BIOPROSPECTING AND DEVELOPMENT OF A BIOPESTICIDE FOR USE AGAINST MOSQUITOES FROM SELECTED PLANTS IN MSAMBWENI SUBCOUNTY, KENYA

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DECLARATION

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

……………………………………………… ………………..

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This thesis has been submitted for examination with our approval as university supervisors

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DEDICATION

To my family and all women and men who are true and honest to fellow mankind.

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ABBREVIATIONS

CAM	Chorioallantoic membrane
CAMVA	The chorioallantoic membrane vascular assay
CDC	Centers for disease control and prevention
CHIK	Chikungunya
CHIKV	Chikungunya virus
DDT	Dichlorodiphenyltrichloroethane
DEET	*N, N*-diethyl-m-toluamide
DHF	Dengue hemorrhagic fever
DNA	Deoxyribonucleic acid
ECVAM	European centre for the validation of alternative methods
EEE	Eastern equine encephalomyelitis
EPA	Environmental protection agency
ESAC	European space astronomy centre
EURL-ECVAM	European union reference laboratory for alternatives to animal testing

ICIPE	International centre for insect physiology and ecology
JE	Japanese encephalitis
KEMRI	Kenya medical research institute
KNH/UoN ERC	Kenyatta National Hospital / University of Nairobi, ethical review committee
MN	Micronuclei
MOH	Ministry of health
NICEATM	NTP Interagency centre for the evaluation of alternative toxicological methods
NRC	National research council
OECD	Organization for economic cooperation and development
PAHO	Pan american health organization
PMD	P-*menthane*-3, 8- *diol*
QSAR	Quantitative structure activity relationships
RhE	Reconstructed human *epidermis*
RR	Ross river virus
RVF	Rift valley fever
SAR	Structure activity relationship

UNICEF	United nations children's fund
USAID	United States agency for international Development
VEE	Venezuelan equine encephalomyelitis
WEE	Western equine encephalomyelitis
WHO	World health organization
YF	Yellow fever

ABSTRACT

Diseases transmitted by mosquitoes affect many people globally with significant numbers of deaths. They are an impediment to economic development in countries where they occur. More than half of the world's inhabitants are threatened with malaria and dengue infections. Another mosquito borne disease, yellow fever lacks cure. Half of those infected by it die and its remedy is personal protection and vaccination. Some mosquito control methods are expensive and toxic. It is difficult to eliminate all aquatic habitats while mosquito predators prey on non target organisms. Mosquitoes have developed resistance to most of insecticides in current use. Plant based insecticides are simple, cost-effective, readily available and culturally acceptable.

The plants studied were selected with regard to ethnobotanical surveys carried on the area in addition to systematic reviews of literature on plants used in Msambweni sub county that have been reported to have various activities against mosquitoes. They were *Plectranthus barbatus* A, *Adansonia digitata* Linn, *Tagetes minuta* L., *Ocimum suave*, *Azadirachta indica* A. Juss. and *Lantana camara* L. They were investigated for phytochemical composition, skin and eye irritability, genotoxicity, mosquito repellency and larvicidal activity. Finally, a mosquito repellent formulation was developed. Phytochemical studies were performed using established procedures to test for the following secondary metabolites: alkaloids, tannins, saponins, flavonoids, sterols, terpenoids and glycosides. Assessment of possible genotoxic activity was by the *Allium cepa* test. Repellency tests were performed according to protocol of world health organization (WHO) using *Aedes aegypti* mosquitoes on human volunteers.

Approval for use of human subjects was obtained from the Kenyatta National Hospital / University of Nairobi, ethical review committee (KNH/UoN ERC). The WHO bioassay method was used to determine knockdown effects of the extracts against mosquitoes.

Acute dermal and ocular irritation potential was assessed using New Zealand white rabbits. Thereafter, the plants' extracts were made into formulations of 10% and 20% which were also subjected to repellency and knockdown tests as outlined above. *A. digitata* and *P. barbatus* had alkaloids, tannins, saponins, sterols and flavonoids. *O. suave* had alkaloids, tannins, saponins and sterols. *A. indica* extracts had saponins, sterols and flavonoids while *T. minuta* had flavonoids, alkaloids, saponins and tannins.

L. camara had alkaloids, saponins, flavonoids, glycosides while tannins, sterols, terpenoids, and were absent. Larvicidal activity was tested on 4th instar larva of *A. aegypti*. A hundred percent larval mortality was achieved by all extracts except aqueous extract of *L. camara* that killed 90% of the larvae. Acetone extracts of *T. minuta* and hexane extract of was *O. suave* were most active as they caused 100% larval mortality at all tested concentrations thus LD_{50} and LD_{95} could not be determined for these extracts.

Mitotic inhibition was dose dependent. The most active was *A. Indica* causing significant mitotic inhibition at 67.95±0.72 for aqueous extracts, 70.36±0.68 for hexane extracts and 71.53±1.18 for acetone extracts while the least active was aqueous extract of *A. Digitata* at 1.42±0.68. *A. indica* produced binucleate and ghost cells.

Regarding repellency activity, percent protection for *L. camara* was 98.33±1.67, 94.86±0.53 and 77.63±1.04 for acetone hexane and aqueous extracts respectively, $P<0.001$.

In *T. minuta*, percent protection was 98.33±1.67 for acetone extracts' 98.33±1.67 for hexane extracts and 94.86±0.53 for aqueous extract, $P=0.182$. Percent protection for *A. indica* acetone extract was 98.33±1.67 for hexane 94.86±0.53 and aqueous 94.86±0.53 and P=

0.070. *A. digitata* had percent protection of 84.58±1.58, 75.27±4.07 and 80.00±4.71 for acetone, hexane and aqueous extracts with P=0.259. *O. suave* extracts had percent protection of 100.00±0.00 for acetone extracts, 94.86±0.5394 for the hexane extracts and 86±0.53 for aqueous extracts, P<0.001.

The percent protection for *P. barbatus* was 84.47±2.70 for acetone extracts, 74.14±3.59 for hexane extracts and 80.53±2.81 for aqueous extracts, P=0.107. The positive control was *N, N*-diethyl-m-toluamide (DEET) and its percent protection was 98.33±1.67. Extracts of acetone had knockdown effects like citronella oil. In acute dermal and ocular irritation tests, the extracts did not cause any toxicity even after 14 days and 21 days of observation on the skin and eyes respectively.

The two formulated products exhibited a repellency effect greater than or similar to DEET. When compared to Ballet® mosquito repellent jelly (which consists of paraffinum liquidum, cera microcrystalline, paraffin, *tacromanthus camphoratus, pelargonium graveolens*, isopropyl myristate, glycerol, myristyl myristate, citronellal, tocopherol acetate, *cymbopogon nardus*, geraniol, *eucalyptus globulus, rosemarinus officinalis*, citral, benzophenone-3, linalool and limonene) they showed a better repellency and knockdown activity P<0.001.

In conclusion, all the plants in this study caused appreciable mortality on *Aedes aegypti* larvae after twenty four hours and did not produce dermal or eye irritation. The two formulated products can offer suitable substitutes to most non-naturally used agents for repelling mosquitoes. Further research is needed to evaluate the activity of the formulated products in field trials. These plants should be evaluated for development of efficacious and environment friendly larvicides. Conservation measures should be put in place to curb over-exploitation of the plants.

CHAPTER ONE

INTRODUCTION

1.1. Background information

Mosquitoes are nearly ubiquitous and inhabit most regions except Antarctica. They exist in regions more than five thousand metres above sea level and almost one thousand three hundred metres below sea level. Mosquitoes belong to family *Culicidae* that has about 3500 species belonging to 41 genera (Service, 1986). Only about 100 mosquito species have been implicated as intermediate hosts of vertebrate parasites since 1878 (Foster and Walker, 2002). Mosquitoes breed in water, moist surfaces, tree holes, and containers (Jahn *et al*., 2010). Their breeding areas have been increased by human activities that increase debris and other material that hold water, (Tolle, 2009).

The life cycle of mosquitoes comprises eggs, larvae, pupae and adults. Female mosquitoes mate once but lay eggs during their entire adult life. They must bite vertebrates to obtain a blood-meal which they use as nourishment for developing eggs (Foster and Walker, 2002). They transmit diseases as some pathogens complete their life cycles within the mosquito and they are passed onto humans during the time of biting (Tolle, 2009).

Males do not suck blood but feed on plant juices (Foster and Walker, 2002). Mosquitoes are of major public health concern since they are important disease vectors (Lee *et al*., 2003). These diseases infect over one billion people worldwide annually with over one million resultant deaths (Marimuthu, 2014; WHO, 2014).

Malaria affects 500 million people worldwide with resultant 1.5 million deaths (Ramazana *et al*., 2010). Dengue virus hemorrhagic fever epidemics now occur in the regions from which it was eliminated in the mid-20th century while West Nile virus is endemic throughout the Americas. Chikungunya virus affects millions in Indian Ocean basin and Japanese encephalitis is endemic in the Indian subcontinent and Australasia. Filariasis is a subject of a global eradication campaign (Tolle, 2009).

Rift Valley Fever is prevalent in Africa and the Middle East where it causes illnesses in human and domestic animals (LaBeaud *et al*., 2011). Mosquito borne diseases also contribute to poverty and mortality thereby affecting socio-economic development in tropical and subtropical countries (Becker *et al*., 2003). Apart from transmitting diseases, their bites cause considerable annoyance, can lead to allergic reactions, dermatitis and secondary infections (Kitchen *et al*., 2009).

Mosquito control can be realized by various ways. These are biological, physical and chemical methods (Jahn *et al*., 2010). Biological control includes use of larvae predators like fish; parasites and/or pathogens. It does not lead to rapid control of the larvae. The predators can also feed on beneficial organisms. They cannot be used in polluted water and temporary water areas such as puddles and vehicle ruts that form only in rainy seasons (Maniafu *et al*., 2009).

Physical methods such as habit change pose a great challenge because it is unfeasible to eradicate all aquatic habitats where mosquitoes breed including vehicle ruts, puddles, irrigation ditches, burrow pits, foot/hoof prints, edges of boreholes, swamps and rice fields (Service, 1986; Mwangangi, 2006).

Maniafu *et al*., (2009) observed that frequent use of chemicals for mosquito control using insecticides and organochlorine larvicides has resulted in vector resistance. They persist in soil, plant and animal tissues and cause death to fish and other aquatic life (Matasyoh *et al*., 2008). Insecticide treated bed nets are effective in controlling mosquitoes but affect ventilation leading to breathing problems (Ogoma *et al*., 2010). Temporary protection from mosquitoes can be achieved with repellents.

Synthetic repellents are expensive and there are concerns about their toxicity and safety (Jahn *et al*., 2010). Another disadvantage is that some of them require electricity for their usage which is not available in many rural poor settings (Karunamoorthi *et al*., 2008). Mosquito control cannot be effectively achieved using one single method. There is therefore the need for integrated mosquito management which includes habitat modification, use of larvicides, pathogens and predators that feed on larvae and depriving the larvae of food sources. Mosquito densities are also reduced by use of natural and synthetic insecticides, ITNs and genetic control methods (Rose, 2001).

Plants have been used since ancient times all over the world for control of mosquitoes. *Artemisia absinthium*, *Ferula asafetida*, *Cassia* spp, *Ficus carica*, *Allium sativum*, *Urgenia maritima* and *Citrus medica* were used as insect deterrents and for personal protection (Moore and Lenglet, 2004). The Chinese used powdered chrysanthemum as an insecticide (Seyoum *et al*., 2002).

In Tanzania, fresh twigs of *Ocimum* spp are placed in the corners of rooms to prevent mosquitoes from entering the houses or burned for the same purpose. *Azadirachta indica* is burned to repel mosquitoes (Moore and Lenglet, 2004). Plants parts are burned to repel mosquitoes or mosquito coils made from dried plants and combustible material are burnt (Seyoum *et al*., 2002).

Naturally derived insecticides were in frequent use before the advent of DDT. These included nicotine anabasin, lupinine and pyrethrum (Campbell *et al*., 1993). Insecticides and repellents from plants reduce transmission of mosquito borne diseases by minimizing contact between the vector and host (Potter and Beavers, 2005).

Natural products are a promising option because they don't impact negatively on the environment and organisms which are not the intended targets (Hemalatha *et al*., 2015). Ghosh *et al*., (2012) explain that insecticides from plants have several compounds that act synergistically to provide their activity as opposed to synthetic insecticides that rely on single constituent thus increasing chances of resistance development by the vectors.

In developing countries, people are unable to purchase conventional insecticides but there exists a long history of use of plants. According to Forget *et al*., (1993) in instances where the insecticides have been made less costly through subsidies from governments, illiteracy and absence of protective gear leads to many unintended toxicities from improper use of these insecticides. Compared to synthetic repellents, traditionally-used insecticides are simple, cost-effective and readily available (Seyoum *et al*., 2002).

Secondary metabolites of plant extracts are toxic to the insects affecting a wide range of molecular targets in the insect. Wiseman and Chapagain, (2005), describe saponins as having insecticidal activity. Others with similar activity have been listed as steroids by Chowdhurry *et al*., (2008), isoflavonoids by Joseph *et al*., (2004), alkaloids and tannins by Khana *et al*., (2007) and essential oils by Cavalcanti *et al*., (2004).

In Kenya, *O. forskolei* and *O. fischeri* essential oils had greater repellent activity than DEET (Odalo *et al*., 2005). *P*-menthane-3, 8-diol (PMD) also called *Quwenling*, is as effective as DEET in repelling mosquitoes (Govere *et al*., 2000). *Cymbopogon nardus* (citronella) is commercially used as a repellent for children because natural repellents are safer than DEET.

Rattan, (2010) postulated activity of plants' secondary metabolites as being due to physiological disruptions including acetyl cholinesterase inhibition by essential oils blocking nerve transmission; thymol blocking gabba amino butyric acid (GABA) transmission and Na^{2+} / K^{+} exchange interruption is caused by pyrethrin while rotenone affects cellular respiration. Ryanodine blocks calcium channels and sabadilla inhibits nerve cell membrane action. Azadirachtin causes disruption of the insects' hormones and damages cells.

Despite this knowledge about plants, there have been minimal endeavors to evaluate and encourage use of plant-derived agents with activity against mosquitoes. This study was done to evaluate activity against different mosquito stages of six plants from Kenya's south coast. Collection of plants was done in Msambweni Sub County which has monsoon climate being hot and dry in the months of January to April. June to August is a cool period.

Short rains are in the months of October to December and long rains from April to July. The mean annual temperatures are 23 ˚C - 34 ˚C and relative humidity is 60% - 80% (Muthaura *et al*., 2011). Conventional health care systems are weak and the climate is warm and humid enabling most mosquitoes to thrive (UNICEF, 2000). Many people in the area don't use bed nets despite extensive campaigns for bed net use (Mutuku *et al.,* 2011).

Even when plant derived agents have been used for many years, it is important to carry out toxicological test to assess potential toxicity (Gbolade *et al*., 2004). These tests include acute dermal absorption and toxicity, immunotoxicity, dermal irritation/sensitization and genotoxicity (Moore, 2004).

The *Allium cepa* test is a measure of potential toxicity to human and environment. It is of short term duration, lasting 4 days, with advantages of being easy, less expensive and avails a suitable investigating platform for damage to cells (Fiskesjö, 1985). The current study was thus designed to broadly assess the activity of the selected plants against mosquito larvae and adults and formulate a safe and efficacious mosquito repellent preparation from these plants.

1.2 Statement of the problem

Mosquito bites cause loss of sleep, allergies and secondary skin infections. Mosquito transmitted diseases are currently uncontrolled. Malaria kills 1.5 million people while dengue affects 50 million people annually as Rift valley fever outbreaks cause human illness and livestock losses. Mosquito control methods like habitat change are hard to realize. Insecticides and larvicides are hazardous since they persist in soil, plant and animal tissues.

Mosquitoes have become resistant to all classes of insecticides used for bed nets and indoor spraying. Repellents supplement bed net use and are essential in areas where mosquitoes bite early in the evening or when people are outdoors. Synthetic repellents like DEET are expensive for daily use, are toxic and cannot be used in children. They also destroy plastic, glasses and wrist watches.

Natural methods of mosquito control and protection are easy, accessible and inexpensive. Plants have been used since antiquity for this purpose but interest to investigate and support use of agents for mosquito control is lacking.

1.3 Study objectives

1.3.1 General objective

This was to evaluate the plants' activity against mosquito larvae and adults and to formulate a safe and efficacious mosquito repellent preparation from these plants.

1.3.2 The specific objectives were:

1. To asses acute dermal irritation potential of the plants
2. To evaluate potential for genotoxicity of plants
3. To evaluate mosquito repellent activity of the selected plants
4. To establish the larvicidal activity of the selected plants
5. To evaluate efficacy of a formulated plant based mosquito repellent

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

Worldwide, there are over 3500 mosquito species (Service, 1986) sometimes existing in altitudes of up to 5500 M above sea level and underneath excavations of 1250 M lower than sea level. They are found in all parts of the world except the Antarctica (Service, 1986; Foster and Walker, 2002).

Those that bite man are of great public health importance. They are *Anopheles*, *Culex*, *Aedes*, *Mansonia*, *Haemagogus* and *Sabethes* (Foster and Walker, 2002; Service, 1986; Tolle, 2009). Nectar and other plant secretions are sources of food. However, female mosquitoes bite hosts to obtain nutrients for egg development (Jahn *et al*., 2010). Mosquito breeding sites have been increased by human activities (Tolle, 2009).

2.2 Overview of mosquitoes

Mosquitoes are slender and measure 2 mm-10 mm in length with a prominent frontward proboscis (Service, 2004). *Anopheles* mosquitoes transmit mostly malaria and filariasis (Rueda *et al*., 2014) while *Culex* mosquitoes transmit *Wuchereria bancrofti* and West Nile Virus (Ciota and Krammer, 2013), Rift Valley Fever (Turrel *et al*., 2007) and other arboviruses (Phasomkusolsil and Soonwera, 2011).

Aedes mosquitoes transmit mostly yellow fever, dengue, encephalitis viruses, *Brugia malayi* and *Wuchererería bancrofti* (Ramar *et al*., 2014). *Mansonia* mosquitoes transmit *Brugia malayi*, *Wuchereria bancrofti* and some arboviruses. *Hemagogus* mosquitoes and *Sabethes* mosquitoes transmit yellow fever, some arboviruses in South and Central America (Service, 1986).

Mosquitoes mate only once after emergence from pupae. Females bite hosts to acquire nutrients for egg development followed. Thereafter, oviposition follows and on subsequent blood meal, another set of eggs matures completing a gonotrophic cycle. Females lay 30-300 eggs per oviposition. *Anopheles* mosquitoes lay eggs singly that float on water while *Culex* and *Mansonia* mosquitoes lay multiple eggs attached to each and float on the water. Their eggs die when they are out of water. *Aedes* and *Hemagogus* mosquitoes deposit their eggs above waterline and on damp substrates. The eggs withstand desiccation and hatch when submerged in water (Service, 1986; 2004).

Mosquito larvae are usually legless with a globular thorax which is broader than both the head and abdomen. They have mouth brushes that take up water together with small particles into the mouth. They require water to develop but also feed on yeasts, bacteria and protozoa (Service, 2004). Larval habitats are usually permanent water collections but may also transitory ones like small pools, tree holes, rock pools, bamboo stumps, leaf axils in banana, split coconut husks, snail shells and manmade container habitats (Mwangangi, 2006).

Larvae are absent in uninterrupted water masses like lakes because fish feed on them. They are also absent in fast flowing water (Service, 2004). Development of pupae takes 2-3 days. They are comma shaped, aquatic and don’t feed but take in air, (Service, 2004). Despite presence proboscis in male mosquitoes, they lack maxillae and mandibles or when present, they are very small. This prevents the males from biting hosts and so males cannot bite and are unable to transmit disease to man.

The saliva of a female mosquito contains anticoagulants and haemagglutins which ensure that the blood does not clot and obstruct the mouth parts during sucking. Due to their elongated mouth parts, female mosquitoes can bite through clothing. Therefore clothing should be of close weave. Female mosquitoes obtain energy from natural sugary substances (Service, 1986; 2004). Body odours, carbon dioxide and heat serve as attractants for female mosquitoes to hosts (Jahn *et al*., 2010).

Mosquitoes are either anthropophagic or zoophagic. Anthropophagic mosquito species bite human beings while zoophagic bite other animals. Those that bite human while inside houses are endophagic while exophagic ones bite hosts outside the houses. After biting and obtaining a blood meal, they seek resting places during digestion of blood meal. Those that rest in houses during this period are endophilic while those resting outside are exophilic.

Aedes aegypti females are anthropophagic, exophagic and exophilic while *Anopheles* mosquitoes are anthropophagic, endophagic and endophilic (Bayoh *et al*., 2010; Reddy *et al*., 2011, Service, 2004; Tiawsirisup and Nithiuthai, 2006). However, some *Aedes aegypti* have been noticed to be biting and resting indoors (Chavasse and Yap, 1997). Mosquitoes' life span is 2-3 weeks and females live longer than males (Service, 2004).

Mosquitoes are classified into subfamilies. Subfamily *Toxorhynchitinae* has about 70 species and the adults are large, colourful having bluish green with orange tufts of hair on the abdomen. They are found on tree holes, tin cans and bamboo stumps. The proboscis is curved and are incapable of biting thus are of no medical importance except that they are predacious on larvae.

The *Anophelinae* subfamily is of medical importance. Eggs of *Anopheles* cannot survive desiccation. Larvae lie parallel to the water surface and are surface feeders. The adults have blackish and whitish scales on the wing veins. They land on surfaces at angles that allow both the proboscis and abdomen to be in straight line.

In subfamily *Culicinae,* the *Aedes, Culex, Mansonia, Sabethes* and *Haemagogus* are of most significance. Their eggs form rafts that enable them to float on water surface. Larvae hung facing downwards but at a slant position to the water surface to enable them get air. The adults land and rest parallel to surfaces. The wings are of consistent brown or black colour (Service, 1986; 2004).

2.3 The mosquito problem

Of all the arthropods, mosquitoes are of most public health importance (Eldridge, 2008). They cause annoying flight and buzzing sound. Their bites are irritating and a nuisance. Their bites can damage human skin leading to secondary bacterial infections. At the same time, substances within the saliva induce allergic reactions, localized irritation and the ensuing scratching may cause wouns that can be secondarily infected with bacteria (Kitchen *et al*., 2009).

As female mosquitoes feed on vertebrate blood, they acquire and transmit of disease-causing microorganisms that can affect human and animals. These diseases include malaria, filariasis, yellow fever, dengue, Japanese encephalitis, Chikungunya, O'Nyong nyong, rift valley fever and west Nile virus (Ogoma *et al*., 2010).

They afflict many people and cause many deaths especially in developing countries. Dengue virus hemorrhagic fever epidemics now occur in areas it was previously eradicated while West Nile virus is now prevalent across the Americas. Chikungunya virus affects millions in Indian Ocean basin and Japanese encephalitis is endemic in the Indian subcontinent and Australasia. Filariasis has now been made a subject of a global eradication campaign (Tolle, 2009).

The world population is currently estimated at 7.3 billion people (The World Bank, 2015). Malaria is of major global public health concern with almost half of the world's population in danger of getting infected with it in 106 endemic countries (Karunamoorthi *et al*., 2013). It affects about half a billion million people causing about two million deaths annually worldwide (Ayoola *et al*., 2008; Dapper *et al*., 2007). Malaria infection due to *P. falciparum* is life threatening and 2% of those infected by it end up dying, (Dikasso *et al*., 2006).

More than half of Kenya's population is at risk of contracting malaria. Infection with malaria and associated dangers is significant among the rural poor, especially young children and the expectant mothers. It accounts for about a third of those who are attended to as outpatients and a fifth of those admitted in health facilities. A fifth of deaths in the under five years is attributed to malaria (MOH, 2007). In Kenya almost two hundred million working hours are lost annually, due to infection with malaria (KEMRI, 2011; USAID 2011).

Figure 1: Global occurrence of malaria. Areas in red indicate malaria is endemic. Source https://wwwnc.cdc.gov/travel/yellowbook/2016/infectious-diseases-related-to-travel/malaria#4653

Dengue fever transmission is by *Aedes aegypti* mosquitoes from those infected with the dengue virus to others who are vulnerable (Fredricks and Fernadez-Semma, 2014). Mosquitoes acquire dengue virus from infected humans during the early stages of infection usually by the fourth day after the infection. While inside the mosquito, the dengue virus proliferates rapidly and reaches the salivary glands then the mosquito is now able to transmit the virus within a week to two weeks from the time it obtained the virus from the infected human being.

For the remainder of its life, the mosquito continues to transmit the disease during any subsequent blood feeding. Dengue fever presents with acute symptoms of fever, headache, arthralgia and rash. In its severe it is either dengue hemorrhagic fever or dengue shock syndrome (Garcia *et al*., 2011).

Currently, about 2.5 billion people live in regions with increased chances of contracting dengue (WHO, 2012). Dengue is endemic in about a hundred countries spread across Africa, the Americas Asia, the Pacific and the Caribbean. Annually, about one hundred million infections occur throughout the globe with a half a million (500,000) dengue hemorrhagic fever incidences and over twenty thousand (22,000) deaths usually in children (Rigau-Perez *et al*., 1998).

Figure 2: Global Dengue. Source: CDC website http://www.cdc.gov/Dengue/

Infection with yellow fever is due to yellow fever virus. Yellow fever is widespread in lowland equatorial Africa and South and Central America where sometimes it occurs as outbreaks or individual cases (Jentes *et al*., 2011). The virus thrives as an enzootic form in monkey populations propagated by forest mosquitoes which leads to isolated human cases otherwise referred to as jungle yellow fever. The other form causes outbreaks in human and is by the domestic form of *Aedes aegypti* (Foster and Walker, 2002).

Yellow fever kills 20% -50% of those who suffer from it. There is no antiviral treatment at present for yellow fever and the only remedy is personal protection against mosquito bites and vaccination to mitigate infections and disease transmission (Jentes *et al*., 2011).

