156552-3, Geneva, Switzerland.

World Health Organization (2018) Pesticide residues in Food: Key facts

World Health Organization (2018). 10th anniversary, malaria consortium, disease control, better health. WHO, Geneva, Switzerland.

World Health Organization (2018). Framework conversion on tobacco control (FCTC): The Health, social, environmental and economic consequences of tobacco consumption and exposure to tobacco smoke worldwide WHO, Geneva, Switzerland.

World Health Organization (WHO) (2018). Responding to antimalarial drug resistance. World Health Organization, Geneva, Switzerland.

World Health Organization (WHO) (2013). Larval Source Management: A supplementary measure for malaria vector control, an operational manual 2013.

World Health Organization (WHO) (2017). Prevent malaria – save lives: WHO Push for Prevention on World Malaria Day, 25th April, 2017. WHO, Geneva, Switzerland

World Health Organization (WHO) and UNCTAI) (2015) United Nations. Conference on Trade and Development (UNCTAD) (2015). Status of Tobacco Production and trade in Africa: Factsheets. WHO (NMH) Department, Switzerland.

World Health Organization (WHO), (2011). Position statement on the use of DDT to achieve sustainable malaria control in the context of the Stockholm convention published in 2007 and revised in 2011.

World Health Organization (WHO), (2012), Global Malaria Report, Geneva, Switzerland, December, 2012.

World Health Organization (WHO), (2013), World Malaria Report 2013. Geneva, Switzerland: December, 2013.

World Health Organization (WHO), (2013). Decision adopted by the Conference of the parties to the Stockholm convention at its 6th meeting concerning the continued use for DDT for disease vector control (25th June 2013). Pdf 23/cb, WHO Ref. No.: WHO/HTM/GMP/2011.

World Health Organization (WHO), (2013). Malaria Entomology and Vector Control, guide for participants: 2.2. Distinguishing anophelines rom culicines. WHO Library cataloguing – in-publication. Data ISBN 9789241505810 (Guide for participants Geneva, Switzerland 2013.

World Health Organization (WHO), (2013). Malaria in pregnancy: http://www.who.int/malaria/high-risk-groups/pregnacy/en/index.html. Retrieved 10.10.2008.

World Health Organization (WHO), (2014). Four species of human malaria. *Malaria facts sheet No. 94 World TB Day*, 24th March, 2014. In:www.who.int/media centre/factsheets/fs094/en. Retrieved 6.6.14.

World Health Organization (WHO), (2014). WHO Media centre fact sheet No. 94. Malaria. In:www.who. int/mediacentre/factsheets/fs094/en. Retrieved 6.6.2014.

World Health Organiztion (2015). World malaria report. WHO NLM classification:WC765, ISBN 9789241565158.

Woudneh, M.B., and Oros, D.R. (2006). Quantitative determination of Pyrethroids, Pyrethrins, and piperonyl butoxide in surface water by High-Resoluton Gas Chromatography / High Resolution Mass Spectrometry. *Journal of Agriculture and Food Chemistry* 54 (19), pp. 6957 – 6962.

Wu, X., Zhou, S., Lu, Y. and Xu, B. (2015). Impact of climate change on human infections diseases: Empirical evidence and human adaptation. Environmental International 86:14-23.

Xu, Huang, S., Wei, S., Yang, M., CaOC., Jiang, R. Zhu, F. and Onyang, G. (2016). Study on Diffusion – Dominated solid-phase microextraction kenetics in semi-solid sample matrix. (Analytical Chemistry 88(18), pp. 8921 – 8925.

Yankanchi, S.R. Yadav, O.V. and Jadhav, G.S (2014). Synergistic and individual efficacy of certain plant extracts against Dengue vector mosquito, *Aedes aegypti*. *Journal of Biopest.*;7 (1) : 22-2.

Zareen, S. Khan, S.N. Adnan, M., Rehman, H.U., Asim, M. Sana, Waheed, M.A. Khan, J., Khan, T. and Rehman, M.U (2016). Larvicidal activity of medicinal plant Eucalyptus leaf extracts against Anopheles mosquitoes collected from district Kohat. *Journal of Entomology and Zoology Studies* 4(4): 696-697.

Zareen, S., Khan, S.N., Adnan, M., Rehman, H.U., Asim, M., Sana, Waheed, M.A., Khan, J., Khan, T. and Rehman, M.U. (2016). Larvicidal activity of medicinal plant Eucalyptus leaf extracts against Anopheles mosquitoes collected from district Kohat. *Journal of Entomology and Zoology studies* 4(4): 696-697.

Zhong, D., Chang X, Zhou, G., He, Z., Fu, F., Yan, Z., Zhu, G., Xu, T., Bonizzoni, M., Wang, M.H., Cui, L., Zneng, B., Chen, B. and Yan, G. (2013). Relationship between knockdown resistance, metabolic detoxification and organizational resistance to pyrethroids in Anopheles Sirensis. PLOSONE 8(2): e55475.https://doi:org/10.1371/journal.pon.0055475.

Zoology.44 (4): 945 -949. *Journal ofAnopheles Laboratory Biology and Culture*

Zuhara,W. F., Factzly, N., Ali, Y. , Zakaria, R., Juperi, S., Asyraf, M. and Dieng, H. (2014). Larvicidal efficacy screening of Anacaridaciae crude extracts on the dengue haemorrhagic vector, Aedes aegypti. *Journal ofTropical Biomedicine*.31(2) : 297-304.