Figure 3: Yellow fever countries and risk. Source: CDC website http://www.cdc.gov/Yellow fever/

Rift Valley fever (RVF) is endemic to Africa and the Middle East where its outbreaks cause severe illness in livestock. The vectors for RVF virus are *Cx. pipiens* and *Aedes* species (Foster and walker, 2002). Humans become infected through mosquito bites, exposure to blood containing the virus or inhaling the virus during animal slaughter.

In man, the disease is rarely fatal but causes fever, headache, joint pains and involvement of the liver and eyes (CDC, 2008). In Africa, outbreaks have been reported since the 1950's in South Africa, Zimbabwe, Egypt, Mauritania Kenya and Somalia. Recent outbreaks led to human affliction and death of livestock in Kenya (LaBeaud *et al*., 2011).

West Nile virus (WNV) results in the most extensive viral disease transmitted by any arthropod globally it singly represents the major epidemic of neuro-invasive infection that has been so far observed. Of all the arboviruses, it is the only one which has a worldwide distribution unavailable only in Antarctica. It exists in an enzootic form sustained by ornithophilic *Culex* mosquito species and vulnerable birds whose susceptibility is of varying geographic locations (Ciota and Krammer, 2013).

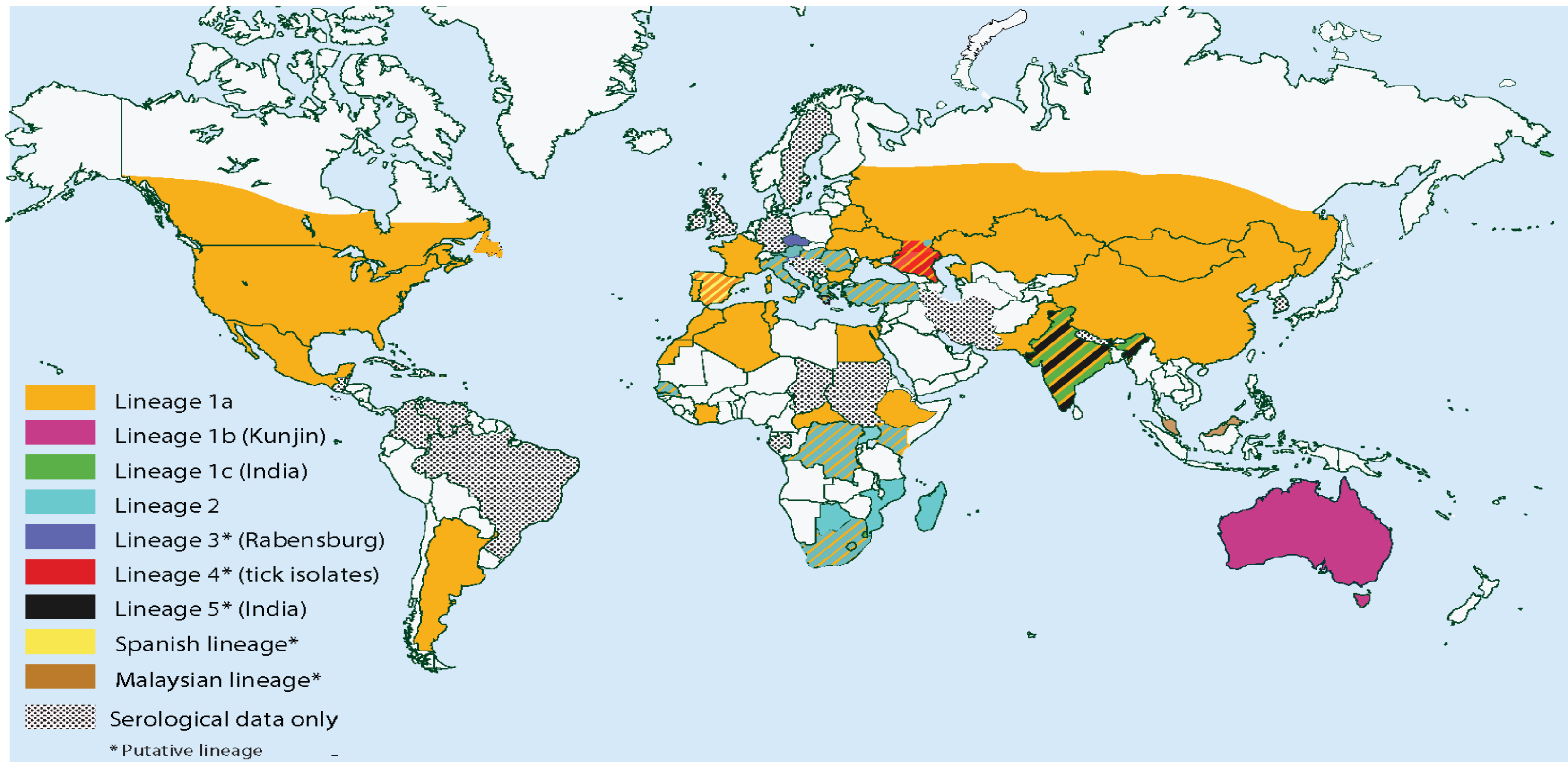


Figure 4: West Nile virus global distribution. Source: Ciota and Krammer, 2013

Chikungunya (CHIK) virus was discovered in Tanzania in 1953 and is found in eastern Africa some areas in India and southeasterly regions of Asia (Jupp *et al*., 2002). *Aedes* mosquitoes especially *Ae*. *albopictus* have been identified as its vectors (Rezza *et al.,* 2007). The regions in which *Ae. albopictus* occur have increased in the last twenty years to include the Pacific and Indian Oceans and southern Europe, especially Romagna region of Italy, in addition to the ones mentioned above (Sambri *et al*., 2008).

CHIK virus also infects baboons and vervet monkeys via *Ae. africanus*, *Ae. luteoccphalus*, *Ae. opok*, *Ae. furcifer*, *Ae. taylori* and *Ae. cordellieri*. It can also be transmitted across human beings by *Ae. aegypti* (Foster and Walker, 2002). Chikungunya (CHIK) virus infection manifests with arthralgia, rash, joint pains but encephalitis is absent. These symptoms can last up to two weeks but arthralgia persists in majority of those infected for several months (Sambri *et al*., 2008).

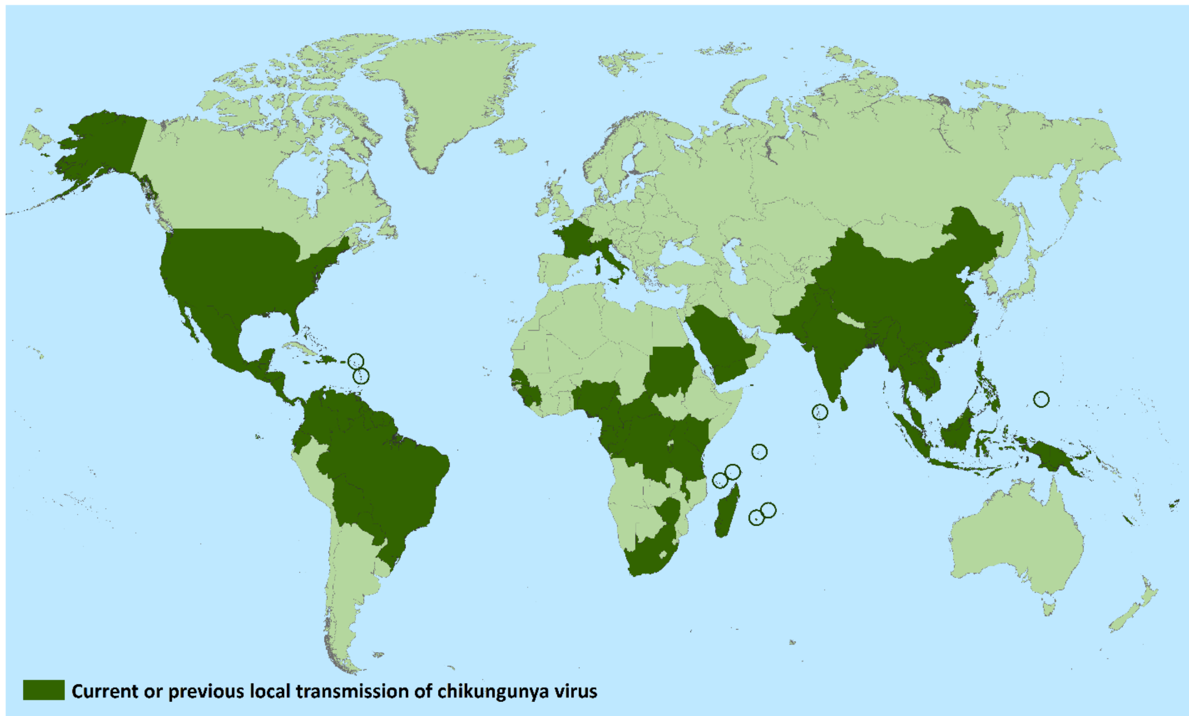


Figure 5: Global distribution of Chikungunya virus by March 10, 2015

Source: CDC website http://www.cdc.gov/chikungunya/

Filariasis is due to filarial nematodes (family: *Onchocercidae*) affecting vertebrate tissues. It can be acute or chronic disease presenting as lymphatic filariasis and is common in tropical and subtropical regions (Grove, 1990). Lymphatic filariasis is caused by *W. bancrofti*, *Brugia* spp. More than forty million (40million) people suffer from elephantiasis, hydrocoele, or lymphoedema (Foster and Walker, 2002). Almost one billion people are in danger of contracting this disease with about 130 million active infections spread about 73 countries throughout the tropics and sub-tropics (WHO, 2013 c).

Figure 6: Global distribution of lymphatic filariasis indicated by red marks. Source: CDC website http://www.cdc.gov/parasites/ lymphatic filariasis

Japanese encephalitis (JE) virus was first isolated from the brain of a person who died of encephalitis in 1935 and from *Cx. tritaeniorhynchus* in 1938 (Foster and Walker, 2002). It is characterized by acute encephalitis but most of the times, it present with no symptoms. The disease kills 25% of those with the acute illness and those who survive manifest with neurologic sequelae (CDC, 2015 a). It occurs mostly in Asia (Foster and Walker, 2002).

Zika virus was discovered in 1947 in Uganda's Zika forest and associated with human disease in 1953 in Nigeria. In over five decades, there were few reported. It has spread to over sixty countries as of 2016 (Petersen *et al.*, 2016, WHO, 2016a). Its major vectors are *Aedes* mosquitoes. It is transmitted from infected person to another through the mosquito vectors, sex, from mother to child and blood transfusion. It leads to microcephaly, pregnancy losses, increases incidences of Guillain-Barré syndrome and neurological disorders such as meningitis and meningoencephalitis (ECDC, 2016; WHO, 2016a).

There is no vaccine available for Zika virus and prevention is through avoiding mosquito bites by use of repellents, wearing long sleeved shirts and trousers during the time when *Aedes* mosquitoes are most active and use of mosquito nets. Pregnant women should avoid travelling to areas where there are reported cases of Zika virus infections (ECDC, 2016; Petersen *et al.,* 2016).

Other mosquito borne viruses include the eastern equine encephalomyelitis (EEE) virus which occurs in the Americas and Caribbean and leads to increased morbidity especially in the very young, the elderly and those with lowered immunity. There is sudden onset of encephalitis, fever, convulsions, coma and half of those with the severe symptoms die. Those who don't die have long-term neurologic complications.

The western equine encephalomyelitis (WEE) virus is common in North America where it causes acute encephalitis in horses and humans. It presents with acute onset of meningitis or encephalitis and accompanying symptoms such as fever headache and coma. About 5% of those affected die and survivors experience long lasting problems of the nervous system.

The Venezuelan equine encephalomyelitis (VEE) virus affects humans and equids in north of South America, Central America and Mexico since the 1930s. Humans may be asymptomatic, have mild symptoms or present with encephalitis which may be worse in the very young. During epidemics, the mortality rate is about 1%.

Ross River (RR) virus causes epidemic polyarthritis in Australia, Fiji, and the Cook Islands. The symptoms are fever, rash, and arthralgia. The only known bacterial transmission by mosquitoes to human is the mechanical transmission of the causative agents of tularemia (*Francisella tularensis*) and anthrax (*Bacillus anthracis*) (Foster and Walker, 2002; Kay and Aaskov, 1988 Sambri *et al*., 2008; PAHO, 1972)

2.4 Control and prevention of mosquitoes

2.4.1 Control by use of biological methods

This implies increasing predators, parasites or pathogens in habitats or their introduction into habitats where they were initially not present. Biological mosquito control methods are almost exclusively directed against larvae (Eldridge, 2008). The advantage is that it doesn't introduce pollution. Predators mostly used are fish of genera *Gambusia, Poecilia, Santherodon* and *Panchax* (Poopathi and Tyagi, 2006).

The predators may also feed on beneficial organisms. Fish are unsuitable for controlling mosquitoes which breed in small containers and temporary water areas. They are not suitable for use in polluted water (Maniafu *et al*., 2009). Other larval and pupae predators are tadpoles (Kweka *et al*., 2011). The larvae of *Toxorhynnchites* mosquito species also feed on other larvae (Service, 1986). Bats have also been used to control mosquitoes by feeding on adult mosquitoes (Kalko, 1995).

Pathogens and parasites include cytoplasmic polyhedrosis viruses, bacteria especially *Bacillus thuringiesis* Var. *Israelis*, protozoa such as *Nosema vavraria*, fungi (*Culicinomyces, Agenidium*) and nematodes, the most promising being *Romanomermis culicivorax* (Lacey and Lacey 1990). They are not harmful to man. The most useful is *Bacillus thuringiesis* var. *Israelis* which is easy to mass produce and formulate into powder form. The powder consists of dead bacteria which kill mosquito larvae when ingested (Talisuna *et al*., 2004).

Use of larvae predators, such as fish, parasites or pathogens is useful but does not lead to rapid control of the larvae. Generally, biological control is complex to execute and sustain (Maniafu *et al*., 2009). Genetic control is another form of biologic control.

Mosquitoes can be genetically manipulated or selectively reared to beget mosquitoes that are refractory to infection with human diseases. Once released to the environment they effectively replace the natural populations. Genetic control is usually combined with insecticides. The danger is that if resistance exists in the wild population, replacement will not be complete (Alphey *et al*., 2010; Dyck *et al*., 2005).

Other methods that have been experimented upon are selection of genes to produce more males than females and also vector replacement with a non vector species. There is also sterile male release in which males are made sterile in the laboratory by radiochemical irradiation, chemosterilants or production of infertile hybrid males (Alphey *et al*., 2010).

Genetic methods overcome the increasing problem of resistance. The main draw backs of genetic methods are that they are difficult to implement, require high levels of expertise technology. Besides, resistance changes may take place enabling parasites to thrive in strains that were initially chosen for their refractoriness (Burattini *et al*., 2008).

2.4.2 Physical control methods

Physical methods include draining of water in ponds and filling of such water masses with soil. This clears water and prevents any mosquitoes from breeding. It is effective but is also unfeasible to eradicate all aquatic habitats where mosquitoes breed such as vehicle ruts, puddles, irrigation ditches, burrow pits, foot/hoof prints, edges of boreholes, swamps and rice fields (Mwangangi, 2006; Service, 1986).

Filling in with sand / rubble, source reduction and drainage completely eradicates breeding sites. It also involves removal of container habitats as well as covering the openings of water jars (Walker, 2002). Reducing use of water storage containers through availing of dependable piped water helps reduce reliance on these containers thus lower rates of breeding of species like *Aedes aegypti* (Tolle, 2009).

Drainage of aquatic masses where larvae breed such as marshes leads to lasting control. However difficulties arise as it is impossible fill all water collections. Also some aquatic habitats like rice fields, fish farms and burrow pits are sources of drinking water and food. Moreover, it is expensive to drain large and permanent swamps (Mwangangi, 2006; Service, 1986).

Habitat change makes the habitats unsuitable for mosquito breeding. They include altering the course of streams to avoid development of puddles (WHO, 1982). Rafatjah, (1988), recommends that overhanging vegetation can be cut so that more sunlight can penetrate hence reduce breeding by species that prefer habitats with little sunlight. Trees can also be planted around larval breeding areas to reduce the breeding of species that prefer areas of much shade such as some *Anopheles* species.

Rooted or floating vegetation can be removed to eliminate breeding sites of mosquitoes. Small and large marshy areas may be dug out to cause development of vertical banks and deep water. This reduces formation of pools around the large expanses of shallow water where mosquitoes could breed (Service, 1986; Thevasagayam, 1985). Whenever aquatic habitat is modified but not eliminated, there is a danger that the change can promote thriving of other mosquitoes that were initially not present (Service, 1986).

2.4.3 Chemical control methods

Chemical methods for mosquito control include use of larvicides, and insecticides such as mosquito adulticides. Organochlorine larvicides and insecticides lead to vector resistance. They persist in soil, plant and animal tissues and cause death to fish and other aquatic life (Matasyoh *et al*., 2008). Insecticide treated bed nets are effective but affect ventilation (Ogoma *et al*., 2010).

Paris green (copper aceto-arsenic) is applied as fine dust and acts as stomach poison to the mosquito larvae. It is effective against surface feeding mosquitoes like the anopheles (Alphey *et al*., 2010; Service, 1986). Diesel and kerosene can be sprayed on water to kill larvae. They physically block trachea and suffocate the larvae besides also interfering with availability of air at the water surface. The disadvantage is that a lot of oil is needed (300-500 L/Ha). Chemical control measures are directed at killing larvae because they will not kill eggs and pupae and they will not prevent mosquito oviposition. They have little residual effects and must be sprayed every 7-10 days (Service, 1986).

Personal protection reduces chances of being bitten by mosquitoes. Screens having 6-8 meshes per centimeter can be used to cover spaces such as in windows and doors and ventilators to prevent mosquito entry. Mosquito nets with 9-10 meshes per centimeter are also used (Service, 1986). They are tucked in under the mattresses or beddings and prevent mosquitoes from biting. The nets can be treated with insecticides for better effects. Bed nets reduce ventilation (Ogoma *et al*., 2010; Walker, 2002).

Repellents one applied provide temporary protection from mosquitoes (Maharaj *et al*., 2010). They reduce vector-host contact (Katritzky *et al*., 2008). Insect repellents are normally applied on exposed skin areas and supplement bed net use. They are also useful outdoors or indoors before people go to sleep (Moore *et al*., 2007).

However, most mosquito repellents are usually effective for about two hours and need reapplication. The most effective mosquito repellent is DEET usually used in concentrations of 20% and is the standard used to compare activity of other repellents (WHO, 2009; Jahn *et al*., 2010).

There is a likelihood of reliance on ineffective repellents among rural poor because most of the synthetic repellents are unaffordable and not culturally acceptable (Logan *et al*., 2010). *N, N*-diethyl-m-toulamide (DEET) causes severe reactions in some people, melts plastic including damaging glasses and mobile phones and has unpleasant odour (Qui *et al*., 1998). Most synthetic repellents are not indicated for use in children (Jahn *et al.*, 2010). Currently, a proportion of *Aedes aegypti* mosquitoes have started developing resistance to DEET (Stanczyk *et al*., 2013).

Other ways of controlling mosquitoes is by having insecticides formulated as mists, fogs or aerosols and spraying directly to the mosquitoes which is useful for mosquitoes that bite man indoors and rest inside houses after feeding (Chavasse and Yap, 1997). Indoor residual house spraying using insecticides that have residual effect such as DDT kills mosquitoes that rest inside houses before or after a blood meal (Guyatt *et al*., 2002).

Due to the above reasons, researchers are looking for plant based repellents of natural sources with promising repellent activities (Tiawsirisup and Nithiuthai, 2006). Many components of plant based repellents have a disadvantage of being very volatile hence the need for repeated applications (Logan *et al*., 2010). It has become necessary for discovery and development of novel compounds to address the above shortcoming (Katritzky *et al*., 2008).

2. 5 Studying insect repellents

Insect repellents can be studied through *In vitro* methods such as use of artificial membrane feeders (Novak *et al*., 1991). Repellency can also be investigated through *in vivo* methods involving use of animals (Moore, 2004). These methods do not adequately mimic the real situation as can be manifested with human skin (WHO, 2009).

Laboratory studies are conducted on human volunteers for greatest accuracy in comparison to DEET (*N, N*-diethyl-m-toluamide) and ethanol or acetone as negative control. The results obtained relate to the real circumstances of use (WHO, 1996, 2009). In these studies, laboratory reared *Ae. aegypti* in cages of approximately 40cm×40cm×40cm are employed as they have less difficulty in breeding while under laboratory conditions and bite effortlessly (Moore, 2004).

Forearms, from the elbow to the wrist (~625cm^2) are treated with the test repellent and the hand from the wrist to the fingers covered by a glove to protect it from the mosquitoes. The forearm is introduced into a cage with approximately 50 mosquitoes and the number of mosquito bites, probes and landings on the forearm are recorded. Repellents are also evaluated in field studies in and around houses and assessments are by catches on human volunteers with both legs bared from knee to the ankle (WHO, 1996, 2009).

2.6 Toxicological evaluation of repellents

Even when traditional repellents have been used for many years, it is important to carry out toxicological test to assess potential toxicity (Gbolade *et al*., 2004). These tests include acute dermal absorption and toxicity, immunotoxicity, dermal irritation/sensitization and genotoxicity (Moore, 2004).

2.6.1 Genotoxicity testing

According to Maurici *et al*., 2005, genotoxicity is the potential of a substance to negatively affect DNA and any other the cell components that assure genome fidelity. The purpose of these *in vitro* and *in vivo* tests is to recognize possible and exclude mutagenic risks to man and for those which show genotoxicity potential, the mechanism of action is determined. OECD, 1997a, indicates these tests are important in understanding carcinogenicity studies. Some of the tests include the following:

2.6.1.1 Bacterial reverse mutation test

It uses *Salmonella typhimurium* and *Escherichia coli* strains which need amino-acid to identify point mutations involving substitution, addition or deletion of DNA base pairs.

Mutations which revert the mutations in the test strains and reinstate the ability of the bacteria to synthesize an essential amino acid are identified. It is fast, affordable and simple (Ames *et al*., 1975).

It is of limited value because bacteria cells are different from mammalian cells and the results cannot be extrapolated to human. Besides, the test is not appropriate for the evaluation of compounds that kill bacteria or those that affect replication in mammalian cells. It is an initial test in genotoxic evaluation of mutation-inducing activity (Maron and Ames, 1983; OECD, 1997b).

2.6.1.2 ***In vitro* mammalian cell micronucleus test (MNvit)**

It is used in identifying micronuclei in the cytoplasm of interphase cells. It forms a basis to further explore potential of substances to damage chromosome *in vitro*. Formation of micronuclei causes cell death and is indicative of damage that has been transmitted to daughter cells. This test does not mimic *in vivo* conditions and false positive results can be obtained because of pH, osmolality or the chemical interacting with the cell culture media (ESAC, 2006; OECD, 2014a).

2.6.1.3 *In vitro* mammalian chromosomal aberration test

This test was adopted in the year 2014. It aids in determining substances which form structural chromosomal aberrations in cultured mammalian cells. The cell cultures are allowed to interact with the test chemical. At preset intervals the cells are treated with a metaphase-arresting substance and analyzed for chromatid-type and chromosome-type aberrations. There is a likelihood of false positive outcomes (Honma, 2011; OECD, 2014b).

2.6.1.4 Mammalian erythrocyte micronucleus test (OECD 474)

This test was adopted in 2014 for evaluating genotoxicity. It identifies chromosomal and mitotic damage to erythroblasts through assessment of micronuclei formation with lagging or chromosomal fragments in erythrocytes of rodents whose presence is indicative of induced chromosomal aberrations (Parton *et al*., 1996; OECD, 2014 c).

2.6.1.5 The *Allium cepa* test

Levan, 1938, introduced the use of *Allium* root system as a tool for determining factors for environmental pollution, potential toxic effects of compounds and investigation of anticancer activities of plants, (Majewska *et al*., 2003; Babatunde and Bakare, 2006).

It has been authenticated by many researchers through test methods carried out on animals with obtained similar outcomes, (Vicentini, *et al*., 2001) and therefore information obtained can be extrapolated to human and animals.

The *Allium cepa* test is also used to investigate the possible genotoxicity of medicinal plants (Akaneme and Amaefule, 2012; Camparoto *et al*., 2002). It last for a short duration, usually 4 days and is simple, cost effective enabling study of chromosome or cell damage (Fiskesjö, 1985).

The roots grow in contact with the test substance and possible DNA damage is predicted. There exists a high percentage of correlation between this test and testing methods for carcinogenicity on rodents. This test has been evaluated for sensitivity in comparison with Ames test with significant positive outcomes (Rank and Nielsen 1994).

2.6.2 Dermal irritation/ corrosion tests

Any material to be applied on human skin must be assessed for irritability and corrosion potential. This serves as a way of assessing and evaluating toxic characteristics of a substance whose route of administration is topical and on the skin (EPA, 1998). There exist several methods for assessing and evaluating possible toxic effects of a substance when applied to human skin.

2.6.2.1 Acute dermal irritation/corrosion test

It is also called Draize test (Draize *et al*., 1944). This is performed to assess the irritancy level of a dilution of a test material on the skin of New Zealand white rabbit, usually three per dilution of test substance (OECD, 2002). The major principle for this test involves application of the test substance initially as one dose to the skins of experimental animals which are usually New Zealand white rabbits.

The test substance is applied on one part of dorsal area of the New Zealand white rabbit while the other part serves as control. Observations are made for the effects of the substance on the skin usually for a period of between fourteen (14) and twenty one (21) days (EPA 1998, OECD 2002).

2.6.2.2 *In vitro* skin irritation: Human skin model test

It does method does not require animal use. It uses human reconstructed tissue models. The test substance is spread across a reconstructed human epidermis model that mimics biochemical and physiological characteristics of the epidermis.

It avoids pain to the animals as well as differences encountered with the traditional animal test such as inter-species variation with regard to responses (OECD, 2013). The information generated is inadequate regarding as substances that are coloured can affect cell viability parameters (OECD, 2002; 2004, 2013)

2.6.3 *In vitro* skin corrosion

2.6.3.1 Reconstructed human epidermis (RhE) test method

It mimics the histomorphology, biochemical and physiological characteristics of the epidermis. Its basis is that substances that corrode the skin shall permeate the *stratum corneum* and to an extent they are toxic to the cells beneath. It is mainly used as a predictor of *in vivo* dermal corrosion effects to be investigated in rabbits (OECD 2002, 2012).

2.6.3.2 Transcutaneous electrical resistance test method (TER)

Skin discs are used to determine substances that are corrosive and hence cause loss of *stratum corneum*. Test substances are applied to the discs' surfaces and the corrosion potential is determined by the ability of the test substance to cause effects the normal *stratum corneum* permeation barrier function (OECD 2013).

The method is cumbersome requiring the rats to be clipped of hair at age of twenty two (22) days, then washing with antibiotics for three (3) days before finally harvesting the skin at age of 28-30 days. It also requires many chemical substances including antibiotics, magnesium sulphate solution, ethanol; electrodes and dyes.

Other tests employ structure-activity relationship (SAR) models and human skin models such as EPISKIN™ and EpiDerm™. Measurement of irritation potential can also be done through *in vitro* cell and tissue culture assays based on cell lines, primary mono layer cultures, organ cultures, and reconstituted human skin equivalents and SAR models.

The *in vitro* tests incorporate numerous endpoints, including cell morphology, viability, membrane damage, metabolic effect, and the release of various inflammatory mediators.

Of importance is that no satisfactory *in vitro* methods are currently available to substitute for the Draize skin irritancy test.

2.6.4 Acute eye irritation tests

The eye is continually exposed to potential hazardous substances in the environment on a daily basis. It is therefore necessary to evaluate substances for their eye irritation potential. The human response should be the ultimate in for eye irritation testing (Bagley *et al*., 2006) but carrying out such experiments on human is unethical and unrealistic because it would require many human subjects so as to represent human diversity and is immoral to develop such a test method.

Data from human on possible risks to the eye is derived from accidental exposures and is not representative of the most serious eye effects since the exposure is too brief occasioned by tearing and blinking. According to Freeberg *et al*., 1986, tests on effects of substances on human eye do not evaluate irritation potential. Due to absence of human database, available rabbit data forms basis for comparison on *in vitro* ocular toxicity tests (Bagley *et al*., 2006; OECD, 2012; Huhtala *et al*., 2008).

2.6.4.1The chorioallantoic membrane vascular assay (CAMVA)

Luepke, 1985; Luepke and Kemper, 1986 described this test. It is also known as Hen's egg test (HET) or simply chorioallantoic membrane vascular assay (CAM assay). The chorioallantoic membrane has a vasculature and inflammatory process similar to the conjunctival tissue of rabbit's eyes.

The test provides information on the possible effects that can occur in the conjunctiva after chemical exposure (NICEATM, 2006). It is classified together with other isolated organ models but it differs in criteria for evaluation (Barile, 2010) since it has the addition of vasculature (Curren and Harbell, 2002).

2.6.4.2 Other tests for ocular irritation determination

These include low-volume eye-irritation test by Griffith *et al*., (1999), the isolated chicken eye as described by Prinsen and Koëter (1993), the bovine cornea opacity assay that was developed by Gautheron *et al*., (1992) that has found use in cosmetics and drug companies (Esekes *et al*., 2005) and is now accepted as OECD TG 437 (OECD, 2013).

The ocular irritectional assay is founded on EYTEX^{TM} system (Eskes *et al*., 2005, 2014) and acts on the principle that irritation to the eye and corneal opacity arises from exposure to irritating chemicals that alter the fundamental function of the proteins that make up corneal tissue. Spectroscopic methods are used to measure the turbidity of the reagent at 405 nm.

Griffith *et al*., (1999) developed the first ever equivalent of a human cornea using immortalized human corneal cells as a way of establishing the failure of cornea to heal laser eye surgery. It is more sensitive compared to the human eye-bank corneas. Reichl *et al*., (2005) also developed a human corneal equivalent for *in vitro* drug permeation studies.

Computer generated models (Lo Piparo and Worth, 2010) also called *In silico* models employ repositories of *in vitro* and *in vivo* data on toxic substances to project the toxicity of test substances (Simon-Hettich *et al*., 2006). They are rapid and inexpensive and are useful at predicting precise endpoints (Nigsch *et al*., 2009). Despite all these studies and developments, none has been demonstrated to be as effective in projecting ocular irritation as the Draize test (Huhtala *et al*., 2008).

2.7 Plants used as mosquito control agents

According to Fradin and Day, (2002), Marco *et al*., (1987) and Perry *et al*., (1998), excessive utilization of conventional insecticides has caused much harm that was unanticipated at the onset. These include direct toxicity to human, other organisms and environment. Most pests have also become resistant to the pesticides because of uncontrolled and continuous use.

The use of plant derivatives has been in existence long before innovations of current conventional insecticides (Isman, 2006). Fresh or dried plants are added to fires to enhance repellent activity of smoke or mosquito coils made from dried plants and combustible material are burnt (Seyoum et al., 2002).

In Tanzania, fresh twigs of *Ocimum* spp are placed in the corners of rooms to prevent mosquitoes from entering the houses or burned for the same purpose. *Azadirachta indica* is burned to repel mosquitoes (Moore and Lenglet, 2004). Important plant products used to keep away mosquitoes are pyrethrum, rotenone, neem, ryania, nicotine and sabadilla (Isman, 2006).

P-*menthane*-3, 8- *diol* (PMD) also called *Quwenling* is as effective as DEET in repelling mosquitoes. Citronella from *Cymbopogon nardus* is commercially used as a repellent for children because natural repellents are believed to be safer than DEET (Govere *et al*., 2000).

Non polar extracts of pyrethrum yield active principles for repelling mosquitoes (Casida and Quistad, 1995; Glynne-Jones, 2001) of which pyrethrins I and II are very potent against many insects (Casida and Quistad, 1995).

Natural pyrethrins are neurotoxic to insects just like DDT but they are easily degraded by sunlight and thus synthetic pyrethroids were developed which are photostable and are now available for indoor and outdoor use (Isman, 2006).

Azadirachta indica also called neem tree has compounds with insecticidal activity (Schmutterer, 2002). The oil obtained from the seeds has activity against insects and mites. Extracts of the same plant contain a triterpene called azadirachtin and triterpenoids salannin and nimbin (Isman, 2006). Azadirachtin is an antifeedant, affects hormones of the insects and may cause sterility in the females (NRC, 1992).

At the moment, it is impractical to synthesize azadirachtin. Besides, it rapidly degraded by sunlight (Isman, 2006). Neem has less commercial viability because the cost of producing its final products is very high (Isman, 2004). According to Bomford and Isman (1996), the activity of azadirachtin may vary across different insect species and there are chances of resistance development or desensitization to azadirachtin by insects.

Essential oils contain monoterpenes, phenols, and sesquiterpenes (Isman, 2006). Those with anti-insect effects are 1, 8-cineole from *Rosmarinus officinale* and *Eucalyptus globus*, eugenol from *Syzygium aromaticum* and thymol from *Thymus vulgaris* (Isman, 1999). Most affect the nervous system of the insects (Dev and Koul, 1997; Kostyukovsky *et al*., 2002). They are very volatile and therefore not effective when insects reinvade treated surface several hours later (Isman, 2006). Nicotine has long history for use as an insecticide. It is an anticholinesterase with similar activity like organophosphate and carbamate insecticides (Hayes, 1982). However, pure nicotine is toxic to mammals and is not frequently used (Isman, 2006).

Despite long use and advantages of plants for mosquito control including as repellents, there is little effort in investigation and promotion of plant based repellents (Seyoum *et al*., 2002).

2.7.1 Locally available mosquito repellents of plant origin

Currently in Kenya, plant derived products with insecticidal activity are few. They include Mozigone® repellent cream and coil developed from leaves of eucalyptus through steam distillation (ICIPE, 2007). Another is Ballet Mosquito Repellent® jelly from Buyline industries in Nairobi. It is composed of paraffinum liquidum, cera microcrystalline, paraffin, *tacromanthus camphoratus, pelargonium graveolens*, isopropyl myristate, glycerol, myristyl myristate, citronellal, tocopherol acetate, *cymbopogon nardus*, geraniol, *eucalyptus globulus,*

rosemarinus officinalis, citral, benzophenone-3, linalool and limonene. The exact process by which Ballet Mosquito Repellent® is prepared is hard to determine.

Kapi Limited, based at Nakuru town of Kenya manufacture natural liquid mosquito vaporizer that consists of essential oils of eucalyptus, palmarosa, basil, cedarwood, turmeric, thyme, peppermint and deodorized kerosene (solvent). It is used with vaporizer gadgets that require electricity and is thus limited to indoor use.

2.7.2 Limitations and safety issues related to development of biopesticides

The major barriers to development, production and commercialization of biopesticides revolve around whether the sources of the raw materials can last for long, establishing a consistency in procedures and processes of obtaining the extracts as well as assuring quality of the extracts and issues related with registration and regulation of their use.

Regarding sustainability, the sources must be preferably agricultural and be able to be produced throughout the year (Isman, 2006). The procedures and processes of obtaining the active ingredients as well as the quality of those ingredients must be standardized (Leatemia and Isman, 2004).

Regulatory approval including registration is important in order to avoid the basic assumption that natural products are entirely safe (Trumble, 2002) even though most of have minimal toxic effects to mammals and the environment. Besides, they don't affect non target organisms as much and persist less in the environment compared to synthetic derivatives (Isman, 2006).

CHAPTER THREE

PHYTOCHEMICAL COMPOSITION AND LARVICIDAL ACTIVITY OF SIX PLANTS USED FOR MOSQUITO CONTROL IN MSAMBWENI SUB COUNTY

3.1 Introduction

Mosquitoes have almost a worldwide distribution occurring in all continents except in Antarctica (Service, 1986; Foster and Walker, 2002). Their bites cause considerable annoyance, can lead to allergic reactions, dermatitis and secondary infections (Kitchen *et al*., 2009). Mosquitoes are vectors in transmission of malaria, filariasis, yellow fever, dengue, West Nile virus among others (Ogoma *et al*., 2010).

Mosquito control can be realized by biological, physical and chemical methods (Jahn *et al*., 2010). Biological control is difficult as mosquito and predators such as fish, parasites or pathogens do not lead to rapid control of the larvae (Chandra *et al*., 2008; Service, 2004; WHO, 2013a). Predators also feed on beneficial organisms and cannot be used in polluted water and temporary water areas such as puddles and vehicle ruts that form only in rainy seasons (Maniafu *et al*., 2009).

Physical control and habit change are unattainable since it is impractical to eliminate all aquatic habitats of the mosquitoes like vehicle ruts , puddles, irrigation ditches, burrow pits, foot/hoof prints, edges of boreholes, swamps and rice fields. Other mosquito habitats are sources of water and/or food such as rice paddies (Service, 1986; Mwangangi, 2006).

Mosquito breeding sites are increased by human activities that increase water trapping (Tolle, 2009). Insecticide treated bed nets affect ventilation by reducing airflow making it hot to sleep under them (Ogoma *et al*., 2010). They have also been implicated as a factor for respiratory problems for those who sleep under them (Alaii *et al*., 2003; Galvin *et al*., 2011; Nnodim *et al*., 2014).

Frequent insecticides' use for mosquito control has resulted in vector resistance and undesirable outcomes on unintended organisms (Maniafu *et al*., 2009). Repellents provide temporary protection from mosquitoes but synthetic repellents such as *N, N*-diethyl-m-toluamide (DEET), are expensive and concerns abound on their toxicity and safety (Jahn *et al*., 2010). Organochlorine larvicides and insecticides lead to vector resistance. They persist in soil, plant and animal tissues and cause death to fish and other aquatic life (Matasyoh *et al*., 2008).

Plants have long history on use for mosquito control since antiquity. *Artemisia absinthium*, *Ferula asafetida*, *Cassia* spp, *Ficus carica*, *Allium sativum*, *Urgenia maritima* and *Citrus medica* were used as insect deterrents and for personal protection (Moore and Lenglet, 2004). Few plants have been studied for their larvicidal activity. Several plant families have been studied for their use in larval control (Ghosh *et al*., 2012; Maharaj *et al*., 2012; Innocent *et al*., 2010). The aim of this study was to determine phytochemical composition as well as evaluate larvicidal effects from the six plants used for mosquito control in Msambweni sub county, Kwale County, Kenya.

3.2 MATERIALS AND METHODS

3.2.1 Selection of the plants and collection of plant material

The plants for this study were selected on their ethnomedicinal information on their use for control of mosquitoes (Nguta *et al.*, 2010). This was in addition to review of relevant literature on plants used for mosquito larval control. Six plants were identified for this study. Plants were collected after initial field identification with the aid of traditional herbal practitioners from Msambweni Sub County. Further identification was done by a plant taxonomist at the Department of Land Resource Management and Agricultural Technology (LARMAT), University of Nairobi where voucher specimens were deposited.

Harvesting of the plants' parts was done on the months of September and November 2012 when there is adequate foliage following the rains and material of best quality is ensured (Muthaura *et al.*, 2007). The harvested plant parts were first cleaned with water then dried off the water and stored in dry sacks. The material was then taken to the Department of Public Health, Pharmacology and Toxicology, University of Nairobi. The study area is presented in figure 7 and the selected plants are listed in table 1 and shown in plate 1.

3.2.2 Plant material preparation and extraction

Further preparation was done at the Department of Public Health, Pharmacology and Toxicology, University of Nairobi. The plant material was dried under shade and upon drying, was ground to powders and stored in air tight papers (Gakuya, 2001; Wagate, 2008).

3.2.3 Extraction

3.2.3.1 Water extraction

One kilogram (1000 grams) of each plant powder was extracted separately with water in conical flasks in which frequent stirring of the mixture was done to ensure proper mixing. The conical flasks were corked tightly with stoppers. Shaking was done regularly to allow for maceration for four days. On the fifth day filtration was done using Whatman No.1 filter paper.

The filtrate was stored in sterilized beakers, covered tightly with aluminium foil and stored in a refrigerator at $^{+}4^{0}$ C pending freeze-drying. Freeze drying was done using Virtis Bench Top 3® Model freeze drier (The Virtis Company, Newyork), at the Department of Veterinary Anatomy and Physiology, University of Nairobi. The freeze dried material was used for subsequent larvicidal laboratory tests.

3.2.3.2 Acetone extraction

One kilogram (1000 grams) of each plant powder was extracted separately with analytical grade acetone in conical flasks. To ensure mixing, stirring was done frequently and then the conical flask corked with appropriate stopper. Thereafter, shaking was done regularly to allow maceration.

On the fifth day, the extracts were filtered using Whatman No.1 filter paper into another conical flask. Acetone was removed in a rotary evaporator at 40^{0}C and the substance obtained was allowed to dry off the acetone then stored in $^{+}4^{0}$C pending larvicidal laboratory tests.

3.2.3.3 Extraction using hexane

One kilogram (1000 grams) of the plant powder was extracted with hexane in a conical flask and then the conical flask corked with appropriate stopper. Stirring was done to ensure proper mixing and percolation and on the fifth day filtration was done with Whatman No.1 filter paper. Hexane was removed in a rotary evaporator at 40^0C and the substance obtained dried then stored in amber coloured bottle in a refrigerator at $^+4^0$C pending larvicidal laboratory tests.

3.2.4 Phytochemical Screening

Qualitative Phytochemical screening was performed using established procedures to determine presence or absence secondary metabolites.

3.2.4.1 Determining presence of alkaloids

Half a gram of the plant extract was mixed with 5 ml of 1% hydrochloric acid and warmed. Filtration was done and 1 ml of the filtrate obtained in which 3 drops of Dragendorff's reagent were added. Appearance of reddish orange precipitation was indicative of the presence of alkaloids (Kisangau, 1999; Orech *et al.*, 2005).

3.2.4.2 Establishing presence of sterols and triterpenes

Half a gram of the extract was defatted with hexane (for aqueous and acetone extracts only. The hexane extract did not need defatting). Further extraction was with dichloromethane and then the solution was dehydrated with magnesium sulphate anhydride followed by treatment with half of a milliliter of acetic anhydride then 2 drops of concentrated sulphuric acid. Appearance of green blue colour was indicative of sterols while colour change from pink to purple indicated triterpenes (Chetri *et al.*, 2008; Parekh and Chanda, 2007).

3.2.4.3 Test for saponins

Five millilitres of distilled water was added to about half a gram of the plant extract and then shaking was done. Frothing that persisted for half an hour was indicative of saponins (Fawole *et al*., 2009; Kisangau, 1999; Parekh and Chanda, 2007).

3.2.4.4 Determining presence of flavonoids and flavones

About two hundred milligrams of the extract was dissolved in 4 ml of 50% methanol and warmed then metal magnesium added. Five drops of concentrated sulphuric acid were added. Red colour formation indicated presence of flavonoids and while orange colour indicated presence of flavones (Orech *et al*., 2005; Parekh and Chanda, 2007; Siddiqui and Ali 1997).

3.2.4.5 Establishing presence of tannins

Two milliliters of distilled water was added to about half a gram of the extract, shaken and filtered to a test tube. Two drops of ferric chloride were added to the filtrate. Development of a blue black precipitate indicated presence of tannins (Chetri *et al*., 2008; Kisangau, 1999; Parekh and Chanda, 2007).

3.2.4.6 Determining presence of cardiac glycosides

The Keller Killian test was used. About hundred milligrams (100 mg) of extract was dissolved in 1ml of glacial acetic acid having a drop of ferric chloride solution. This was then under-layered with 1 ml of concentrated sulphuric acid. The appearance of a brown ring at the interface of the two layers with the lower acidic layer turning blue green upon standing indicated the presence of cardiac glycosides (Ngbede, *et al*., 2008; Parekh and Chanda, 2007).

3.2.4.7 Establishing presence of anthraquinones

The Bonträger Test was used to test for anthraquinones. A gram (1 gm) of the extract was dissolved in 70% acetone to make a final concentration of 50mg/ml. Two milliliters was then collected and shaken with 4 ml of hexane to defat. When the upper lipophilic layer was separated it was treated with 4ml of dilute ammonia. The lower layer changed to violet, then pink and indicated the presence of anthraquinones (Hettiarachchi, 2006; Ngbede, *et al*., 2008; Siddiqui and Ali 1997).

3.2.5 Determination of larvicidal activity

3.2.5.1 Mosquito Species

Aedes aegypti were used due to their advantages (Moore, 2004). They were obtained from a colony of *A. aegyptiae* mosquitoes reared under standardized conditions in insectary section of at the School of Biological Sciences, University of Nairobi. Larvae were hatched from the eggs of *Aedes aegypti* and bred in trays containing tap water with temperatures of 28±2°C. On hatching, the larvae were fed on yeast powder and glucose (Koech and Mwangi, 2013; 2014). Tests were carried out on 4th instar larvae.

Extracts' dilutions of 1, 0.5, 0.25 and 0.125% were made to stock solutions according to Shanmugasundaram *et al*., 2008; Singh *et al*., 2006; Tinneke and Puput, 2015; Wiseman and Chapagain, 2005. The aqueous extracts were made in distilled water, acetone extracts in analytical grade acetone while hexane extracts in 3% DMSO (WHO, 2005). One (1) ml of each dilution added to 249 ml of distilled water to make 250ml of the test substance and distilled water. Twenty five (25) larvae introduced to each concentration of the extracts.

Deltamethrin 5% was positive control while tap water was the negative control. Dead larvae were those that remained immobile, could not reach the water surface and head to tail flexion was absent when the beaker was tapped (Maniafu *et al*., 2009). Larvae were considered moribund when they were incapable of rising to the surface and lacked a diving reaction when the water is disturbed (WHO, 2005). Mortality was recorded during 24 hours and determined using Abbott's formula (Ansari *et al*., 2005).

3.2.6 Statistical analysis

The SPSS V22 software (IBM, 2013) was used to determine average larval mortality and LD_{50}, LD_{95} at 95% confidence limits of upper confidence limit and lower confidence limit. Results with $P < 0.05$ were considered to be statistically significant.

3.3 RESULTS

The map of Kenya in Figure 7 shows the location of Shimoni area, that of Msambweni sub county and Kwale County. The selected plants are listed in table 1.

Table 1: Plant species collected from Msambweni sub-county

Family	**Plant species, voucher specimen**	**Local name**	**Life form**	**Part used**
Asteraceae	*Tagetus minuta* L. (JM 17)	Bangi ya shambani	Herb	Whole plant
Bombacacea	*Adansonia digitata* Linn. (JM 09)	Mbuyu / Mbamburi	Tree	Leaves
Labiatae	*Ocimum suave* Willd (JM 05)	Kirihani/Kivumbani	Herb	Whole plant
Labiatae	*Plectranthus barbatus* Andr.(JM 03)	Kizimwilo	Shrub	Leaves
Meliaceae	*Azadirachta indica* (L) Burm. (JM 10)	Mwarobaini/ Mkilifi	Tree	Leaves
Verbenaceae	*Lantana camara L* (JM 11)	Mjasasa	Shrub	Leaves

Plate 1: Photographs of six plants used for mosquito control in Msambweni sub county, Kwale County, Kenya

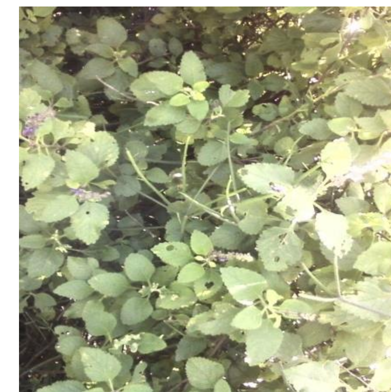
Ocimum suave whole plant

Aerial parts of *Azadirachta indica*

Aerial parts of *Lantana camara*

Adansonia digitata tree

Tagetes minuta (whole plant)



Aerial parts of *Plectranthus barbatus*

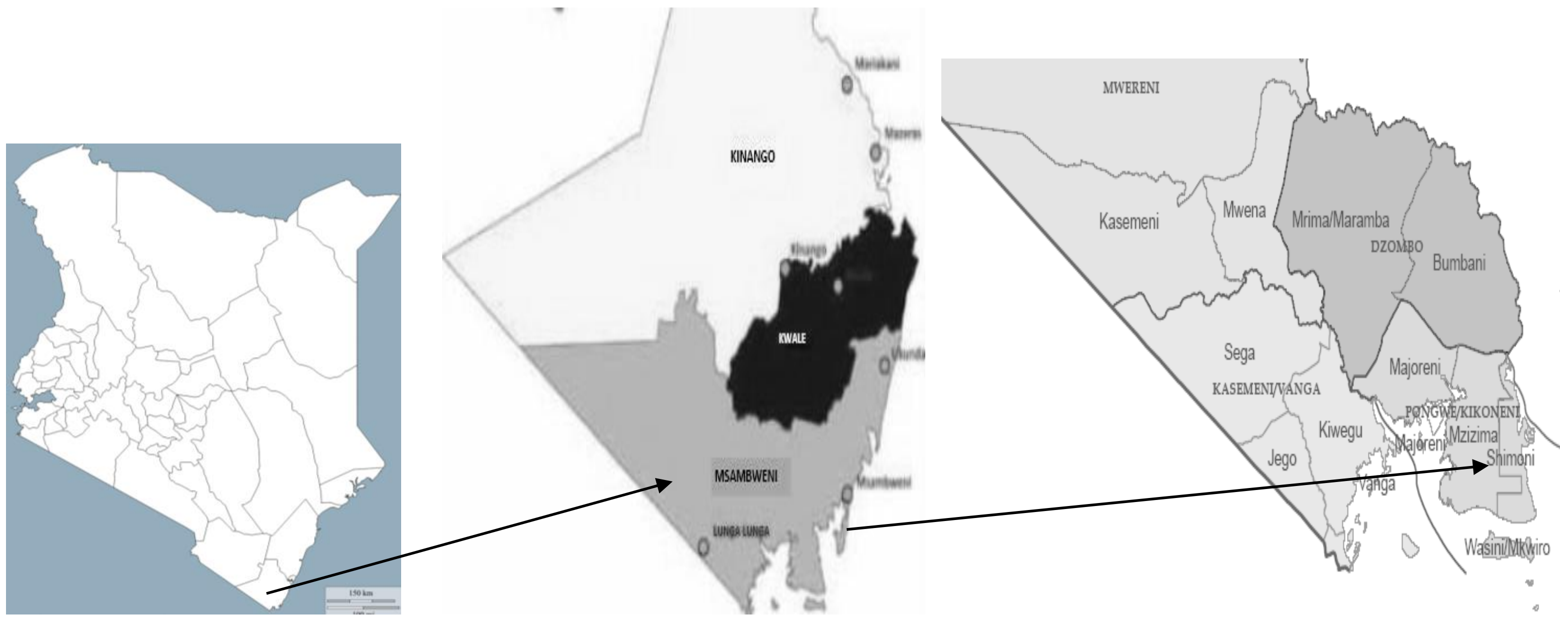


Figure 7: Map of Kenya showing Kwale County, Msambweni Sub County and Shimoni area

3.3.1 Results of phytochemical screening

Among the aqueous extracts, saponins and flavonoids were present in all the plants' parts. Alkaloids were present in five plant species namely *A.digitata* (leaves), *O. suave* (whole plant), *T.minuta* (whole plant), *P. barbatus* (leaves), *L. camara* (leaves). They were absent in *A. indica* (leaves).

Tannins were present in *A.digitata* (leaves), *O. suave* (whole plant), *T.minuta* (whole plant), *P. barbatus* (leaves) while they were lacking in *L. camara* (leaves) and *A. indica* (leaves). Sterols were present in *O. suave* (whole plant), *A.digitata* (leaves) and *A. indica* (leaves). They were absent in *T.minuta* (whole plant), *P. barbatus* (leaves) and *L. camara* (leaves).

Terpenoids were present in *T.minuta* (whole plant), *P. barbatus* (leaves) and *O. suave* (whole plant). They were absent in *A.digitata* (leaves), *L. camara* (leaves) and *A. indica* (leaves). Glycosides were present in *O. suave* (whole plant), *T.minuta* (whole plant), *P. barbatus* (leaves) and *L. camara* (leaves) while they were absent in *A.digitata* (leaves) and *A. indica* (leaves).

All acetone extracts contained alkaloids, flavonoids and saponins. Only *A. indica* (leaves) lacked tannins. Sterols were present *A.digitata* (leaves), *O. suave* (whole plant) and *A. indica* (leaves) while they were not detected in *T.minuta* (whole plant), *P. barbatus* (leaves) and *L. camara* (leaves).

Terpenoids were detected in *O. suave* (whole plant), *T.minuta* (whole plant) and *P. barbatus* (leaves). They were absent in *A.digitata* (leaves), *L. camara* (leaves) and *A. indica* (leaves). Glycosides were present in *O. suave* (whole plant), *P. barbatus* (leaves) and *P. barbatus* (leaves). They were not detected in *A.digitata* (leaves), *A. indica* (leaves) and *T.minuta* (whole plant).

For the hexane extracts, saponins and flavonoids were present in all plants. Only *A. indica* (leaves) did not have alkaloids while *L. camara* (leaves) and *A. indica* (leaves) lacked tannins. Sterols were present *A.digitata* (leaves), *A. indica* (leaves) and *O. suave* (whole plant) while they were absent in *T.minuta* (whole plant), *P. barbatus* (leaves) and *L. camara* (leaves).

Terpenoids were present in *O. suave* (whole plant), *T.minuta* (whole plant) and *P. barbatus* (leaves). They were absent in *A.digitata* (leaves), *L. camara* (leaves) and *A. indica* (leaves). Only *L. camara* (leaves) contained glycosides. The results are presented in the table 2.

Table 2: Results of the phytochemical determination

Extract	Plant species and part tested		Alkaloids	Tannins	Saponins	Sterols	Terpenoids	Flavonoids	Glycosides
Aqueous	Bombacaceae	*A.digitata* (leaves)	+	+	+	+	-	+	-
	Labiatae	*O. suave* (whole plant)	+	+	+	+	+	+	+
	Meliaceae	*A. indica* (leaves)	-	-	+	+	-	+	-
	Asteraceae	*T.minuta* (whole plant)	+	+	+	-	+	+	+
	Labiatae	*P. barbatus* (leaves)	+	+	+	-	+	+	+
	Verbenaceae	*L. camara* (leaves)	+	-	+	-	-	+	+
Acetone	Bombacaceae	*A.digitata* (leaves)	+	+	+	+	-	+	-
	Labiatae	*O. suave* (whole plant)	+	+	+	+	+	+	+
	Meliaceae	*A. indica* (leaves)	+	-	+	+	-	+	-

	Asteraceae	*T.minuta* (whole plant)	+	+	+	-	+	+	-
	Labiatae	*P. barbatus* (leaves)	+	+	+	-	+	+	+
	Verbenaceae	*L. camara* (leaves)	+	-	+	-	-	+	+
Hexane	Bombacaceae	*A.digitata* (leaves)	+	+	+	+	-	+	-
	Labiatae	*O. suave* (whole plant)	+	+	+	+	+	+	-
	Meliaceae	*A. indica* (leaves)	-	-	+	+	-	+	-
	Asteraceae	*T.minuta* (whole plant)	+	+	+	-	+	+	-
	Labiatae	*P. barbatus* (leaves)	+	+	+	-	+	+	-
	Verbenaceae	*L. camara* (leaves)	+	-	+	-	-	+	+

+ = The secondary metabolite is present

- = The secondary metabolite is absent

3.3.2 Larvicidal activity

3.3.2.1 LD_{50} and LD_{95} at 95% confidence interval

Acetone extracts of *A.digitata* exhibited great toxicity to the mosquito larvae followed by its aqueous and the hexane extract. Aqueous extracts of *O. suave* had greater toxicity than the acetone extracts. However, at the tested hexane concentrations, both LD_{50} and LD_{95} could not be determined since larvae were killed in all the concentrations. Acetone and aqueous extracts of *A. indica* were more toxic than the hexane extracts of the same.

In *T. minuta*, the aqueous extract of was more toxic than its hexane extract while the acetone extract exhibited greater toxicity than both the aqueous and hexane extracts. For *P. barbatus* acetone extracts had greater toxic effect followed by the hexane extracts and then aqueous extracts. Acetone extracts of *L. camara* were more toxic followed by hexane and then aqueous extracts. Generally, for all plants' extracts, acetone extracts had greater larvicidal effect followed by the aqueous then the hexane extracts.

3.3.2.2 Larval mortality

All the extracts had appreciable lethality effect on the mosquito larvae. This toxicity was dependent on the type of the extract and the concentration. At concentration of 1 mg/ml, all the extracts had a hundred (100) percent mortality of the tested larvae except aqueous extract of *Lantana camara* that killed ninety (90) percent of the larvae. Acetone extracts had most toxicity on the larvae. They caused a hundred percent larval mortality at 0.5 mg/ml of the extracts except for *Ocimum suave* where its mortality effect was only fifty (50) percent

Table 3: Larvicidal efficacy of plants used to control mosquitoes in Msambweni Sub County

Plant family and species		Aqueous		Acetone		Hexane	
		LD_{50}	LD_{95}	LD_{50}	LD_{95}	LD_{50}	LD_{95}
Bombacaceae	*Adansonia digitata*	0.743	2.123	0.64	1.135	1.101	3.223
Labiatae	*Ocimum suave*	2.598	3.987	2.453	4.492	-	-
Meliaceae	*Azaridachta indica*	1.366	3.784	1.363	3.524	1.858	3.31
Asteraceae	*Tagetes minuta*	0.61	2.256	-	-	1.053	2.601
Labiatae	*Plectranthus barbatus*	1.914	4.065	1.501	2.47	2.694	3.33
Verbenaceae	*Lantana camara*	2.227	5.436	1.638	3.32	2.823	4.216

LD_{50}: The dose of a substance that kills 50% of test population
LD_{95}: The dose of a substance that kills 95% of test population

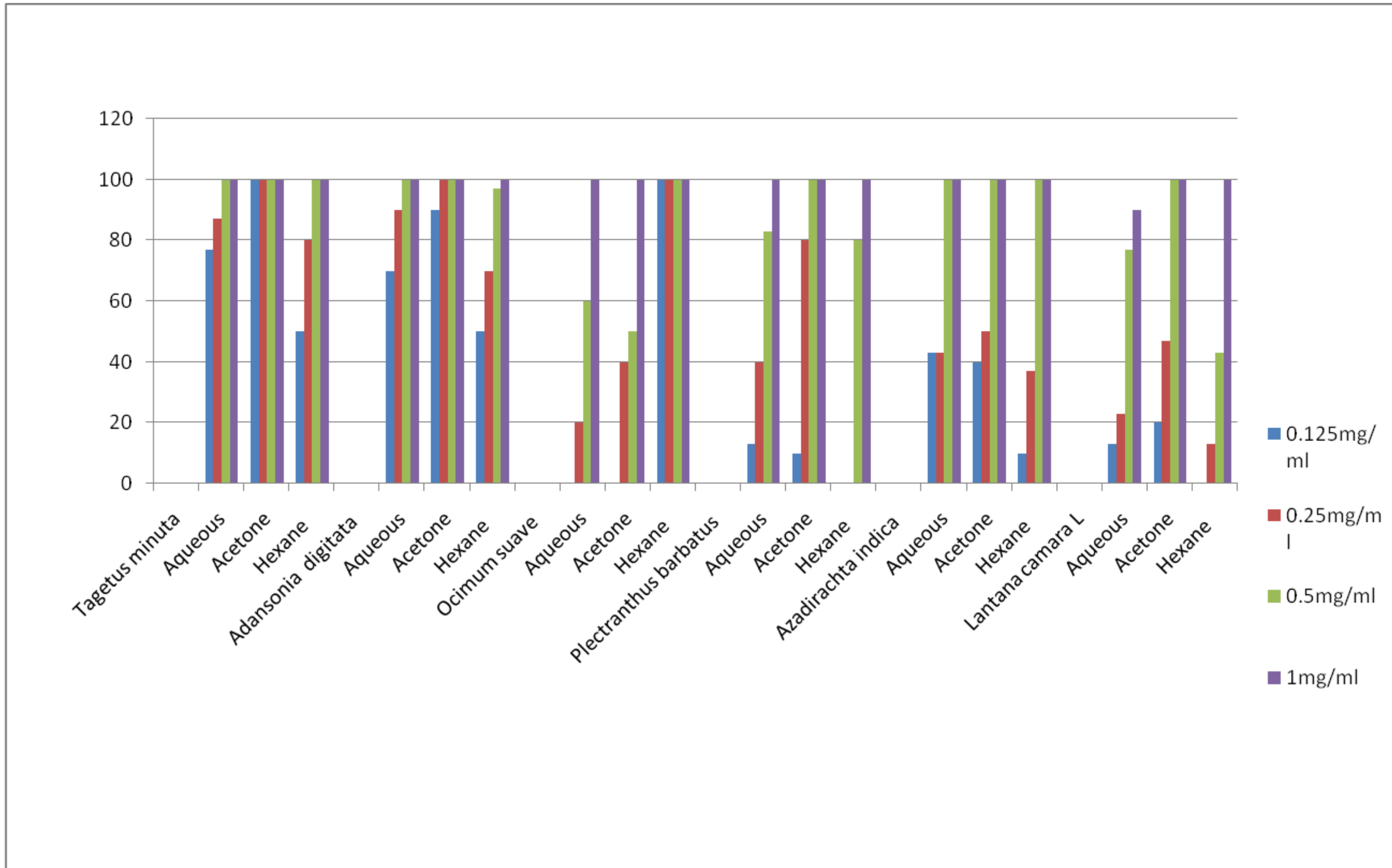


Figure 8: Mortality of *Aedes aegypti* larvae after 24 hr exposure

3.3.4 DISCUSSION

This study investigated the activity of plants' extracts against larvae of *Aedes aegypti* mosquito. Larval control is easier and effective compared to other methods of mosquito control which have serious limitations (Howard *et al*., 2007; Maniafu *et al*., 2009). The larvicidal activity was dose dependent and at the highest concentration of 1mg/ml, all extracts exhibited 100% mortality. This could be due to presence of secondary metabolites especially saponins, alkaloids and flavonoids. Alkaloids have long history of use as insecticides (Hayes, 1982.)

Rotenone is a flavonoid with insecticidal properties (Hollingworth *et al*., 1994). Thus the presence of alkaloids and flavonoids in all the plants studied could have contributed to their larvicidal activity. Other secondary metabolites which have been previously studied and found to have larvicidal activity include isoflavonoids according to Joseph *et al*., (2004), saponins according to Wiseman and Chapagain, 2005, steroids as mentioned by Chowdhurry *et al*., (2008), essential oils according to Cavalcanti *et al*., (2004), and tannins according to Khana *et al*., (2007) whose presence in the study plants could have contributed to larvicidal activity.

Lantana species have been shown to have larvicidal activity in previous studies. Dose dependent larvicidal effect of methanol and ethanol extract of *Lantana camara* on the larvae of *Aedes aegypti* has been demonstrated. Kumar and Maneemegalai (2008), showed a dose dependent larvicidal effect of methanol and ethanol extract of *L. camara* on larvae of *Aedes aegypti*. They recorded a maximum effect against the larvae at concentration of 1mg/ml even though the methanol extract had lesser activity.

This study showed dose and extract type dependent larvicidal effect of *L. camara*. A study done earlier established that aqueous leaf extracts of *A. indica* exhibited 100% mortality on 3rd instar larvae after twenty four hours (Rathy *et al*., 2015). Results from this study showed that aqueous leaf extracts of *A. indica* had 100% mortality on the tested mosquito larvae after twenty four (24) hours.

This supports findings of that previous study. This study showed that *Tagetes minuta* has larvicidal activity with LD_{50} values of 0.61 to 2.6 mg/ml. Perich *et al*., 1995 established larvicidal activity of fractions *Tagetes minuta* L. against larvae of *Anopheles stephensi*. Their findings are greater than the LD_{50} and LD_{95}values reported in the present study.

Larvicidal activity of *Tagetes minuta* is probably due to presence of saponins, alkaloids and flavonoids. This study records larvicidal activity of *Ocimum suave*, *Plectranthus barbatus* and *Adansonia digitata* as causing 100% mortality at 1 mg/ml which was the highest concentration tested. Kokwaro, 2009 lists that an infusion of *O. suave* is traditionally used as an insecticide and a disinfectant.

Information is scant concerning these three plants' use as larvicides, and to the best of our knowledge, this study reports for the first time mosquito larvicidal activity from *Ocimum suave*, *Plectranthus barbatus* and *Adansonia digitata*.

3.3.5 CONCLUSIONS

All the plants in this study had hundred percent (100%) mortality on *Aedes aegypti* larvae after twenty four hours. The studied plants should further be evaluated for possible development of efficacious and environment friendly larvicides that are not harmful to humans.

CHAPTER FOUR

REPELLENCY PROPERTIES OF SIX PLANTS USED FOR MOSQUITO CONTROL IN MSAMBWENI SUB COUNTY

4.1 INTRODUCTION

Mosquito-borne diseases pose a great risk to humankind as they are a cause of very high rates of morbidity and mortality worldwide. These diseases are also a major impediment to socio-economic development in countries where they are endemic (Karunamoorthi and Bekele, 2009; Karunamoorthi and Sabesan, 2010). Malaria is of major global public health concern with almost half of the people in the world being in danger of infection (Karunamoorthi *et al*., 2013). Infection with *Plasmodium falciparum* is life threatening and 2% of those infected by the parasite die (Dikasso *et al*., 2006).

Dengue and yellow fever infections are also of major concern. Currently, almost 2.5 billion people live in areas where dengue transmission is rife (WHO, 2012) while almost half of those affected by yellow fever die. Since there is no cure for yellow fever, personal protection, avoidance of mosquitoes and vaccination are crucial to lower disease risk and mortality (Jentes *et al*., 2011). Rift Valley fever (RVF) is endemic to Africa and the Middle East and causes human illness and livestock death (Foster and Walker, 2002).

Emergence of resistance by mosquitoes to pyrethroids in malaria-endemic settings worldwide contributed by heavy reliance on pyrethroids poses a challenge to public health (Karunamoorthi and Sabesan, 2013). Plant-derived insecticides are specific in activity and safe to environment. In this regard, many plant species have been evaluated for their potential as insecticidal agents and the extent to which they exert this activity (Karunamoorthi, *et al*., 2012a). Most of commercially available insect repellents consist of pyrethrum or its derivatives (Karunamoorthi *et al*., 2012b).

This study aimed to evaluate repellency of six plants used to control mosquitoes in Msambweni sub county, Kwale County, Kenya. The six plants and their parts are *Lantana camara* (leaves), *Tagetes minuta* (whole plant), *Azadirachta indica* (leaves), *Adansonia digitata* (leaves), *Ocimum suave* (whole plant) and *Plectranthus barbatus* (leaves).

For ethical considerations on use of human subjects in repellency testing of the extracts, the study protocol was submitted to the Kenyatta National Hospital / University of Nairobi, ethical review committee (KNH/UoN ERC) where approval was granted under protocol number P357/05/2015.

4.2 MATERIALS AND METHODS

4.2.1 Preparation of plant material and extraction

The plants' parts were prepared as outlined in chapter three above. Extraction was by use of water, acetone and hexane and was carried out as described in chapter three of this thesis. For aqueous extracts, freeze drying was done and the freeze dried material was stored at $+4^0$C pending repellency testing. The respective resultant viscous substances for acetone and hexane extracts were dried and stored in amber coloured bottles at $^+4^0$C awaiting repellency tests.

4.2.3 Mosquito rearing

Aedes aegypti mosquitoes were reared in the Insectary unit located in the School of Biological Sciences, Chiromo campus of the University of Nairobi. The colony has been bred continuously over the last twenty years without exposure to insecticides, repellents and pathogens. The eggs stored on filter papers were obtained from a desiccator. These were used to develop a temporary colony for the study.

The eggs were then floated in rearing trays, half filled with tap water. At larval stage, they were fed daily with 100 mg of active dry yeast that was sprinkled on the water surface. Water in rearing trays was refreshed every two days in order to avoid scum formation that might kill the larvae. Trays were washed in clean tap water and the larvae sieved out of the trays and cleaned with tap water before being returned to the fresh water in rearing trays. On pupation, the pupae were collected using a Pasteur pipette and transferred in a container, three-quarters full of water that was then inserted in a wooden cage.

The emerged adults were continuously provided with 10% sugar solution as an energy source. The female adults used for the egg production were given access to blood meal by use of a live rabbit that was restrained in a wooden box, its head inserted in a mosquito cage and mosquitoes allowed to feed for one hour.

Whatman No 1 filter paper rolled in a cone-shape was inserted in a plastic container, three-quarter full of tap water and provided to the engorged females. Every three days, the eggs were collected and dried on a soft cotton wool before being stored in the desiccator until when required (Koech and Mwangi, 2013,2014).

4.2.4 Test mosquitoes

The mosquitoes used for the laboratory repellent bioassay were 3-7 day old, laboratory-bred and starved adult females of *Aedes aegypti*. Tests were conducted in triplicate using female *A. aegyptiae* mosquitoes. Prior to the time of tests, they were deprived of blood meal for 18 hours.

4.2.5 Cage tests

Cage tests were performed in 40 x 40 x 40 cm cages made of aluminium sheet at the bottom, Pyrex window screen on sides and top, and a cotton stockinet sleeve for access on the front (Kweka *et al*., 2008; Innocent *et al*., 2010). Tests were performed in a 12:12 (Light: Dark) photoperiod and room temperature of 27 ± 2° C with relative humidity of 80% (WHO 1996, 2009).

Only female mosquitoes bite humans to suck blood necessary for egg development (Foster and Walker, 2002; Kim *et al*., 2011). Active female host-seeking *A. aegyptiae* mosquitoes aged 5-7 days were collected from stock population using an aspirator and starved for the preceding 12 hours. Fifty female mosquitoes were utilized in these tests (Innocent *et al*., 2010; WHO 1996, 2009).

One hundred (100) milligram of each sample was dissolved in a 100 ml mixture of analytical grade acetone (99.95%) and liquid paraffin (1:1). Serial five-fold dilutions were made using acetone/liquid paraffin mixture (1:1) to obtain concentrations in the range of 0.125mg/ml-1mg/ml. Acetone/ liquid paraffin mixture (1:1) was used as negative control while 20% DEET (*N, N*-diethyl-m-toluamide), the positive control.

Volunteers who had avoided use of fragrance, any mosquito repellent, perfumed soap or tobacco for 12 hours prior and during the experiment (WHO, 2009; Innocent *et al*., 2010) were used for the experiments. The forearm, from the elbow to the wrist (~696.6 cm^2) was cleaned with water dried and then one millilitre of test sample applied as evenly as possible. The rest of the hand from the wrist to the fingers was covered with latex glove to prevent the mosquitoes from biting (WHO, 2009).

One (1) ml of acetone/liquid paraffin mixture was applied on the other forearm that had been prepared as above, and served as negative control. The initial test to determine the readiness of the mosquitoes to land or bite involved the use of the negative control forearm which was first inserted into the cage and the number of mosquitoes that landed or probed the skin within 30 seconds counted.

The negative control forearm was carefully withdrawn and the positive control arm with 20% DEET (*N, N*-diethyl-m-toluamide) inserted in the cage for 30 seconds and the number of mosquitoes that land or probe counted. The test proceeded when there over ten landings and/or probings within the thirty seconds period in the negative control forearm. The volunteer's forearm was prepared as above and 1 ml of the lowest concentration of the test sample applied and inserted into the cage for about five minutes.

Mosquitoes that landed or probed during this period were counted and shaken off before they could imbibe any blood (Kweka *et al*., 2008; WHO, 2009; Innocent *et al*., 2010). The volunteers' arms were exposed to progressively higher concentrations of the extracts every time in clean cages with fresh mosquitoes (Innocent *et al*., 2010).

Percent protection efficacy (PE) was calculated as PE = (C-T)/C x 100

Where C and T are the mean numbers of mosquitoes that landed on the control and test arm respectively (Ansari *et al*., 2005)

4.3 RESULTS FOR REPELLENCY TESTING

4.3.1 Percent protection at concentration of 0.125 mg/ml

There was no significant difference across different extracts of *Lantana camara* and *Tagetes minuta*. The *P* values were 0.852 and 0.718 respectively. *Azadirachta indica* extracts differed significantly from each other and *P* value was 0.012. Hexane extract differed significantly from acetone and aqueous extracts.

The protection efficacy of hexane extract of *Azadirachta indica* was similar to DEET. No significant difference across the different types of extracts of *Ocimum suave* observed. The *P* value was 0.099. All extracts were similar to *N, N*-diethyl-m-toluamide (DEET) in terms of protection efficacy.

The hexane extract of *Ocimum suave* was not significantly different from the hexane extract of *Azadirachta indica*. All extracts of *Adansonia digitata* did not differ significantly from each other. The *P* value was 0.846. The hexane and aqueous extracts of *Adansonia digitata* were similar in terms of protection efficacy to the hexane and aqueous extracts of *Lantana camara*.

The acetone extracts of *Adansonia digitata* did not differ significantly from the acetone extracts of *Lantana camara, Tagetes minuta* and *Plectranthus barbatus*. The *P* value for *Plectranthus barbatus* extracts was 0.697. The hexane and aqueous extracts *Plectranthus barbatus* did not differ significantly from the hexane and aqueous extracts of *Lantana camara* and *Adansonia digitata.*

The acetone extract of *Plectranthus barbatus* did not differ significantly from the acetone extract of *Lantana camara, Tagetes minuta* and *Adansonia digitata*. It also did not differ significantly from the aqueous extract of *Lantana camara* and the hexane extract of *Tagetes minuta*.

4.3.2 Percent protection at concentration of 0.25mg/ml

The *P* value for *Lantana camara* extracts was 0.008. There was no significant difference across the different extracts of Tagetes *minuta Azadirachta indica* and *Adansonia digitata* whose *P* values were 0.664, 0.104 and 0.846 respectively. The extracts of *Adansonia digitata* were not significantly different from those of *Tagetes minuta* at this concentration. P value for *Ocimum suave* extracts was 0.005.

The acetone extracts of *Ocimum suave* differed significantly from its hexane and aqueous extracts. Its hexane and aqueous extracts did not differ significantly from one another. The acetone extract of *Ocimum suave* had activity like that of DEET, the positive control. At this concentration, the extracts of *Plectranthus barbatus* did not differ significantly from those of *Tagetes minuta* and *Adansonia digitata* in terms of percent protection.

4.3.3 Percent protection at the concentration of 0.5mg/ml

The *P* values for *Lantana camara* and *Tagetes minuta* were 0.55 and 0.008 respectively. Acetone and hexane extracts of *Tagetes minuta* differed significantly from its aqueous extracts. The *P* value was 0.545 for *Azadirachta indica* extracts and acetone extract of *Azadirachta indica* was not significantly different from the hexane extract of *Lantana camara*. Generally, the hexane extract of *Azadirachta indica* had similar activity to that of positive control *N, N*-diethyl-m-toluamide (DEET). *P* value was 0.081 for *Adansonia digitata* extracts

The aqueous extract of *Adansonia digitata* was not significantly different from the aqueous extract of *Tagetes minuta*. All the extracts of *Ocimum suave* had similar activity like for DEET. *P* value was 0.125 for *Ocimum suave* extracts.

The extracts of *Plectranthus barbatus* did not differ significantly from each other and the *P* value was 0.08. The aqueous extract of *Plectranthus barbatus* was not significantly different from the acetone and aqueous extract of *Tagetes minuta* and aqueous extract of *Adansonia digitata*.

4.3.4 Percent protection at the concentration of 1mg/ml

Extracts of *Lantana camara* differed from each other in terms of activity, $P < 0.001$. Extracts of *Tagetes minuta* and *Azadirachta indica* did not exhibit difference and the *P* values were 0.182 and 0.07 respectively. Extracts of *Azadirachta indica* were not significantly different from those of *Tagetes minuta* at this concentration.

The extracts of *Adansonia digitata* did not differ significantly from each other and the P value was 0.259. For *Ocimum suave*, extracts differed from each other and $P < 0.001$. The acetone extract had similar activity to that of the positive control. The *P* value *Plectranthus barbatus* extracts was 0.107. The extracts of *Plectranthus barbatus* were not significantly different from those of *Adansonia digitata*.

Acetone and hexane extracts of *Lantana camara*, all extracts of *Tagetes minuta* and *Azadirachta indica*, hexane and aqueous extracts of *Ocimum suave* had same activity like the positive control. Acetone extract of *Ocimum suave* had a greater activity than the positive control although this activity was not significantly different. Tables 4-7 show the percent protection of the different concentrations of the plants' extracts and the controls together with the *P* values.

Table 4: Percent protection of controls and that of selected six plants at concentration of 0.125 mg/ml

Treatment			**Acetone Mean±SE**	**Hexane Mean±SE**	**Aqueous Mean±SE**	**P-value**
Verbenaceae	*Lantana camara*	Leaves	39.29±7.48^{b}	47.54±19.36^{bc}	37.37±10.29^{b}	0.852
Asteraceae	*Tagetes minuta*	Whole plant	42.24±10.52^{b}	41.63±7.97^{b}	50.71±7.10^{bc}	0.718
Meliaceae	*Azadirachta indica*	Leaves	70.30±3.74^{Ac}	84.05±3.78^{Bcd}	64.02±3.69^{Ac}	0.012
Bombacaceae	*Adansonia digitata*	Leaves	48.60±5.27^{b}	48.60±5.27^{bc}	44.55±6.41^{bc}	0.846
Labiatae	*Ocimum suave*	Whole plant	86.80±2.69^{cd}	79.44±2.11^{bcd}	84.58±1.58^{d}	0.099
Labiatae	*Plectranthus barbatus*	Leaves	44.60±6.31^{b}	55.80±15.95^{bc}	44.55±6.41^{bc}	0.697
DEET (Positive control)			98.33±1.67^{d}	98.33±1.67^{d}	98.33±1.67^{d}	-
Ethanol (Negative control)			0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	-
P-value			<0.001	<0.001	<0.001	

Values of mean followed by similar letter/ capital letter(s) in the same row are not significantly different from one another (One –way ANOVA, SNK-test, α=0.05)

Table 5: Percent protection of controls and that of selected six plants at concentration of 0.25 mg/ml

Treatment			**Acetone Mean±SE**	**Hexane Mean±SE**	**Aqueous Mean±SE**	**P-value**
Verbenaceae	*Lantana camara*	Leaves	69.58±2.20[Bc]	63.63±2.26[Bb]	45.96±6.51[Ab]	0.008
Asteraceae	*Tagetes minuta*	Whole plant	48.60±5.27[b]	55.60±7.64[b]	48.60±5.27[b]	0.664
Meliaceae	*Azadirachta indica*	Leaves	74.30±2.63[c]	84.58±1.58[c]	82.85±4.63[c]	0.104
Bombacaceae	*Adansonia digitata*	Leaves	48.60±5.27[b]	48.60±5.27[b]	44.55±6.41[b]	0.846
Labiatae	*Ocimum suave*	Whole plant	89.72±1.05[Bd]	79.44±2.11[Ac]	84.58±1.58[Ac]	0.005
Labiatae	*Plectranthus barbatus*	Leaves	52.49±4.80[b]	48.18±7.18[b]	44.55±6.41[b]	0.675
DEET (Positive control)			98.33±1.67[d]	98.33±1.67[d]	98.33±1.67[c]	-
Ethanol (Negative control)			0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	-
P-value			<0.001	<0.001	<0.001	

Values of mean followed by similar letter/ capital letter(s) in the same row are not significantly different from one another (One –way ANOVA, SNK-test, α=0.05)

Table 6: Percent protection of controls and that of selected six plants at concentration of 0.5 mg/ml

Treatment			**Acetone Mean±SE**	**Hexane Mean±SE**	**Aqueous Mean±SE**	**P-value**
Verbenaceae	*Lantana camara*	Leaves	73.58±1.72^{b}	73.63±1.49^{c}	64.60±11.03^{b}	0.550
Asteraceae	*Tagetes minuta*	Whole plant	88.75±2.09^{Bc}	82.52±2.60^{Bd}	74.30±2.63^{Ab}	0.008
Meliaceae	*Azadirachta indica*	Leaves	89.03±3.90^{c}	90.28±3.67^{de}	84.52±3.70^{cd}	0.545
Bombacaceae	*Adansonia digitata*	Leaves	71.96±2.31^{b}	64.02±3.69^{b}	74.30±2.63^{bc}	0.081
Labiatae	*Ocimum suave*	Whole plant	98.33±1.67^{d}	93.19±2.18^{e}	94.86±0.53^{d}	0.125
Labiatae	*Plectranthus barbatus*	Leaves	69.16±3.16^{b}	61.27±4.92^{b}	75.44±3.24^{bc}	0.080
DEET (Positive control)			98.33±1.67^{d}	98.33±1.67^{e}	98.33±1.67^{d}	-
Ethanol (Negative control)			0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	-
P-value			<0.001	<0.001	<0.001	

Values of mean followed by similar letter/ capital letter(s) in the same row are not significantly different from one another (One –way ANOVA, SNK-test, α=0.05)

Table 7: Percent protection of controls and that of selected six plants at concentration of 1mg/ml

Treatment			**Acetone Mean±SE**	**Hexane Mean±SE**	**Aqueous Mean±SE**	**P-value**
Verbenaceae	*Lantana camara*	Leaves	98.33±1.67^{Bc}	94.86±0.53^{Bc}	77.63±1.04^{Ab}	<0.001
Asteraceae	*Tagetes minuta*	Whole plant	98.33±1.67^{c}	98.33±1.67^{c}	94.86±0.53^{c}	0.182
Meliaceae	*Azadirachta indica*	Leaves	98.33±1.67^{c}	94.86±0.53^{c}	94.86±0.53^{c}	0.070
Bombacaceae	*Adansonia digitata*	Leaves	84.58±1.58^{b}	75.27±4.07^{b}	80.00±4.71^{b}	0.259
Labiatae	*Ocimum suave*	Whole plant	100.00±0.00^{B}	94.86±0.53^{Ac}	94.86±0.53^{Ac}	<0.001
Labiatae	*Plectranthus barbatus*	Leaves	84.47±2.70^{b}	74.14±3.59^{b}	80.53±2.81^{b}	0.107
DEET (Positive control)			98.33±1.67^{c}	98.33±1.67^{c}	98.33±1.67^{c}	-
Ethanol (Negative control)			0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	-
P-value			<0.001	<0.001	<0.001	

Values of mean followed by similar letter/ capital letter(s) in the same row are not significantly different from one another (One –way ANOVA, SNK-test, α=0.05)

4.4 KNOCKDOWN EFFECTS OF PLANTS USED TO CONTROL MOSQUITOES IN MSAMBWENI SUB COUNTY

The WHO bioassay method was used to determine knockdown effects of the extracts of the six plants with slight modifications (Avicor *et al*., 2015; WHO, 1981; 2013b). The tests were done in triplicates using filter papers were treated with plant extracts at concentrations of 75 mg/m^2, 100 mg/ m^2, 200 mg/m^2, 250 mg/m^2 and 500 mg/m^2. The negative controls were untreated filter papers while papers treated with citronella oil at concentrations as above were positive control (WHO, 1996).

Each of the treated filter paper was inserted into a chamber shown holding twenty five female active *Ae. aegyptiae* mosquitoes aged between 5-7 days that had not been blood-fed. They had been selected using an aspirator from stock populations of adult mosquitoes and they were placed in each of the chamber with filter papers with different plant extracts for one hour.

After one hour the mosquitoes were transferred from the chamber to recovery chambers that had cups with 10% sucrose solution for the mosquito to feed. Mortality and recovery within 24 hours was scored. Data was analyzed using SPSS v22 and was considered significant if $P<0.05$.

4.4.1 RESULTS

The knockdown effects of the plant extracts were determined at concentration of 1mg/ml which was the highest concentration as shown in table 8. Activity of acetone extracts was similar to that of citronella oil which was the positive control. However, the extracts' activity was significantly different from the negative control which was untreated filter papers. Among the hexane extracts, that of *Tagetes minuta* was least active compared to the other extracts had which had similar activity to citronella oil.

All the hexane extracts differed significantly from the negative control which was untreated filter papers. For the aqueous extracts, that of *Tagetes minuta* had least knockdown effect followed by those of *Ocimum suave, Adansonia digitata* and *Plectranthus barbatus*.

The knock down effect of *Plectranthus barbatus* was significantly different from that of *Adansonia digitata* and similar to that of *Lantana camara* and *Azadirachta indica* thus not significantly different from that of citronella oil, the positive control. Aqueous extracts of *Tagetes minuta* and *Ocimum suave* had least knockdown effect on the mosquitoes.

Table 8: Knockdown effect of controls and plants' extracts

Extract	Acetone Mean±SE	Hexane Mean±SE	Aqueous Mean±SE
Lantana camara leaves	83.33 ± 8.82^{b}	80.00 ± 5.77^{bc}	77.50 ± 4.79^{cde}
Tagetes minuta whole plant	66.67 ± 6.67^{b}	60.00 ± 5.77^{b}	36.67 ± 8.82^{b}
Azadirachta indica leaves	86.67 ± 8.82^{b}	90.00 ± 5.77^{bc}	83.33 ± 3.33^{de}
Ocimum suave whole plant	63.33 ± 14.53^{b}	80.00 ± 11.55^{bc}	46.67 ± 8.82^{bc}
Adansonia digitata leaves	80.00 ± 5.77^{b}	70.00 ± 10.00^{bc}	56.67 ± 12.02^{bcd}
Plectranthus barbatus leaves	83.33 ± 12.02^{b}	80.00 ± 11.55^{bc}	65.00 ± 25.00^{bcde}
Untreated filter papers (Negative Control)	13.33 ± 3.33^{a}	13.33 ± 3.33^{a}	13.33 ± 3.33^{a}
Citronella oil (Positive control)	93.33 ± 3.33^{b}	93.33 ± 3.33^{bc}	93.33 ± 3.33^{e}
P-value	<0.001	<0.001	<0.001

4.5 DISCUSSION

From the results, all plants in the present study had appreciable repellency. At 0.125 mg/ml (the lowest concentration) acetone, hexane and aqueous extracts of *Ocimum suave* and hexane extracts of *Azadirachta indica* exhibited repellency activity similar that of DEET. The repellency activity increased with dose. At concentration of 1mg/ml, most of the extracts except *A. digitata* and *P. barbatus* had activity similar to that of DEET.

Acetone extract of *Ocimum suave* had greater repellency than DEET. Extracts of *Plectranthus barbatus, Adansonia digitata* and aqueous extract of *Lantana camara* had activity that was lower than that of DEET even though it was appreciable. Comparing the extracts, the acetone extracts of the six plants had greater activity than the hexane and aqueous extracts. This could be attributed to the capability of acetone to extract both polar and non polar compounds.

The repellency effect of *Lantana camara* has been applied in traditional medicine practice. Seyoum *et al*., (2002) noted that the plant is already commonly used for hedges around the huts in many villages around Lake Victoria to control mosquitoes while Dua *et al*., (1996; 2010) determined that essential oil from the leaves of *L. camara* had mosquito adulticidal activity.

In this study, *L. camara* had appreciable repellency and knockdown effect against the study mosquitoes. The aromatic essential oil of *Tagetes minuta* is used against malaria because of its insecticidal activity (Batish *et al*., 2007). Maradufu *et al.,* (1978) reported a terpenoid, 5-E-ocimenone from *Tagetes minuta* possessed larvicidal activity.

Perich *et al*., (1995) reported that the whole plant of *Tagetes minuta* was repellent to *Ae. aegypti* and *An. stephensi*. The findings of this study corroborate those findings that *T. minuta* has activity against both the larvae and adult mosquitoes.

According to Monzon *et al*., (1994), leaf and seed extracts of *Azadirachta indica* exhibited repellency on *Ae. aegypti* and *Cx. Quinquefasciatus*. The activity of *Azadirachta indica* against insects is due to the presence of azadirachtin which has been demonstrated against numerous insect pests (Schmutterer and Singh 1995).

Azadirachtin is larvicidal (Maradufu *et al.,* 1978). Azadirachtin disrupts feeding, reproduction, or development of insects (Walter, 1999). This study was able to demonstrate that leaf extracts of *Azadirachta indica* have larvicidal and repellency effects on mosquitoes. Information about insecticidal activity of *A.digitata* is scanty. However, this study established that *A. digitata* leaves have repellent activity against laboratory bred *Aedes aegypti* mosquitoes.

Genus of *Plectranthus* has been evaluated for mosquito repellent activity. The tested oil of leaves of *Plectranthus incanus* link had stronger repellent activity than citronella oil, which was used as a positive control (Pal *et al*., 2011). *Plectranthus barbatus* was shown to have *in vitro* antiprotozoal effects (Al-Musayeib *et al*., 2012). This study was able to show that *Plectranthus barbatus* had repellent activity against *Ae. aegypti* mosquitoes.

Essential components of *ocimum* species have insect repellant activities (Perez-Alonso *et al*., 1995). According to Maurya *et al*., (2009) extracts of genus *Ocimum* are larvicidal In Nigeria it was found to have mosquito-repellent and mosquitocidal potential (Oparaocha *et al*., 2010). This study determined that *Ocimum suave* leaves have mosquito repellent activity.

Generally aqueous extracts had least knockdown effect while acetone extracts had greatest knockdown effect.

The knockdown effects of *Plectranthus barbatus* and *Adansonia digitata* were appreciable compared to their repellency effects. Aqueous extracts of *Tagetes minuta* and *Ocimum suave* had least knockdown effect on the mosquitoes. This implies that the mechanisms of repellency and knockdown differ.

CHAPTER FIVE

TOXICITY TESTING

5.1 GENOTOXICITY TESTING

5.1.1 INTRODUCTION

Maurici *et al*., (2005) explains the purpose of genotoxicity as being to recognize possible mutagenic dangers of substances to human and defining the mechanism of action of those that have that capability. The tests are of various types and include those conducted using organisms or simulations of body structures and those that are conducted outside organisms. According to OECD, (1997a), this testing is necessary in order to project possible cancer causes and study how they come about. The purpose of this study was to evaluate genotoxicity of the plants' parts.

5.1.2 MATERIALS AND METHODS

Plants for this study had been identified from evidence that they are traditionally utilized for control of mosquitoes among the people of Kwale County (Nguta *et al*., 2010; Nguta, 2011). The six plants and their parts are *Lantana camara* (leaves), *Tagetes minuta* (whole plant), *Azadirachta indica* (leaves), *Adansonia digitata* (leaves), *Ocimum suave* (whole plant) and *Plectranthus barbatus* (leaves).

Extraction was by use of water, acetone and hexane and was carried out as described in chapter three of this thesis. For aqueous extracts, freeze drying was done and the freeze dried material was stored at $+4^0$C pending genotoxicity testing. The respective resultant viscous substances for acetone and hexane extracts were dried and stored in amber coloured bottles at $^+4^0$C awaiting genotoxicity tests.

5.1.2.1 The *Allium cepa* test

This was performed according to Çelik, and Aslantürk, (2010) and Olusegun *et al*., (2010). Sixteen (16) *Allium cepa* bulbs (2.5–2.8 cm diameter) were used per concentration per test sample. They were grown in small cups with water at room temperature for 3 days for emergence of roots. Root lengths were measured periodically and scored. When roots lengths were 3 cm, the bulbs were treated with the extracts and the controls at concentrations of 125, 250, 500, and 1,000 µg/ml. Positive control was vincristine and negative control was water (Ping *et al*., 2012).

Roots were assessed for root length, turgescence, form and colour change. About 2 mm of the root tips were fixed in ethanol: glacial acetic acid (3:1 v/v) and hydrolyzed in 1N HCL at 60 °C for five minutes then cleaned with distilled water. They were compacted on a microscope slide and stained with aceto-orcein for 10 min. Excess stain was removed and cover slips placed on the smear. Cover slips were sealed on the slides with clear fingernail polish according to Grant, (1982).

Observation of the slides was at 40× magnification under a light microscope. Photomicrographs were made and analyzed for mitotic index, early anaphases, chromosomal bridges/fragments, stickiness and c-mitosis. Mitotic index was calculated as = Number of cells in mitosis/Total number of cells (Fiskesjo, 1985; Ping *et al*., 2012)

5.1.3 Statistical analysis

Data obtained was analyzed using the SPSS V22 (Statistical Package of Social Sciences) software (IBM,2013) for means, standard deviations of means followed by one way ANOVA and Student Newman Keul (SNK) test. Results with $P < 0.05$ were considered significant.

5.1.4 RESULTS

5.1.4.1 Root length

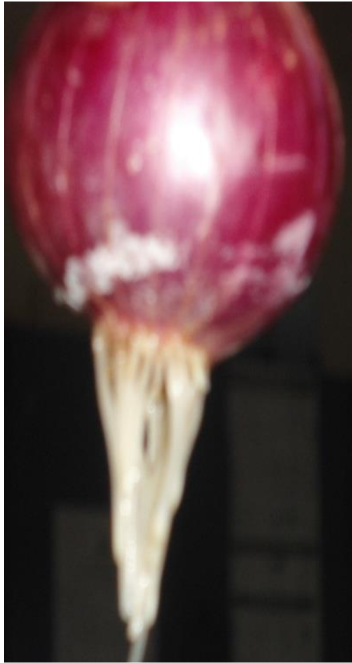
The effects of *L. camara* crude extracts on *Allium cepa* root growth are shown on table 9. Root length was dependent on dose with the highest concentration (1 mg/ml) showing least root growth. Significance difference did not exist on root growth among the different concentrations of *Tagetes minuta* extracts, table 10 below. Root growth was dose dependent.

Among the different concentrations of *Azadirachta indica* as shown on table 11, there was significant difference on root growth; *P* value for hexane was less than 0.001 that of aqueous extracts was 0.004. There was significant difference on the dose versus root growth for acetone extracts as the *P* value was 0.007. The roots were bent but there was no colour change among the *Azadirachta indica* extracts. Root growth was dose dependent.

The effects of *Ocimium suave* extracts are shown on table 12. There was no significant difference on root growth among the different extracts and the concentrations. Extracts of *Ocimium suave* caused roots of *Allium cepa* to be turgid.

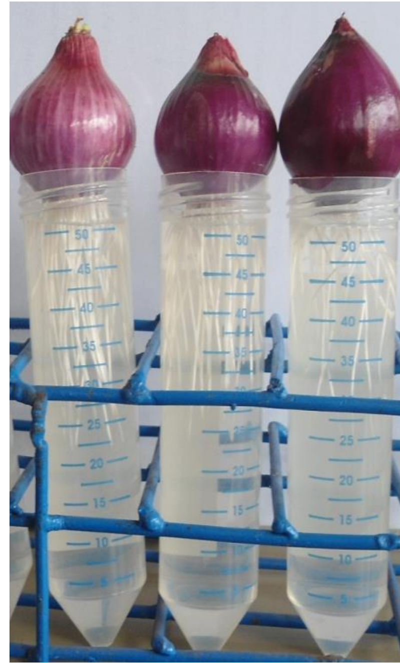
For *Adansonia digitata,* there was significant difference on root growth in different doses of the extracts, table 13. However, there was no significant difference in root growth inhibition across the concentrations. Among the extracts of *Plectranthus barbatus*, there was no significant difference on root growth across the extracts, table 14.

Plate 2 Shows examples of observations on the roots. The mean root lengths of different plant extracts in relation to various concentrations are shown figures below. Figure 9 represents acetone extracts, figure 10 hexane extracts and figure 11, aqueous extracts.



Vincristine sulphate

A. indica



Tap water

O. suave

Plate 2: Effects of some of the plants' extracts and controls on *Allium cepa* roots

Table 9: Mean root lengths of extracts of *Lantana camara*

Extract/ Concentration	Acetone extract Mean±SE	Hexane extract Mean±SE	Aqueous extract Mean±SE	P-value
0.125 mg/ml	4.36±0.16^{c}	4.50±0.13^{d}	4.42±0.17^{c}	0.812
0.25 mg/ml	4.08±0.15^{c}	4.00±0.13^{c}	4.14±0.14^{c}	0.785
0.5 mg/ml	3.66±0.06^{b}	3.58±0.07^{b}	3.70±0.09^{b}	0.553
1 mg/ml	2.92±0.07^{a}	2.98±0.07^{a}	3.00±0.08^{a}	0.711
	P<0.001	P<0.001	P<0.001	

Values of mean followed by similar letter/ capital letter(s) in the same row are not significantly different from one another (One –way ANOVA, SNK-test, α=0.05)

Table 10: Mean root lengths in various extracts of *Tagetes minuta*

Extract/ concentration	Acetone extract Mean±SE	Hexane extract Mean±SE	Aqueous extract Mean±SE	P-value
0.125 mg/ml	4.80 ± 0.07^{c}	4.72 ± 0.09^{c}	4.82 ± 0.07^{b}	0.619
0.25 mg/ml	4.40 ± 0.16^{b}	4.32 ± 0.15^{b}	4.44 ± 0.23^{b}	0.895
0.5 mg/ml/	4.26 ± 0.17^{b}	4.18 ± 0.16^{b}	4.30 ± 0.17^{b}	0.874
1 mg/ml	3.52 ± 0.10^{a}	3.36 ± 0.11^{a}	3.58 ± 0.13^{a}	0.393
P-value	<0.001	<0.001	<0.001	

Values of mean followed by similar letter/ capital letter(s) in the same row are not significantly different from one another (One –way ANOVA, SNK-test, α=0.05)

Table 11: Means of root lengths of various *Azadirachta indica* extracts

Extract/ Concentration	Acetone extract Mean±SE	Hexane extract Mean±SE	Aqueous extract Mean±SE	P-value
0.125 mg/ml	4.16 ± 0.17^{c}	4.22 ± 0.12^{b}	4.28 ± 0.15^{c}	0.850
0.25 mg/ml	3.86 ± 0.17^{bc}	3.96 ± 0.13^{b}	3.96 ± 0.12^{bc}	0.855
0.5 mg/ml/	3.38 ± 0.18^{ab}	3.54 ± 0.19^{a}	3.48 ± 0.20^{ab}	0.837
1 mg/ml	3.12 ± 0.24^{a}	3.14 ± 0.10^{a}	3.20 ± 0.25^{a}	0.960
p-value	P=0.007	p<0.001	P=0.004	

Values of mean followed by similar letter/ capital letter(s) in the same row are not significantly different from one another (One –way ANOVA, SNK-test, α=0.05)

Table 12: Root length in various doses and extracts of *Ocimum suave*

Extract/ concentration	Acetone extract Mean±SE	Hexane extract Mean±SE	Aqueous extract Mean±SE	P-value
0.125 mg/ml	4.32±0.06[b]	4.20±0.07[c]	4.38±0.14[b]	0.442
0.25 mg/ml	4.10±0.04[b]	4.02±0.07[c]	4.16±0.07[b]	0.326
0.5 mg/ml/	3.62±0.16[a]	3.46±0.14[b]	3.66±0.18[a]	0.655
1 mg/ml	3.32±0.11[a]	3.14±0.10[a]	3.36±0.10[a]	0.318
p-value	<0.001	<0.001	<0.001	

Values of mean followed by similar letter/ capital letter(s) in the same row are not significantly different from one another (One –way ANOVA, SNK-test, α=0.05)

Table 13: Means of various extracts of *Adansonia digitata*

Extract/ concentration	Acetone extract Mean±SE	Hexane extract Mean±SE	Aqueous extract Mean±SE	*P*-value
0.125 mg/ml	$4.92±0.19^{b}$	$5.08±0.15^{c}$	-	0.563
0.25 mg/ml	$4.62±0.28^{b}$	$4.60±0.24^{bc}$	$4.78±0.26^{b}$	0.869
0.5 mg/ml/	$4.36±0.22^{ab}$	$4.28±0.20^{ab}$	$4.52±0.19^{b}$	0.709
1 mg/ml	$3.82±0.16^{a}$	$3.74±0.15^{a}$	$3.90±0.10^{a}$	0.725
p-value	0.016	0.002	0.023	

Values of mean followed by similar letter/ capital letter(s) in the same row are not significantly different from one another (One –way ANOVA, SNK-test, α=0.05)

Table 14: Means of various extracts of extracts of *Plectranthus barbatus*

Extract/ Concentration	Acetone extract Mean±SE	Hexane extract Mean±SE	Aqueous extract Mean±SE	P-value
0.125 mg/ml	4.92±0.18[b]	5.02±0.16[c]	4.88±0.19[b]	0.845
0.25 mg/ml	4.40±0.19[b]	4.10±0.10[b]	4.64±0.19[b]	0.106
0.5 mg/ml/	4.34±0.19[b]	3.86±0.33[b]	4.30±0.20[b]	0.349
1 mg/ml	3.00±0.06[a]	3.08±0.13[a]	3.14±0.11[a]	0.657
	P<0.001	P<0.001	<0.001	

Values of mean followed by similar letter/ capital letter(s) in the same row are not significantly different from one another (One –way ANOVA, SNK-test, α=0.05)

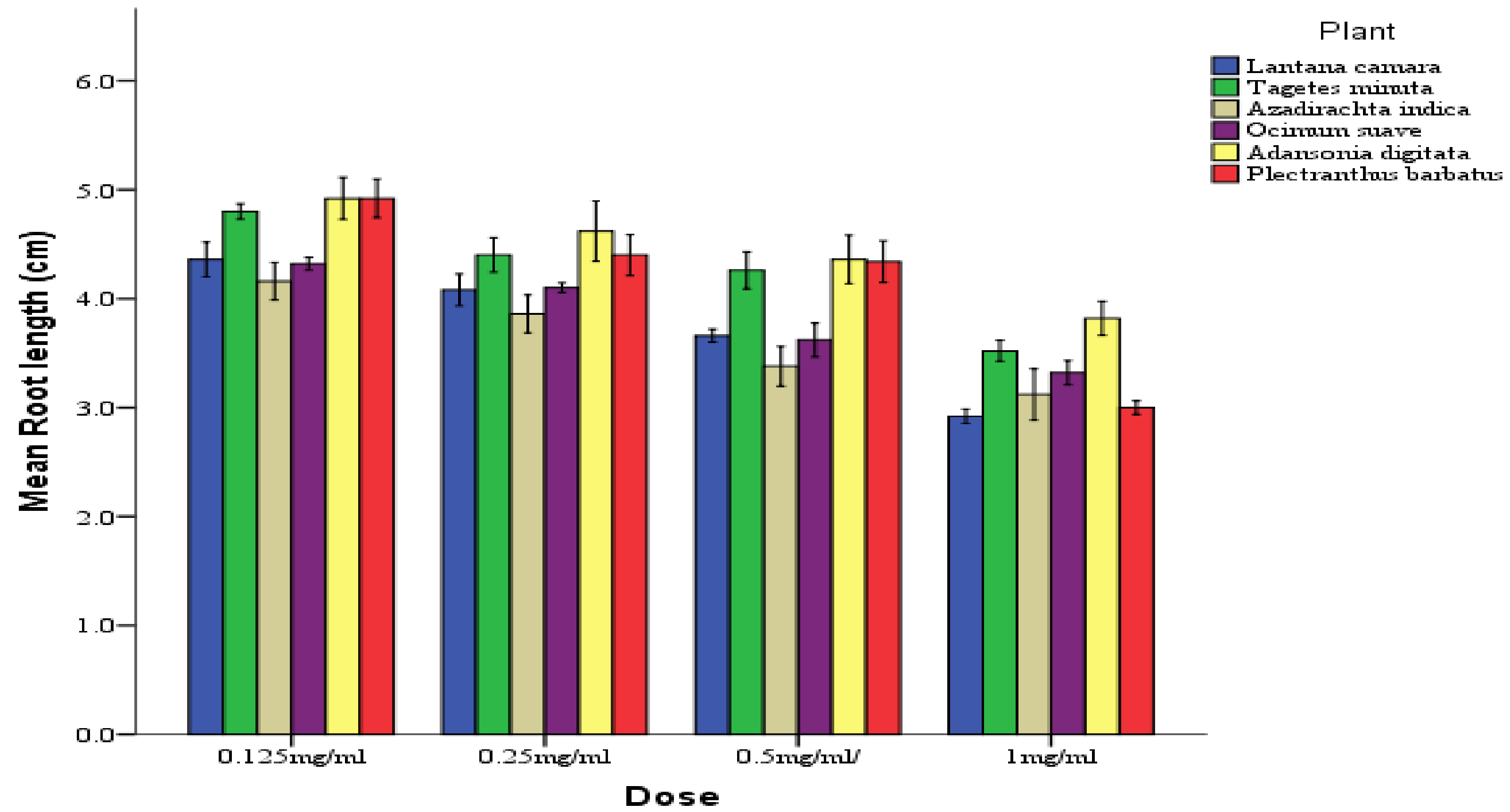


Figure 9: Means of root lengths in various acetone extracts

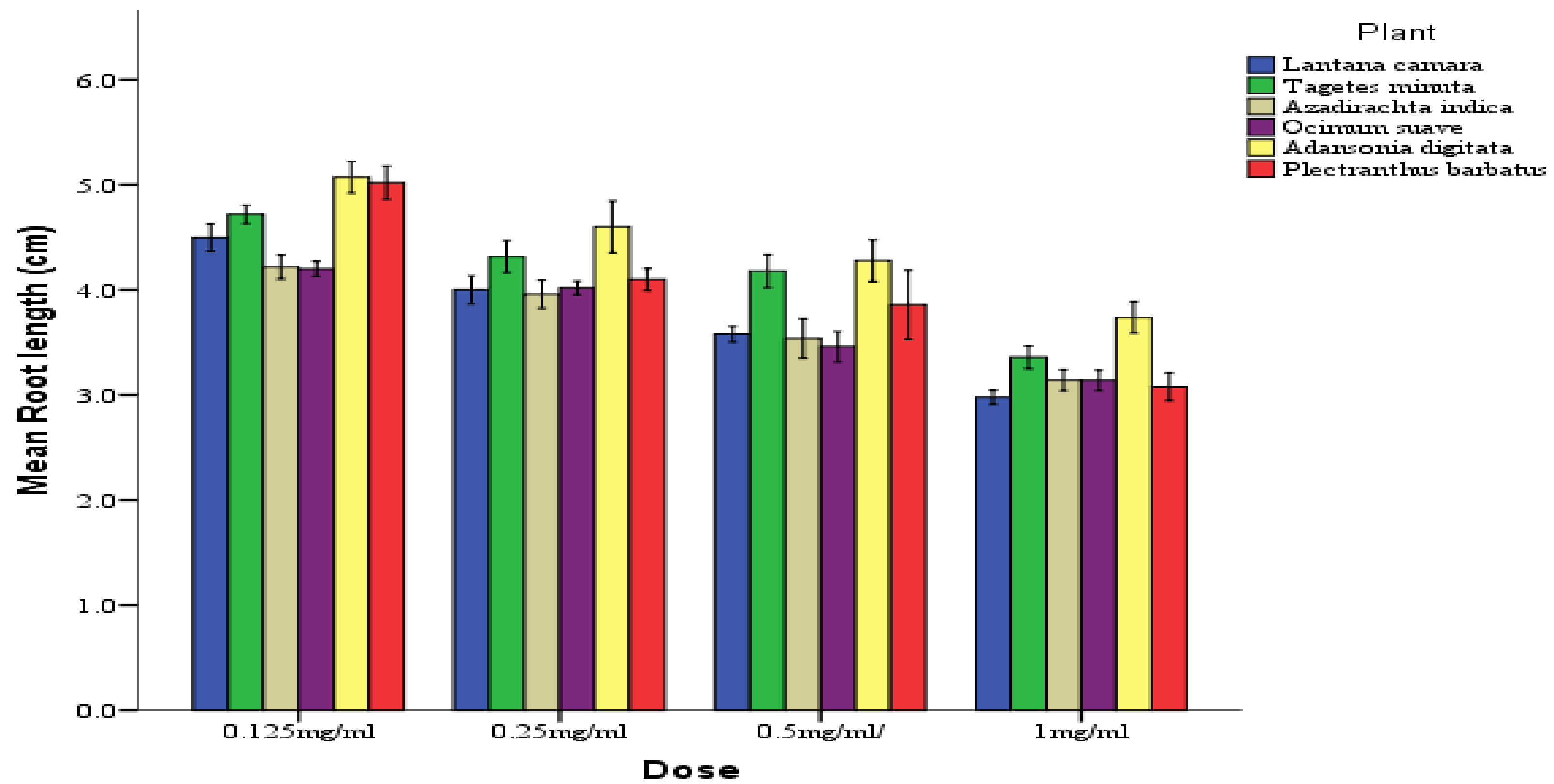


Figure 10: Means of root lengths in various hexane extracts

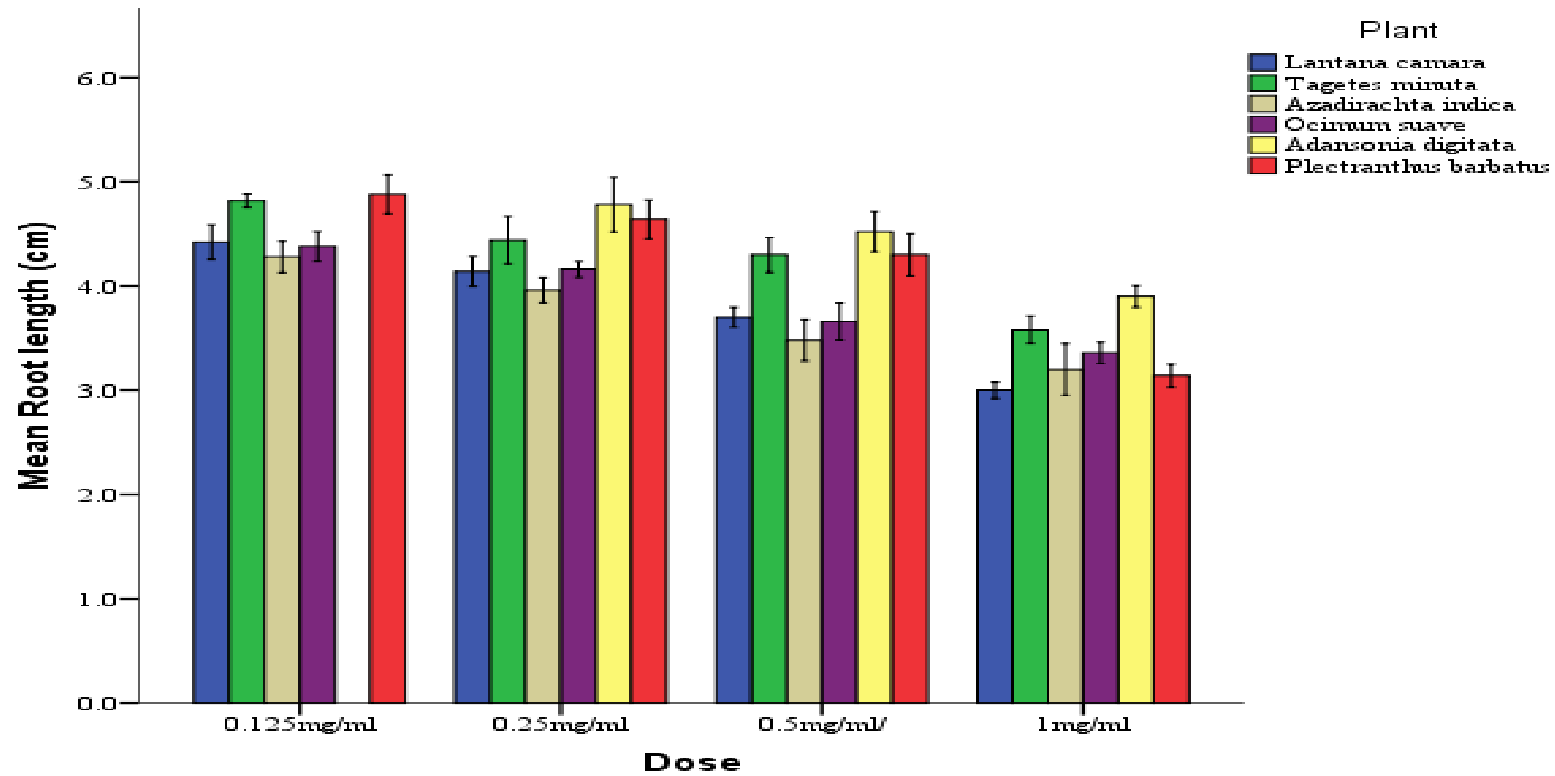


Figure 11: Means of root lengths in various Aqueous extracts

5.1.4.2 Assessment of damage to chromosomes

Plate 3 below shows some of the effects induced by different extracts on the *Allium cepa* meristem cells. Among the acetone extracts, as shown in table 15, there existed a difference in activity among the plants. *Azadirachta indica* showed most mitotic inhibition at concentration of 1mg/ml at 70.36±0.68 which was similar to vincristine.

In table 16 the mitotic inhibition effects of hexane extracts are shown. The most active was *Azadirachta indica* at 1 mg/ml at 71.53±1.18 which was not significantly different from vincristine sulphate while the least active was *Adansonia digitata* at 1.42±0.68 at 0.125 mg/ml. There were significant differences among extracts and concentrations $p<0.001$.

For aqueous extracts (table 17), the most active was *A. indica* at 1mg/ml at a value of 67.95±0.72 which was not significantly different from vincristine sulphate. The least active was *Adansonia digitata* at concentration of 0.125 mg/ml with a value of 2.66±0.76.

Table 15: Mitotic inhibition of acetone extracts

Plant/Treatment	Concentration			
	0.125 mg/ml	**0.25 mg/ml**	**0.5 mg/ml**	**1 mg/ml**
Lantana camara	23.55±0.79^{Ad}	40.76±0.93^{Be}	50.36±0.66^{Cd}	62.45±1.10^{De}
Tagetes minuta	-	27.49±0.68^{Ad}	33.80±0.63^{B}	44.93±0.95^{Cd}
Azadirachta indica	28.91±0.98^{Ae}	41.06±0.92^{Be}	54.57±0.67^{Ce}	70.36±0.68^{Df}
Ocimum suave	13.76±0.51^{Ab}	23.40±1.17^{Bc}	36.01±2.21^{Cc}	37.83±0.70^{Cc}
Adansonia digitata	1.02±0.46^{Aa}	16.27±1.02^{Bb}	23.62±1.02^{Cb}	29.53±0.61^{Db}
Plectranthus barbatus	18.61±2.33^{Ac}	23.59±1.24^{Bc}	33.55±0.73^{Cc}	45.16±1.14^{Dd}
tap water	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
vincristine sulphate	94.99±0.38^{f}	94.99±0.38^{f}	94.99±0.38^{f}	94.99±0.38^{f}
	P<0.001	P<0.001	P<0.001	P<0.001

Values of mean followed by similar letter/ capital letter(s) in the same row are not significantly different from one another (One –way ANOVA, SNK-test, α=0.05)

Table 16: Mitotic inhibition of hexane extracts

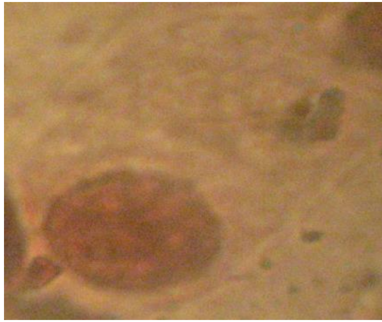
Treatment	Concentration			
	0.125 mg/ml	**0.25 mg/ml**	**0.5 mg/ml**	**1 mg/ml**
Lantana camara	20.02±1.26^{Ac}	27.46±1.07^{Bcd}	53.16±1.10^{Ce}	63.79±0.73^{De}
Tagetes minuta	22.10±0.84^{Ac}	23.85±0.86^{Ac}	33.95±0.82B^{c}	43.49±0.84^{Cc}
Azadirachta indica	29.64±0.84^{Ad}	34.60±1.17^{Be}	57.30±1.47^{Cf}	71.53±1.18^{Df}
Ocimum suave	17.13±0.71^{Ab}	28.08±1.81^{Bcd}	37.66±1.23^{Cd}	48.27±0.87^{Dd}
Adansonia digitata	1.42±0.68^{Aa}	17.49±1.34^{Bb}	25.72±1.16^{Cb}	30.74±0.91^{Db}
Plectranthus barbatus	21.39±0.46^{Ac}	30.92±1.91^{Bd}	36.06±1.11^{Ccd}	42.61±1.31^{Dc}
tap water	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
vincristine sulphate	94.99±0.38^{e}	94.99±0.38^{f}	94.99±0.38^{g}	94.99±0.38^{g}
p-value	< 0.001	< 0.001	< 0.001	< 0.001

Values of mean followed by similar letter/ capital letter(s) in the same row are not significantly different from one another (One –way ANOVA, SNK-test, α=0.05)

Table 17: Mitotic inhibition of aqueous extracts

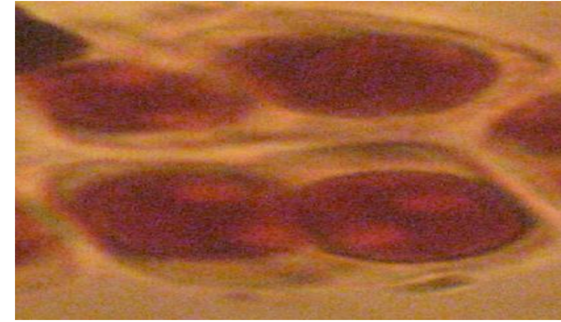
Treatment	Concentration			
	0.125 mg/ml	**0.25 mg/ml**	**0.5 mg/ml**	**1 mg/ml**
Lantana camara	18.43±0.98^{Ab}	34.31±0.78^{Bd}	44.00±1.41^{Cd}	61.53±0.49^{De}
Tagetes minuta	17.70±0.98^{Ab}	22.90±1.44^{Bc}	35.95±1.06^{Cc}	40.51±1.61^{Dc}
Azadirachta indica	28.93±1.54^{Ac}	40.46±0.91^{Be}	51.36±0.81^{Ce}	67.95±0.72^{Df}
Ocimum suave	16.51±0.50^{Ab}	22.57±1.60^{Bc}	35.85±0.80^{Cc}	38.44±2.20^{Cc}
Adansonia digitata	2.66±0.76^{Aa}	15.89±1.36^{Bb}	22.38±1.59^{Cb}	25.64±1.71^{Cb}
Plectranthus barbatus	19.70±1.75^{Ab}	25.04±1.07^{Bc}	34.42±0.79^{Cc}	46.45±0.67^{Dd}
tap water	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
vincristine sulphate	94.99±0.38^{d}	94.99±0.38^{f}	94.99±0.38^{f}	94.99±0.38^{g}
P-value	< 0.001	< 0.001	< 0.001	< 0.001

Values of mean followed by similar letter/ capital letter(s) in the same row are not significantly different from one another (One –way ANOVA, SNK-test, α=0.05)

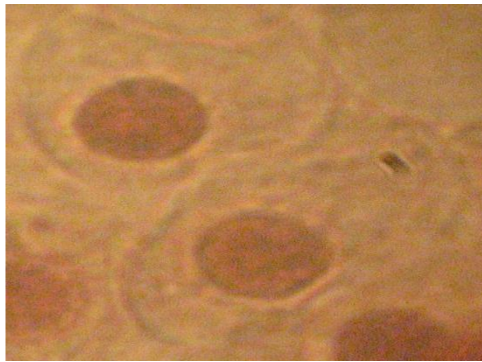


Ghost cell (*A.indica* aqueous extract)

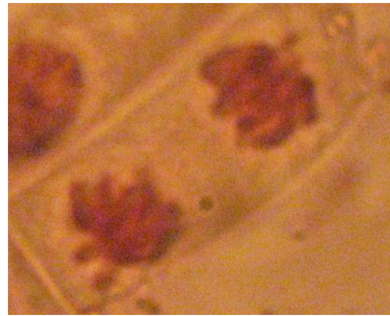
Binucleate cells (*A.indica* acetone extract)



Binucleate cells due to vincristine



High cytoplasm to nucleus ratio (Aqueous extract of *p. barbatus*)



Normal metaphase

Plate 3: Examples of observed effects of plant extracts and controls

5.1.5 DISCUSSION

Root growth inhibition was dose dependent with higher concentrations exhibiting greater inhibition. Greater root inhibition was seen with the highest concentration of 1mg/ml. There was significant difference on root length across the different doses.

Concerning mitotic inhibition, for acetone extracts, significant difference among the extracts existed and mitotic inhibition was dose dependent. The most active was *Azadirachta indica* at 1mg/ml at 70.36±0.68 which was similar to that of vincristine sulphate. In the hexane extracts, the most active was *A. indica* at 1mg/ml at 71.53±1.18 and was not significantly different from vincristine sulphate. The least active was *A. Digitata* at 1.42±0.68 at 0.125mg/ml. There were significant differences among extracts and concentrations $p<0.001$.

In the aqueous extracts, the most active was *A. indica* at 1mg/ml at a value of 67.95±0.72 which was not significantly different from vincristine sulphate. The least active was *A. digitata* at concentration of 0.125mg/ml with a value of 2.66±0.76. There was a significant difference between doses whereby mitotic inhibition increased with concentration such that the higher the dose, the greater the mitotic inhibition.

Of all the plants, *Azadirachta indica* induced greater mitotic inhibition. According to Soliman, (2001), several chromosomal aberrations such as micronuclei, bridges, stickiness, laggards and polyploidy were attributed to *Azadirachta indica*. This study recorded that *Azadirachta indica* induced binucleate cells and ghost cells. Formation of binucleate cells is as a result of interference between chemicals and cell wall formation (Baeshin *et al*., 1999).

Plectranthus barbatus induced least mitotic inhibition but there were cells which had a high cytoplasm nucleus ratio including a bulging cytoplasm. Binucleate cells, ghost cells, an increase in cytoplasm nucleus ratio and bulging cytoplasm occur in malignancies (Dowerah and Borgohain, 2016).

5.1.6 CONCLUSION

This study determined that leaf extracts of *Azadirachta indica* induces mitotic inhibition, and produces binucleate and ghost cells in the *Allium cepa* root meristems. Previous studies have shown similar outcomes including micronuclei, which is a clear indication of mutagenic potential. These studies identified seed extracts of *Azadirachta indica* as having greater genotoxic risk than the leaf extracts.

This study did not identify any form of micronuclei. These plants can be used topically on the skin. The study raises concerns on *Azadirachta indica* and *Plectranthus barbatus* because of the abnormal cells they induced and there is a possibility of inducing the same in human.

5.2 ACUTE DERMAL IRRITATION OF SIX PLANTS USED FOR MOSQUITO CONTROL IN MSAMBWENI SUB COUNTY

5.2.1 INTRODUCTION

During assessment and evaluation of substances, any material that is going to be applied on human skin must be assessed for irritability and corrosion potential through the acute dermal irritation/corrosion test. This is done to determine the degree of irritation that a dilution of a test material can produce on the skin of New Zealand white rabbit, usually three per dilution of test substance (OECD, 2002).

It avails information on possible dangers from short-term exposures through the skin and also provides information on absorption through the skin and the mode of toxic action of a substance by topical route. It is an initial part in determining a dosage regimen for subsequent studies (Chaudhary *et al*., 2008; Draize, 1965).

To guarantee safety and care of the test animals for the acute dermal and ocular irritation testing, the study protocol was submitted to and approval obtained from the Faculty of veterinary medicine biosafety, animal use and ethics committee of the University of Nairobi.

5.2.2 MATERIALS AND METHODS

5.2.2.1 Preparation of the test extracts

Extraction was by use of water, acetone and hexane and was carried out as described in chapter three of this thesis. For aqueous extracts, freeze drying was done and the freeze dried material was stored at $+4^{0}C$ awaiting acute dermal and ocular irritation testing. The respective resultant viscous substances for acetone and hexane extracts were dried and stored in amber coloured bottles at $^{+}4^{0}C$ pending acute dermal and ocular irritation testing.

5.2.2.2 The test animals

According to Sanders, (2007), rabbit is an appropriate model for this study since the results can be extrapolated to human. Three New Zealand white rabbits per plant extract per concentration were used. They weighed 2.5- 3 kg and aged 18-20 weeks. They were housed individually in the animal house at the Department of Public Health, Pharmacology and Toxicology of the University of Nairobi in relative humidity of 50-60% and lighting simulating day and night with conventional laboratory diet and unrestricted access to water (EPA, 1998; OECD, 2002).

5.2.2.3 Acute dermal irritation testing

This was performed on intact and abraded skin of rabbits. Only animals with healthy intact epidermis by gross observation were used for the study, and three rabbits were used per test. For intact skin, prior to the test, fur was removed through shaving the left and right dorsal areas of the trunk of the animals. The skin was cleaned with distilled water and left for 24 hours. This was to allow for recovery of the *stratum corneum* from any disturbance caused by the shaving (Payasi *et al*., 2010).

Testing on abraded skin was done to simulate situations when the skin has wounds, pimples or scratches. The same procedure as for the intact skin was used except that the shaved skin was rubbed with a fine abrasive paper (Amasa *et al*., 2012).

Half a milliliter (0.5 ml) of each concentration of each test extract was spread evenly to about 6 cm^2 of skin on the left dorsal area. It was covered with a gauze patch held in place by a non-irritating tape. The shaved skin on the right side applied with only 0.5 ml of distilled water was the control (Teshome *et al*., 2008; Zhu *et al*., 2009).

Trunks of the animals were wrapped with skin tight corsets to prevent them from interfering with the patches. After four (4) hours, the plant extracts were cleaned off by gentle swabbing with cotton wool soaked in distilled water. The animals were observed for signs of irritation such as erythema and oedema.

Findings were scored at one hour, then at 24 hours, 48 hours and 72 hours after patch removal. The animals were further observed for any signs of dermotoxicity, behavior, general condition, posture and reflexes, attitude towards food, water, and hygiene on days 7 and 14 (OECD, 2002; Payasi *et al*., 2010). They were also weighed on day 0 and the last day of the experiments.

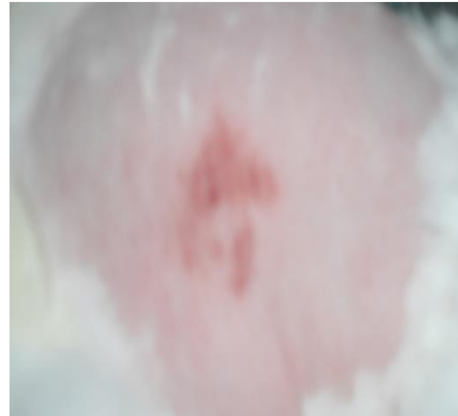
5.2.2.4 Determination of Primary Irritation Index

The primary irritation index (PII):

PII = Σ (erythema grade at 24, 48 and 72 hr) + Σ (oedema grade at 24, 48 and 72 hr)/ total number of observations (Kapoor and Saraf, 2008).

5.2.3 RESULTS

Plate 4 below shows one of the test animals that had just been prepared for the tests using an improvised corset. It also shows an abraded skin area of a rabbit and the rabbit skin healing well. The results showed that the tested six plants did not cause any toxicity even after 14 days of observation, table 18. There were no signs of acute dermal toxicity such as redness, erythema, oedema or eschar. There was no significant change in weight of the test animals during the treatment period and there were no mortalities. Primary Irritation Index was zero for all treatments as the parameters for its determination were absent.



A rabbit with an improvised corset an abraded skin area of a rabbit

Rabbit healing well

Plate 4: A rabbit with an improvised corset, an abraded skin area of a rabbit and a rabbit healing well

Table 18: Effects on intact and abraded rabbit skin

Treatment (0.5ml)	Duration of treatment /observation of signs and symptoms of toxicity					
	1 hr	24 hrs	48 hrs	72 hrs	7 days	14 days
Lantana camara (acetone)						
0.01mg/ml	–	–	–	–	–	–
0.1mg/ml	–	–	–	–	–	–
1mg/ml/	–	–	–	–	–	–
10 mg/ml	–	–	–	–	–	–
Lantana camara (hexane)						
0.01mg/ml	–	–	–	–	–	–
0.1mg/ml	–	–	–	–	–	–
1mg/ml/	–	–	–	–	–	–
10 mg/ml	–	–	–	–	–	–
Lantana camara (aqueous)						
0.01mg/ml	–	–	–	–	–	–
0.1mg/ml	–	–	–	–	–	–
1mg/ml/	–	–	–	–	–	–
10 mg/ml	–	–	–	–	–	–
Tagetes minuta (acetone)						
0.01mg/ml	–	–	–	–	–	–
0.1mg/ml	–	–	–	–	–	–

1mg/ml/	–	–	–	–	–	–
10 mg/ml	–	–	–	–	–	–
Tagetes minuta (hexane)						
0.01mg/ml	–	–	–	–	–	–
0.1mg/ml	–	–	–	–	–	–
1mg/ml	–	–	–	–	–	–
10 mg/ml	–	–	–	–	–	–
Tagetes minuta (aqueous)						
0.01mg/ml	–	–	–	–	–	–
0.1mg/ml	–	–	–	–	–	–
1mg/ml/	–	–	–	–	–	–
10 mg/ml	–	–	–	–	–	–
Azadirachta indica (acetone)						
0.01mg/ml	–	–	–	–	–	–
0.1mg/ml	–	–	–	–	–	–
1mg/ml	–	–	–	–	–	–
10mg/ml	–	–	–	–	–	–
Azadirachta indica (hexane)						
0.01mg/ml	–	–	–	–	–	–
0.1mg/ml	–	–	–	–	–	–
1mg/ml	–	–	–	–	–	–
10mg/ml	–	–	–	–	–	–

Azadirachta indica (aqueous)						
0.01mg/ml	–	–	–	–	–	–
0.1mg/ml	–	–	–	–	–	–
1mg/ml	–	–	–	–	–	–
10 mg/ml	–	–	–	–	–	–
Ocimum suave(acetone)						
0.01mg/ml	–	–	–	–	–	–
0.1mg/ml	–	–	–	–	–	–
1mg/ml/	–	–	–	–	–	–
10 mg/ml	–	–	–	–	–	–
Ocimum suave(hexane)						
0.01mg/ml	–	–	–	–	–	–
0.1mg/ml	–	–	–	–	–	–
1 mg/ml/	–	–	–	–	–	–
10 mg/ml	–	–	–	–	–	–
Ocimum suave(aqueous)						
0.01mg/ml	–	–	–	–	–	–
0.1mg/ml	–	–	–	–	–	–
1 mg/ml/	–	–	–	–	–	–
10 mg/ml	–	–	–	–	–	–

Adansonia digitata (acetone)	–	–	–	–	–	–
0.01mg/ml	–	–	–	–	–	–
0.1mg/ml	–	–	–	–	–	–
1mg/ml/	–	–	–	–	–	–
10 mg/ml	–	–	–	–	–	–
Adansonia digitata (hexane)						
0.01mg/ml	–	–	–	–	–	–
0.1mg/ml	–	–	–	–	–	–
1mg/ml/	–	–	–	–	–	–
10 mg/ml	–	–	–	–	–	–
Adansonia digitata (aqueous)	–	–	–	–	–	–
0.01mg/ml	–	–	–	–	–	–
0.1mg/ml	–	–	–	–	–	–
1mg/ml	–	–	–	–	–	–
10mg/ml						
Plectranthus barbatus (acetone)						
0.01mg/ml	–	–	–	–	–	–
0.1mg/ml	–	–	–	–	–	–
1mg/ml	–	–	–	–	–	–
10 mg/ml	–	–	–	–	–	–
Plectranthus barbatus (hexane)						

0.01mg/ml	–	–	–	–	–	–
0.1mg/ml	–	–	–	–	–	–
1mg/ml	–	–	–	–	–	–
10mg/ml	–	–	–	–	–	–
Plectranthus barbatus (aqueous)						
0.01mg/ml	–	–	–	–	–	–
0.1mg/ml	–	–	–	–	–	–
1mg/ml	–	–	–	–	–	–
10mg/ml	–	–	–	–	–	–

- = no irritation observed
+ = irritation observed

5.3 ACUTE OCULAR TOXICITY TESTING OF SIX PLANTS USED FOR MOSQUITO CONTROL IN MSAMBWENI SUB COUNTY

5.3.1 INTRODUCTION

Products being applied to the skin especially on the face should also be evaluated for their effects on the eyes. According to Huhtala *et al*., (2008), OECD, (2012) and Barile, (2010), Draize testing as developed by Draize *et al*., (1944), is the major test for determination of toxicity to the eyes. It uses New Zealand white rabbits due to the advantage with their eyes having excellent anatomy and physiology (Wilhelmus, 2001).

5.3.2 MATERIALS AND METHODS

Plants' extracts were prepared into 1, 10, and 100 µg/ml then used for this test. Draize test was used with slight modification as adapted by OECD test guideline number 405 (OECD, 2012). Three New Zealand white rabbits were used per extract per concentration. Only rabbits with non-deformed eyes were used. A tenth of a millimeter of test substances was deposited in one eye and lids held together for one minute then left for twenty four hours. The other eye served as control. Observations were made and recorded at 1, 24, 48, and 72 hours days 7, 14 and 21 following test substance application.

The animals were observed and any redness, swelling, cloudiness, oedema, hemorrhage, discharge, excessive blinking, excessive tearing, corneal damage, absence of light reflex and conjuctival ulceration and blindness recorded (Huhtala *et al*., 2008). The observations were recorded and graded according to set guidelines (OECD, 2012).

5.3.3 RESULTS

All the extracts of the six plants did not cause toxicity to the rabbit eye, table 19. There were no signs of eye irritation such as redness, swelling, cloudiness, oedema, hemorrhage, discharge, excessive blinking, absence of light reflex and conjuctival ulceration and blindness even after the 21-day experimental period.

Table 19: Ocular effects of the extracts on rabbit eye

Treatment (0.5ml)	Duration of treatment /observation of signs and symptoms of toxicity						
	1 hr	24 hrs	48 hrs	72 hrs	7 days	14 days	21 days
Lantana camara (acetone)							
1 µg/ml	–	–	–	–	–	–	–
10 µg/ml	–	–	–	–	–	–	–
100 µg/ml	–	–	–	–	–	–	–
Lantana camara (hexane)							
1 µg/ml	–	–	–	–	–	–	–
10 µg/ml	–	–	–	–	–	–	–
100 µg/ml	–	–	–	–	–	–	–
Lantana camara (aqueous)							
1 µg/ml	–	–	–	–	–	–	–
10 µg/ml	–	–	–	–	–	–	–
100 µg/ml	–	–	–	–	–	–	–
Tagetes minuta (acetone)							
1 µg/ml	–	–	–	–	–	–	–
10 µg/ml	–	–	–	–	–	–	–
100 µg/ml	–	–	–	–	–	–	–
Tagetes minuta (hexane)							
1 µg/ml	–	–	–	–	–	–	–
10 µg/ml	–	–	–	–	–	–	–

100 µg/ml	–	–	–	–	–	–	–
Tagetes minuta (aqueous)							
1 µg/ml	–	–	–	–	–	–	–
10 µg/ml	–	–	–	–	–	–	–
100 µg/ml	–	–	–	–	–	–	–
Azadirachta indica (acetone)							
1 µg/ml	–	–	–	–	–	–	–
10 µg/ml	–	–	–	–	–	–	–
100 µg/ml/	–	–	–	–	–	–	–
Azadirachta indica (hexane)							
1µg/ml	–	–	–	–	–	–	–
10 µg/ml	–	–	–	–	–	–	–
100 µg/ml	–	–	–	–	–	–	–
Azadirachta indica (aqueous)							
1µg/ml	–	–	–	–	–	–	–
10 µg/ml	–	–	–	–	–	–	–
100 µg/ml	–	–	–	–	–	–	–

Ocimum suave(acetone)							
1 µg/ml	–	–	–	–	–	–	–
10 µg/ml	–	–	–	–	–	–	–
100 µg/ml	–	–	–	–	–	–	–
Ocimum suave(hexane)							
1µg/ml	–	–	–	–	–	–	–
10 µg/ml	–	–	–	–	–	–	–
100 µg/ml	–	–	–	–	–	–	–
Ocimum suave(aqueous)							
1µg/ml	–	–	–	–	–	–	–
10 µg/ml	–	–	–	–	–	–	–
100 µg/ml	–	–	–	–	–	–	–
Adansonia digitata (acetone)							
1µg/ml	–	–	–	–	–	–	–
10 µg/ml	–	–	–	–	–	–	–
100 µg/ml	–	–	–	–	–	–	–
Adansonia digitata (hexane)							
1 µg/ml	–	–	–	–	–	–	–
10 µg/ml	–	–	–	–	–	–	–
100 µg/ml	–	–	–	–	–	–	–

Adansonia digitata (aqueous)							
1 µg/ml	–	–	–	–	–	–	–
10 µg/ml	–	–	–	–	–	–	–
100 µg/ml	–	–	–	–	–	–	–
Plectranthus barbatus (acetone)							
1 µg/ml	–	–	–	–	–	–	–
10 µg/ml	–	–	–	–	–	–	–
100 µg/ml	–	–	–	–	–	–	–
Plectranthus barbatus (hexane)							
1µg/ml	–	–	–	–	–	–	–
10 µg/ml	–	–	–	–	–	–	–
100 µg/ml	–	–	–	–	–	–	–
Plectranthus barbatus (aqueous)							
1µg/ml	–	–	–	–	–	–	–
10 µg/ml	–	–	–	–	–	–	–
100 µg/ml	–	–	–	–	–	–	–

- = no irritation observed
+ = irritation observed

5.4 DISCUSSION

Material to be applied on human skin should be assessed for irritability and corrosion potential. The acute dermal irritation/corrosion test is used because results obtained can be extrapolated to human (Sanders, 2007). It is useful in determining the mode of toxicity of a substance through the skin (Draize, 1965).

All plants' extracts in this study were not irritating to both the intact and abraded skins of rabbits. They were also not irritating to the eyes of the rabbits. These plants' parts are not likely to produce irritation to the human skin and eyes. Further studies need to be carried out to evaluate whether there exists any other of form irritation in these plants because the Draize test may not fully determine mild irritation on the skin and in the eyes.

5.5 CONCLUSION

The acute dermal irritation and ocular irritation conducted have not shown any toxicity attributable to these plants. The plants are not likely to cause severe irritation on the skin if applied or/on contact and are not also likely to cause severe eye irritation. Further tests are needed to establish long term use of these plants especially with regard to regular contact with the skin or the eyes.

CHAPTER SIX

FORMULATING A PLANT BASED MOSQUITO REPELLENT

6.1 INTRODUCTION

Diseases transmitted by mosquitoes cause morbidity and mortality in humans. The effects of these transcends human affliction to economic loss and social disruption in the poorest countries of the world (Awad and Shimaila, 2003; Becker *et al.*, 2003). Among methods for control of these diseases is the disruption mosquito-host contact for example by use of repellents (Curtis, 1992; Gupta and Rutledge, 1994; Okigbo *et al*., 2010). Besides reducing vector host interaction, it also reduces bites and associated distress (Fradin, 2001).

Unfortunately, most repellents used currently are of synthetic origin and have been associated with toxicities and several adverse effects. Prolonged exposure to pyrethroids has adverse effects on children's nervous system (Sinha *et al*., 2004). *N, N*-diethyl-m-toluamide (DEET) is toxic to human, affects plastics and some fabrics and there are concerns as some mosquitoes have started developing resistance as has been expressed by service members of the US military, its largest number of users (Sanders *et al*. 2005).

Extensive use of chemicals for vector control results in environmental toxicities, non acceptance by users and overall resistance by the insects (Shyamapada, 2011). This calls for search of safe and efficacious repellents and folklore is an important start point. Plants and plant products have repellent activities (Isman, 2006; Krajick, 2006).

Plant derived botanicals do not persist in the environment and do not have major effects in organisms or plants that they are not intended to be used against (Govindarajan *et al*., 2008). They can substitute synthetic insecticides or complement the use of the same (Samidurai *et al*., 2009).

However, only a few of plant derived biopesticides have been investigated for their possible adverse effects (Zhu *et al*., 2009). This study's purpose was to formulate a plant based mosquito repellent that is effective and safe to use.

6.2 MATERIALS AND METHODS

6.2.1 The plants

Utilization of plants in this study was based on their mosquito repellency activity. In comparison, acetone extracts had greater activity than the hexane and aqueous extracts, (table 20). Therefore, for the formulation of the biopesticide, acetone extracts of the plants' species were used.

Table 20: Protection efficacy of extracts and controls

Plant	**Acetone Mean±SE**	**Hexane Mean±SE**	**Aqueous Mean±SE**	***P*-value**
Lantana camara	$98.33±1.67^{Bc}$	$94.86±0.53^{Bc}$	$77.63±1.04^{Ab}$	<0.001
Tagetes minuta	$98.33±1.67^{c}$	$98.33±1.67^{c}$	$94.86±0.53^{c}$	0.182
Azadirachta indica	$98.33±1.67^{c}$	$94.86±0.53^{c}$	$94.86±0.53^{c}$	0.070
Adansonia digitata	$84.58±1.58^{b}$	$75.27±4.07^{b}$	$80.00±4.71^{b}$	0.259
Ocimum suave	$100.00±0.00^{B}$	$94.86±0.53^{Ac}$	$94.86±0.53^{Ac}$	<0.001
Plectranthus barbatus	$84.47±2.70^{b}$	$74.14±3.59^{b}$	$80.53±2.81^{b}$	0.107
DEET	$98.33±1.67^{c}$	$98.33±1.67^{c}$	$98.33±1.67^{c}$	-
Ethanol	$0.00±0.00^{a}$	$0.00±0.00^{a}$	$0.00±0.00^{a}$	-
P-value	<0.001	<0.001	<0.001	

Values of mean followed by similar letter/ capital letter(s) in the same row are not significantly different from one another (One –way ANOVA, SNK-test, α=0.05)

6.2.2 Extraction of plant material and formulation of the test extracts

Extraction was by use of acetone and was carried out as described in chapter three of this thesis. The test extracts were formulated in pure petroleum jelly. The formulations tested were made into concentrations of 10% and 20% of plant extracts in petroleum jelly. To make 10% of the formulation, fifty four (54) grams of the pure petroleum jelly was weighed and transferred to a clean 100 ml beaker.

The beaker with the pure petroleum jelly was warmed in a laboratory water bath at 80 °C and stirred with a stirring rod until it was fully melted. One gram (1 gm) of each of the six plant extracts was then added to the melted jelly and stirred continuously until it mixed fully with the petroleum jelly. Upon complete mixing, the resultant formulation was stored at +4°C awaiting repellency testing.

To make 20% of the formulation, forty eight (48) gram of pure petroleum jelly was melted in a beaker using water bath at 80 °C. Two gram (2 gm) of each of the six plant extracts added to the melted petroleum jelly and stirred continuously until it full mixing. The resultant formulation was stored at +4°C awaiting repellency testing.

6.2.3 Test mosquitoes

The mosquitoes used for the laboratory repellent bioassay were 3-7 day old, laboratory-bred and starved adult females of *Aedes aegypti*. Tests were conducted in triplicate using female *A. aegypti* mosquitoes (Moore, 2004). Prior to the time of tests, they were starved for 24 hours but provided with only water.

6.2.4 Cage tests

They were performed in 40 x 40 x 40 cm cages as described in chapter four of this thesis and according to Innocent *et al*., (2010) with a 12:12 (Light: Dark) photoperiod and controlled temperatures of about 27^0C and a relative humidity of 80% maintained by use of an electric fan heater (WHO 1996, 2009).

Active female host-seeking *A. aegyptiae* mosquitoes aged 5-7 days were collected from stock population using an aspirator and starved for the preceding 24 hours. Tests were performed as set out in chapter four of this thesis and according to Innocent *et al*., (2010) and WHO, 1996, 2009). Acetone/petroleum jelly mixture (1:1) was negative control while 20% DEET (*N, N*-diethyl-m-toluamide) was the positive control.

Volunteers who had avoided use of fragrance, any mosquito repellent, perfumed soap or tobacco for 12 hours prior and during the experiment (WHO, 2009; Innocent *et al*., 2010) were used for the experiments. The forearm, from the elbow to the wrist (~696.6 cm^2) was rinsed with water then dried in air. The extracts were applied as evenly and as thinly as possible. The rest of the hand from the wrist to the fingers was covered with latex glove to prevent the mosquitoes from biting (WHO, 2009).

Acetone/petroleum jelly mixture was applied on the other forearm that had been prepared as above, and served as negative control. The volunteer's forearm that had been prepared as above was introduced into the cage through the sleeve for 5 minutes.

Mosquitoes that landed on or probed during this period were counted and shaken off before they can imbibe any blood (Kweka *et al*., 2008; WHO, 2009; Innocent *et al*., 2010). Percent protection efficacy (PE) determined like in chapter four of this thesis

6.2.5 Determination of knockdown effect of formulated product

Knockdown effect of the formulations was determined using WHO bioassay method (WHO, 1981; 2013 b) with slight modifications (Avicor *et al*.,2105) Tests were done in triplicates with positive and negative controls. Filter papers were treated with the formulated product and then air dried. Each was inserted into a chamber.

Twenty five active *A. aegyptiae* mosquitoes aged 5-7 days that had not been blood-fed were selected using an aspirator from the stock populations of adult mosquitoes and used for this test. They were placed in each of the chamber with filter papers with different concentrations of formulated product for 1 hour.

Untreated filter papers were negative control while citronella oil at concentrations of 500 mg/m^2 was positive control. After one hour the mosquitoes were transferred to different holding chambers that had cups with 10% sucrose solution for the mosquito to feed. Mortality and recovery within 24 hours was scored and the time taken to knock down 90% of the population (KD_{90}) at 95% confidence interval was determined.

6.3 RESULTS

The two formulated products exhibited a repellency effect greater than or similar to DEET, the positive control as shown in tables 21 and 22 below. Product A which contained 10% of the plant extracts had repellent effect similar to DEET. Product B comprised of 20% of the plant extracts and offered 100% protection. This was greater than that was offered by DEET.

The difference in activity of the formulated products and DEET was not statistically significant. In comparison to Ballet® mosquito repellent jelly, the formulated products showed a better repellency effect and the difference in activity was significant, $P<0.001$.

The knockdown effect of product A was similar to citronella oil, plate 4. Product B had greater knockdown effect compared to citronella oil even though the difference in activity was not statistically significant. When compared to a combination of paraffinum liquidum, cera microcrystalline, paraffin, *tacromanthus camphoratus, pelargonium graveolens*, isopropyl myristate, glycerol, myristyl myristate, citronellal, tocopherol acetate, *cymbopogon nardus*, geraniol, *eucalyptus globulus, rosemarinus officinalis*, citral, benzophenone-3, linalool and limonene (**Ballet® mosquito repellent**) the two formulated products had greater activity and the difference in knockdown effect was significant, $P<0.001$.

Table 21: Percent (%) protection of formulated products compared to DEET and Ballet® mosquito repellent

Treatment	**% Protection**
Product A (10%)	98.33±1.67
Product B (20%)	100.00±0.00
A combination of paraffinum liquidum, cera microcrystalline, paraffin, *tacromanthus camphoratus, pelargonium graveolens*, isopropyl myristate, glycerol, myristyl myristate, citronellal, tocopherol acetate, *cymbopogon nardus*, geraniol, *eucalyptus globulus, rosemarinus officinalis*, citral, benzophenone-3, linalool and limonene (**Ballet® mosquito repellent**)	75.00±4.07
Ethanol (Negative control)	0.00±0.00
DEET 20 %(Positive control)	98.33±1.67
P-value	<0.001

Table 22: Knock down effect of formulated products, ballet mosquito repellent, and citronella oil

Treatment	**%Knockdown**
Product A (10%)	93.33±3.33
Product B (20%)	96.67±3.33
A combination of paraffinum liquidum, cera microcrystalline, paraffin, *tacromanthus camphoratus, pelargonium graveolens*, isopropyl myristate, glycerol, myristyl myristate, citronellal, tocopherol acetate, *cymbopogon nardus*, geraniol, *eucalyptus globulus, rosemarinus officinalis*, citral, benzophenone-3, linalool and limonene(**Ballet® mosquito repellent**)	35.67±8.82
Negative Control	13.33±3.33
Citronella oil	93.33±3.33
P-value	<0.001

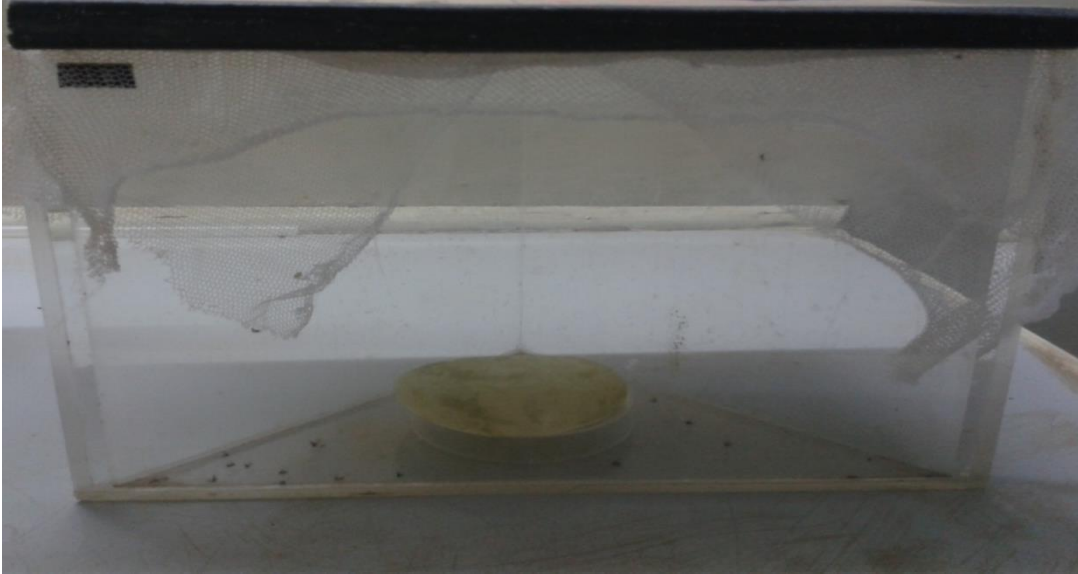


Plate 5: Mosquito knockdown effect of formulation A

All mosquitoes were dead/ immobile in the first three minutes. They never recovered even when they were transferred to the recovery chamber

6.4 DISCUSSION

From the results, the formulated products had repellency and knockdown effect suggesting there was synergism among the plant extracts. The knockdown effect of both formulations was equal or greater than that of citronella oil which was the positive control. Similarly, the percent protection offered by the each of the two formulations was equal or greater than that of DEET, the positive control.

According to Logan *et al*., (2010), the drawback of using plant-derived repellents is that most consist of volatile substances making them only useful for short durations and requiring applications ever so often. Formulation with petroleum jelly contributed to the improved activity as many researchers have also reported improved repellency after addition of a fixative substance to the repellents (Govindarajan, 2014).

Besides if a volatile compound is combined with a non-volatile substance, it is possible to block insect attack both on the air and the skin surface (Oyedele et al., 2002). This study agrees with previous findings that a combination of paraffinum liquidum, cera microcrystalline, paraffin, *tacromanthus camphoratus, pelargonium graveolens*, isopropyl myristate, glycerol, myristyl myristate, citronellal, tocopherol acetate, *cymbopogon nardus*, geraniol, *eucalyptus globulus, rosemarinus officinalis*, citral, benzophenone-3, linalool and limonene (Ballet® mosquito repellent) does not provide complete protection against mosquitoes (Koech and Mwangi, 2013).

The developed products can substitute the more expensive conventional repellents as they offer good protection against mosquitoes in the form of great repellent effect and knockdown activity.

6.5 CONCLUSION

From the results, the two formulated products have excellent repellency and knockdown effect due to synergism among the plants' extracts. The formulated products can be used as mosquito repellent agents as alternatives to synthetic mosquito repellents. Formulating the products with a fixing agent, in this case the petroleum jelly enhanced the activity of the formulations by preventing loss of active compounds as has been observed by other researchers.

Further research is necessary to evaluate the activity of the formulated products in both semi field and field trials. Preservatives, stabilizers, antioxidants should be considered and the formulated products should also be assessed for shelf life and stability. The formulated products should be evaluated for commercial viability. Studies should be carried out concerning conservation of these plants and all the others that have been reported to have activities against different stages of mosquitoes.

CHAPTER SEVEN

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

7.1 GENERAL DISCUSSION

Mosquito are of public health importance among the arthropod vectors. They transmit diseases which lead to serious illnesses and deaths globally. These diseases are obstacles to socio-economic development in countries wherever they are endemic. Cumulatively, these diseases affect over almost one billion people worldwide annually (Karunamoorthi and Sabesan, 2010; WHO, 2014).

Almost 2.5 billion people live in areas where dengue virus thrives (WHO, 2012) causing 22,000 deaths annually (Rigau-Perez *et al.*, 1998) from 390 million infections per year (WHO, 2016b). Yellow fever lacks antiviral treatment and the only means of remedy is personal protection and vaccination (Jentes *et al.*, 2011). Outbreaks of RVF in Kenya result in human illness and livestock losses every time they occur (LaBeaud *et al.*, 2011).

All methods of mosquito control have drawbacks. Physical control and habit change are unattainable since all aquatic habitats cannot be eliminated whereas others are sources of water or food such as rice paddies (Service, 1986). Biological control is an intricate method as mosquito predators especially fish also prey on beneficial insects and larvae. They cannot be used in polluted and/or temporary water areas such as puddles and vehicle ruts that form only in rainy seasons (Maniafu *et al.*, 2009).

Genetic control methods require high expertise and technology which is not available in most settings where mosquitoes transmit diseases. Resistance changes are bound to arise and parasites able to thrive in mosquitoes that were initially chosen due to refractoriness (Burattini *et al.*, 2008).

According to Maniafu *et al*., (2009), mosquitoes have become resistance to all classes of insecticides used for their control while Debboun and Stickman (2013) and Katritzky *et al*., (2008) postulate that utilizing repellents minimizes contacts between mosquitoes and human, and can reduce the rate of disease transmission. However, synthetic repellents like DEET are costly and cannot be used daily due to their toxicity are toxic while others require frequent application (Jahn *et al*., 2010).

Insecticide treated nets (ITNs) provide personal and a community protective effect and exert a level of control on mosquito populations where they are used at high uptake (Mutuku *et al*, 2011). There are shortcomings to use of ITNs such as effects on children (Sinha *et al*., 2004). Even where the ITNs are available, people fail to use them and divert them to economic uses such as fishing and drying fish (Minakawa *et al*., 2008).

The search for new compounds for mosquito control is on and traditional medicine is a first choice. Plants may be used wholly by themselves or serve as blue prints for development of those compounds. Vectors of diseases are unlikely to develop resistance to plant derived insecticides because the mechanism of action is based on multiple components rather than a singular constituent as happens with synthetic insecticides.

In the testing of the six plants' extracts on mosquito larvae, the larvicidal activity was dose dependent and at the highest concentration of 1mg/ml, all extracts exhibited 100% mortality. This is due to presence of secondary metabolites especially alkaloids and flavonoids.

Alkaloids such as sabadilla and nicotine have insecticidal activity (Hayes, 1982). Flavonoids also have insecticidal action by being are mitochondrial poisons (Hollingworth *et al*., 1994). The presence of alkaloids and flavonoids in all the plants studied could have contributed to their larvicidal activity.

Saponins also have larvicidal activity (Wiseman and Chapagain, 2005) and were present. The results of this study confirm previous findings. Kumar and Maneemegalai (2008) showed a dose dependent larvicidal effect of methanol and ethanol extract of *Lantana camara* on larvae of *Aedes aegypti*. Rathy *et al*., (2015) determined leaves of *A. indica* caused 100% mortality on 3rd instar larvae after twenty four hours.

This study was able to show that *A. indica* aqueous leaf extract exhibited 100% mortality on the tested mosquito larvae at twenty four (24) hours and therefore corroborates that previous finding. Larvicidal activity of *Tagetes minuta* is probably due to presence of terpenoids, saponins, alkaloids and flavonoids. This study records larvicidal activity of *Ocimum suave*, *Plectranthus barbatus* and *Adansonia digitata* as causing 100% mortality at 1mg/ml which was the highest concentration tested.

Personal protection is important among methods for controlling mosquitoes. It prevents disease spread by killing or preventing mosquitoes from biting hosts (Okigbo *et al*., 2010). In Africa, people burn plant parts or grind repellent plant parts and sprinkle on floors or hanging the repellent plant leaves on the roof (Karunamoorthi *et al*., 2009).

The drawback of many insect repellents of plant origin is they are highly volatile thus cannot provide protection for a long time (Kitchen *et al*., 2009). This study reports that all plants in the present study had appreciable repellency activity against *Aedes aegypti* mosquitoes.

Natural products cannot be assumed to be without toxicity despite decades of use. They have to be evaluated for their genotoxicity. One of the methods is the *Allium cepa* test whose data can be projected to human and plants. The most active species was *A. Indica* at 1mg/ml at 70.36±0.68 which was similar to from the vincristine sulphate. *Azadirachta indica* produced binucleate cells and ghost cells.

Draize testing is the international standard assay for acute ocular and dermal toxicity (Huhtala *et al*., 2008; Barile, 2010; Draize, 1965). All the plants' extracts in this study were not irritating to both the intact and abraded skins of rabbits. They were also not irritating to the eyes of the rabbits. These plants' parts are not likely to produce irritation to the human skin and eyes.

The formulated products had repellency and knockdown effect suggesting there was synergism among the plant extracts. The knockdown effect of both formulations was equal or greater than that of citronella oil which was the positive control. Similarly, the percent protection offered by the each of the two formulations was equal or greater than that of DEET, the positive control.

Formulation with petroleum jelly contributed to the improved activity as many researchers have also reported improved repellency when fixative agents are added to the repellents (Govindarajan, 2014). Besides if a volatile compound is combined with a non-volatile substance, it is possible to block insect attack both on the air and the skin surface (Oyedele *et al*., 2002).

This study agrees with previous findings that a combination of paraffinum liquidum, cera microcrystalline, paraffin, *tacromanthus camphoratus, pelargonium graveolens*, isopropyl myristate, glycerol, myristyl myristate, citronellal, tocopherol acetate, *cymbopogon nardus*, geraniol, *eucalyptus globulus, rosemarinus officinalis*, citral, benzophenone-3, linalool and limonene (Ballet® mosquito repellent) does not provide complete protection against mosquitoes (Koech and Mwangi, 2013). The developed formulations can substitute other synthetic formulations.

7.2 CONCLUSIONS

1. All the plants in this study had hundred percent (100%) lethality on *Aedes aegypti* larvae after twenty four hours.
2. The acute dermal irritation and ocular irritation conducted did not show any toxicity attributable to these plants
3. The two formulated products have excellent repellency and knockdown effect due to synergism among the plants' extracts may be used as mosquito repellent products.
4. Further research is necessary to evaluate the activity of the formulated products against mosquitoes in both semi field and field trials

7.3 RECOMMENDATIONS

1. These plants should further be evaluated for possible development of efficacious, environment friendly larvicides that are not harmful to the users.
2. People should be advised against chronic ingestion of *Azadirachta indica* plant parts.
3. Further research is needed to evaluate the genotoxicity of these plants.
4. Further tests are needed to establish long term use of these plants especially with regard to regular contact with the skin or the eyes.
5. The formulated products should be evaluated for commercial viability.
6. Studies should be carried out concerning conservation of these plants and all the others that have been reported to have activity against different mosquito stages.

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APPENDICES

APPENDIX 1: DATA COLLECTION TOOL FOR MITOTIC INHIBITION AND INDEX

Study title: Development of a biopesticide for use against mosquitoes from selected plants in Msambweni sub county, Kenya

Study ID**: BioDev** **Date** --------------------------

Investigator: J.K Musau

Instructions

1. Sixteen (16) *Allium cepa* bulbs (2.5–2.8 cm diameter) per concentration per test sample, grown in tap water at room temperature for 3 days will be used for this test.
2. The bulbs' roots will be measured daily till they reach 2–4 cm in length. Bulbs will be removed from the tap water and treated with different concentrations of the test samples of the plant extracts in the ranges of 125, 250, 500, 1,000 µg/mL.
3. Positive control will be Ethylmethane sulfonate in concentrations of 125, 250, 500, 1,000 µg/mL and negative control, *A. cepa* will be grown in water
4. Solutions will be changed daily for 48 hours and roots assessed for length, turgescence, form and colour change.
5. About 1–2 mm of the root tips will be cut and placed on slides and a drop of aceto-orcein placed on the root tip and left for 2 min. They will be mashed with metal rod and another drop of aceto-orcein added then the slides covered with cover slips and sealed with clear fingernail polish.
6. The slides will be observed under light microscope at 400x and 630x magnification and photomicrographs be made then analyzed for mitotic index, early anaphases, chromosomal bridges/fragments, stickiness and c-mitosis.

Mitotic index will be calculated as = Number of cells in mitosis/Total number of cells

CHART FOR SCORING ROOT LENGTH, MITOTIC INDEX, MITOTIC INHIBITION AND CHROMOSOMAL ABERRATIONS

Extract/ Conc mg/ml	**Total cells**	**No. of Dividing cells**	**%mitotic index = [No. Dividing Cells/ Total cells]*100**	**MI= MI in [negative control-MI in treated control/MI negative control]*100**	**Roots**				**Chromosomal aberrations**				
					L	C	T	F	A	B	FR	S	CM

Key: **L**- length, **C**- colour, **T**- turgescence, **F**- form, **A**- early anaphase, **B**- bridges, **FR**- fragments, **S**- stickiness, **CM**-c- mitosis

APPENDIX 2: DATA COLLECTION TOOL FOR EVALUATING SKIN REACTIONS ON TEST RABBITS

Study title: Development of a biopesticide for use against mosquitoes from selected plants in Msambweni sub county, Kenya

Study ID**: BioDev** **Date** --------------------------

Investigator: J.K Musau

Instructions

1. Three New Zealand white rabbits per plant extract will be used (2.5- 3kg, 18-20 weeks old), housed individually in relative humidity of 50-60% and lighting simulating day and night with conventional laboratory diet and unrestricted access to water

2. A day before the test, fur will be removed by clipping the left and right dorsal areas of the trunk of the animals.

3. Plant extracts made into concentrations of 0.1mg/ml, 1mg/ml and 10mg/ml will be used.

4. Half a milliliter (0.5ml) (or 0.5gm) if a solid of each concentration of each test extract will be applied to about 6 cm^2 of skin of the left dorsal area and covered with a gauze patch held in place with non-irritating tape

5. The control will be the same area of the shaved skin on the right side and 0.5ml of distilled water (or 0.5gm if a solid) will be applied

6. After 4 hours, the plant extracts will be removed by gently swabbing with cotton wool soaked in distilled water and animals examined for signs of erythema and oedema

7. Responses scored at 60 minutes and then at 24, 48 and 72 hours after patch removal

8. The dermal reactions will be graded and recorded according to set guidelines (OECD, 2002).

Chart for evaluating skin reactions on rabbits

Plant extract ……………………… conc…….mg/ml

SKIN REACTIONS						
Erythema and Eschar Formation	**Grade**	**<u>Score</u>** **Hours**				
		1	**24**	**48**	**72**
No erythema	0				
Very slight erythema (barely perceptible)..	1				
Well-defined erythema..	2				
Moderate to severe erythema...	3				

Severe erythema (beef redness) to eschar formation preventing grading of erythema... 4				
Oedema Formation				
No oedema 0				
Very slight oedema (barely perceptible)..1				
Slight oedema (edges of area well-defined by definite raising)........................... 2				
Moderate oedema (raised approximately 1 millimetre) 3				
Severe oedema (more than 1 mm and extending beyond area of exposure)… ...4				

APPENDIX 3: DATA COLLECTION TOOL FOR EYE IRRITATION TESTING

Study title: Development of a biopesticide for use against mosquitoes from selected plants in Msambweni sub county, Kenya

Study ID**: BioDev** **Date** --------------------------

Investigator: J.K Musau

Instructions

This test will run concurrently with the above test on acute dermal irritation

1. A tenth of a milliliter (0.1 ml) of test substance using a micro syringe onto the cornea and conjunctival sac of one eye of a rabbit

2. The conjuctival sac is held closed for one minute

3. The other eye serves is the control

4. Test rabbits will be observed at intervals for up to 21 days for signs of irritation including redness, swelling, cloudiness, edema, hemorrhage, discharge and blindness.

5. In severe eye irritation or pain is observed, it is recommended that the animals are euthanized or removed from the study prior to the 21day time point.

Chart for scoring eye irritation

Cornea **Grade**	**score**
Opacity: degree of density (readings should be taken from most dense area) No ulceration or opacity 0	
Scattered or diffuse areas of opacity (other than slight dulling of normal lustre); details of iris clearly visible 1	
Easily discernible translucent area; details of iris slightly obscured 2	
Nacrous area; no details of iris visible; size of pupil barely discernible 3	
Opaque cornea; iris not discernible through the opacity 4	
Iris	
Normal.......... 0	
Markedly deepened rugae, congestion, swelling, moderate circumcorneal hyperaemia; or injection; iris reactive to light (a sluggish reaction is considered to be an effect..........1	
Conjunctivae	
Redness (refers to palpebral and bulbar conjunctivae; excluding cornea and iris)	
Normal.......... 0	
Some blood vessels hyperaemic (injected) 1	
Diffuse, crimson colour; individual vessels not easily discernible.......... 2	
Diffuse beefy red.......... 3	
Chemosis	
Swelling (refers to lids and/or nictating membranes):Normal..........0	
Some swelling above normal.......... 1	
Obvious swelling, with partial eversion of lids 2	
Swelling, with lids about half closed.......... 3	
Swelling, with lids more than half closed.......... 4	

APPENDIX 4: DATA COLLECTION TOOL FOR LARVICIDAL TESTING

Study title: Development of a biopesticide for use against mosquitoes from selected plants in Msambweni sub county, Kenya

Study ID**: BioDev** **Date** ---------------------------

Investigator: J.K Musau

Instructions

1. Larvicidal tests will be carried out on late 3rd and newly emerged 4th instar larvae reared under standard conditions
2. Dilutions of the extracts of 1, 0.5, 0.25 and 0.125% in analytical grade acetone will be made to stock solutions
3. Twenty five larvae will be exposed to test concentration in 250 ml of water
4. Larvae will be considered dead if they are immobile, unable to reach the water surface and lack head to tail flexion in response to tapping the beaker with a probe

Mortality will be recorded after 24 and 48 hours

Chart for larvicidal activity testing

Plant extract &conc mg/ml

24 hr						48 hr			
Conc (mg/ml)	No. of larvae	**Un-affected**	**dead**	**Immobile**	**Recovered**	**Un-affected**	**dead**	**immobile**	**Recovered**

APPENDIX 5: MOSQUITO REPELLENCY TESTS DATA COLLECTION TOOLS

Study title: Development of a biopesticide for use against mosquitoes from selected plants in Msambweni sub county, Kenya

Study ID: BioDev **Date** --------------------------

Investigator: J.K Musau

Instructions for cage tests

1. Cage tests will be performed in 40 x 40 x 40 cm cages made of aluminium sheet at the bottom, Pyrex window screen on sides and top, and a cotton stockinet sleeve for access on the front
2. Only female mosquitoes bite humans to suck blood necessary for egg development
3. Active female host-seeking *A. aegypti* mosquitoes aged 5-7 days will be collected from stock population using an aspirator and starved for the preceding 12 hours.
4. Fifty test mosquitoes will be used in each of five replicates
5. One hundred (100) milligram of each sample will be dissolved in a 100 ml mixture of analytical grade acetone (99.95%) and liquid paraffin (1:1).
6. Serial ten-fold dilutions will be made using acetone/liquid paraffin mixture (1:1) to obtain the other concentrations in the range of 0.01-10% w/v.
7. Acetone/liquid paraffin mixture (1:1) will be negative control while 20% DEET (*N, N*-diethyl-m-toluamide), the positive control.
8. Human participants will have avoided use of fragrance, any mosquito repellent, perfumed soap or tobacco for 12 hours prior and during the experiment (WHO, 2009; Innocent *et al*., 2010).

9. The forearm, from the elbow to the wrist (~696.6 cm^2) will be washed with water and left to dry then 1 ml of test sample applied as evenly as possible.
10. The rest of the hand from the wrist to the fingers will be covered with latex glove to prevent the mosquitoes from biting (WHO, 2009).
11. One (1) ml of acetone/liquid paraffin mixture will be applied on the other forearm that has been prepared as above, and serve as negative control.
12. The initial test to determine the readiness of the mosquitoes to land or bite will involve the use of the negative control forearm which will first insert into the cage and the number of mosquitoes that land on or commence to probe the skin in 30 seconds counted. The negative control forearm will be carefully withdrawn and the positive control arm with 20% DEET (*N, N*-diethyl-m-toluamide) inserted in the cage for 30 seconds and the number of mosquitoes that land or probe counted.
13. The test will proceed when there are ≥10 landings and/or probings in the 30 s period in the negative control forearm.
14. The human participants forearm will be prepared as above and 1 ml of the lowest concentration of the test sample applied and inserted into the cage for 3 minutes.
15. The number of mosquitoes that land on or probe during this period are counted and shaken off before they can imbibe any blood
16. The human participants' arms will be exposed to progressively higher concentrations of the sample, each time to fresh mosquitoes in a clean cage.
17. Percent protection efficacy (PE) will be calculated using the formula:

    PE = (C-T)/C x 100

    Where C and T are the mean numbers of mosquitoes that landed on the control and test arm respectively

Chart for scoring repellency

Plant extract…………….. **Conc……………mg/ml**	**Total No. of mosquitoes**	**probings**	**landings**	**bites**

Instruction for Susceptibility bioassays

Study title: Development of a biopesticide for use against mosquitoes from selected plants in Msambweni sub county, Kenya

Study ID**: BioDev** **Date** --------------------------

Investigator: J.K Musau

1. Tests will be done in four replicates with positive and negative controls
2. Filter papers will be treated with different plant extracts at 75 mg/m^2, 100 mg/ m^2, 200 mg/m^2, 250 mg/m^2 and 500 mg/m^2.
3. The filter papers will be air dried and each inserted into a WHO susceptibility testing tube for adult mosquitoes.
4. Twenty five active *A. aegyptiae* mosquitoes aged 2-5 days that have not been blood-fed will be selected using an aspirator from the stock populations of adult mosquitoes and used for this test. They will be placed in each tube with filter papers with different concentrations of the plant extracts for 1 hour.
5. Untreated filter papers will be negative control while citronella oil at concentration of 500 mg/m^2 will be positive control
6. After one hour the mosquitoes from each tube will be transferred to different holding chambers, depending on the concentration, where mortality and recovery within 24hours will be scored.
7. Time taken to knock down 90% of the population (KD90) and 95% confidence interval will be calculated per treatment.

Chart for scoring knockdown effects

Plant extract…………….. Conc……………mg/ml	Total No. of mosquitoes	Knockdown effects							
		One hour				Twenty four (24) hrs			
		C	I	D	R	C	I	D	R

Key: C- confused, I- immobile, D – dead, R- recovered

APPENDIX 6: PARTICIPANT INFORMATION AND CONSENT FORM

ADULT CONSENT FOR ENROLMENT IN THE STUDY

This participant and consent form will be administered in English, or by translation to any other appropriate language that the participant best and fully understands.

TITLE OF STUDY: Development of a biopesticide for use against mosquitoes from selected plants in Msambweni sub county, Kenya

Study ID: BioDev Date ---------------------------

Principal investigator: Joseph Kisivo Musau

Institution: University of Nairobi, Department of Public Health, Pharmacology and Toxicology

Co-investigators:

1. Prof. James M. Mbaria

   Institution: University of Nairobi, Department of Public Health, Pharmacology and Toxicology

2. Dr Joseph M. Nguta

   Institution: University of Nairobi, Department of Public Health, Pharmacology and Toxicology

3. Prof. Mbaabu Mathiu

   Institution: University of Nairobi, Department Veterinary Anatomy and Physiology

4. Prof Stephen G. Kiama

   Institution: University of Nairobi, Department Veterinary Anatomy and Physiology

INTRODUCTION

I would like to tell you about a study being conducted by the above listed researchers. The purpose of this consent form is to give you the information you will need to help you decide whether or not to be a participant in the study. Feel free to ask any questions about the purpose of the research, what happens if you participate in the study, the possible risks and benefits, your rights as a volunteer and anything else about the research or this form that is not clear.

When we have answered all your questions to your satisfaction, you may decide to be in the study or not. This process is called 'informed consent'. We will give you a copy of this form for your records.

May I continue? YES/ NO

WHAT IS THIS STUDY ABOUT?

The researchers listed above are interviewing individuals who shall take part in laboratory based mosquito repellency studies. The purpose of this study is to find out whether selected plants used in mosquito control in Kenya's south coast repel mosquitoes. This will be assessed through 'arm in cage' testing protocol as set out by WHO. In general terms, this involves applying a test substance on the arm of a participant then inserting it in a mosquito cage. The arm is withdrawn before mosquitoes can imbibe any blood. The numbers of mosquitoes that probe, land or bite are counted. Participants in this study will be asked about their sensitivity and allergy to mosquito bites. Those who are allergic to mosquito bites will not be allowed to take part in this study.

The plant extracts will have been tested for irritability on the skin and genotoxicity potential using established procedures. Those plants' extracts that cause irritation or possess aspects of genotoxicity will not be used for this study. During the days of the repellency testing, the participants will avoid use of fragrance and tobacco. Even though the mosquitoes are laboratory bred over generations and therefore pose no risk of transmission of disease, participants will be put mefloquine prophylaxis to prevent malaria.

Thus those who are allergic to mefloquine will not be allowed to take part in the study. There will be twelve (12) participants in the study randomly chosen. We are asking for your consent to consider participating in this study.

WHAT WILL HAPPEN IF YOU DECIDE TO TAKE PART IN THIS RESEARCH STUDY?

If you decide to take part in this study, you will be interviewed by a trained interviewer in privacy for you comfort. The interview will last about fifteen minutes. The interview will cover topics such as:

1. Sensitivity and allergy to mosquito bites: Those who are allergic to mosquito bites will not be allowed to take part in this study
2. Use of fragrance and tobacco: The participants will avoid use of fragrance and tobacco during the days of testing.
3. Allergy to antimalarial drugs such as mefloquine or related compounds (quinine): Even though the mosquitoes are laboratory bred over years and several

generations and therefore pose no risk of transmission of disease, participants will be put mefloquine prophylaxis to prevent malaria. Thus those who are allergic to mefloquine and quinine will not be allowed to take part in the study.

4. History of depression, generalized anxiety disorder, psychosis, schizophrenia, and other major psychiatric disorders: mefloquine is contraindicated in these conditions

5. History of seizures: mefloquine is contraindicated

6. Cardiac diseases abnormalities will not be included in the study since mefloquine is contraindicated in these individuals: mefloquine is contraindicated.

7. Pregnancy and breastfeeding: safety of mefloquine in these groups is not guaranteed.

ARE THERE ANY RISKS DISCOMFORTS ASSOCIATED WITH THIS STUDY?

The major risk is loss of privacy. However, we will keep anything you tell us as confidential as we can. We will use a code number to protect your identity in a password protected computer data base and all our paper records in a locked cabinet. However no system is completely secure so someone somehow could find out you were in this study and hence find out information about you.

Even though at the outset you may not be allergic to mosquito bites, some degree of allergy may develop. In this case, you will be put on a course of antihistamines and given an opportunity to opt out of the study. In case you develop side effects related to mefloquine, you will be managed by trained clinicians and be withdrawn from the study.

ARE THERE ANY BENEFITS RELATED TO THIS STUDY?

You will benefit by receiving free medical checkup in a hospital to determine your suitability for this study. This will include but not limited to assessment of heart function, psychiatric tests, pregnancy tests (for the females) and neurologic test. The outcomes of this study will help in understanding use of plants for mosquito control and aid in development of a plant based mosquito repellent that is safe, cost effective, acceptable and efficacious to use.

WILL BEING IN THIS STUDY COST YOU ANYTHING?

In monetary terms, no, but you will need to create time for the tests

WILL YOU GET REFUND FOR ANY MONEY SPENT AS PART OF THIS STUDY?

Yes. You will be reimbursed money for transport to the venue of the tests and given allowance for tea and lunch.

WHAT IF YOU HAVE ANY QUESTIONS FOR THE FUTURE?

Any questions or concerns will be handled by the study team. The mobile number of the principal investigator and a functioning E-mail will be at the bottom of this page.

For more information about your rights as a research participant, contact: **Prof A.N Guantai,** The chairperson, Kenyatta National Hospital/University of Nairobi Research and Ethics Committee, telephone number 2726300 ext 44355/44102. The study will pay for your charges to these numbers if the call is for study related communication.

WHAT ARE YOUR OTHER CHOICES?

Your decision to participate in this research is voluntary. You are free to decline participation in the study and you can withdraw from the study at any time without injustice or loss of benefits

Informed consent agreement

I ----------------------------------aged--------a) male------ b) female ----- have read the foregoing information (or it has been read to me). I have had the opportunity to ask questions about it, and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to participate as a participant in this research and understand that I have the right to withdraw from the research at any time.

I hereby agree to participate in this study with my full consent and declare to the best of my knowledge, and to what I have been explained about the repellency studies by the researcher regarding this study that I shall in no way be harmed. That it is voluntary with no coercion, inducement or false pretence.

Signature/ thumb print------------------------------- Date-----------------------------

Witness if the participant is illiterate

I have accurately read or witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Print name of witness: ______________________

Signature of researcher: ______________________

Date: ___________________________

A copy of this Informed Consent Form has been provided to participant _____ (initialed by the researcher/assistant).

RESEARCHER'S STATEMENT

I, the undersigned have fully explained the relevant details of this research study to the participant named above and believe that the participant has understood and has freely given his/her consent.

Researcher's name: ___________________________ **Date** _______________

Signature ____________________

Role in the study: principal investigator [ ] co investigator [ ] *tick **one** as appropriate*

For more information, contact Joseph Kisivo Musau at (+254) 0723103154 E-mail: jaymus2007@yahoo.com/ jmus2007@gmail.com. This can be done at any time of the day.

WITNESS TO THE ABOVE

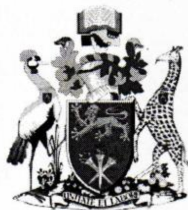
Name: ____ **Contact**_____________________________

Signature/ thumb stamp ___________________________ **Date** _______________________________

APPENDIX 7: PUBLICATIONS ASSOCIATED WITH THIS THESIS

1. **Musau J. K., Mbaria J. M., Nguta J. M., Mbaabu M.** and **Kiama S. G.** Evaluation of genotoxicity potential of plants traditionally used for mosquito control in Kenya's South coast. Merit Research Journal of Medicine and Medical Sciences (ISSN: 2354-323X) 2016, 4(4): 178-182
2. **Musau, J. K., Mbaria, J. M., Nguta, J.M., Mbaabu, M., Kiama S.G.** Mosquito repellency and knockdown effect of a plant based formulation. IOSR Journal of Pharmacy 6(5): 09-14
3. **Joseph K. Musau, James M. Mbaria, Joseph M. Nguta, Mbaabu Mathiu** and **Stephen G. Kiama.** Phytochemical composition and larvicidal properties of plants used for mosquito control in Kwale County, Kenya. International Journal of Mosquito Research, 2016, 3(3):12-17

APPENDIX 8: APPROVALS FOR THIS STUDY



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Twitter: @UONKNH_ERC https://twitter.com/UONKNH_ERC

KENYATTA NATIONAL HOSPITAL
P O BOX 20723 Code 00202
Tel: 726300-9
Fax: 725272
Telegrams: MEDSUP, Nairobi

Ref: KNH-ERC/A/413

15th October 2015

Joseph Kisivo Musau
Reg. No.J87/83561/2012
Dept. of Public Health, Pharmacology and Toxicology
University of Nairobi

Dear Joseph

RESEARCH PROPOSAL: DEVELOPMENT OF A BIOPESTICIDE FOR USE AGAINST MOSQUITOES FROM SELECTED PLANTS IN MSAMBWENI DISTRICT, KENYA (P357/05/2015)

This is to inform you that the KNH/UoN-Ethics & Research Committee (KNH/UoN-ERC) has reviewed and **approved** your above proposal. The approval periods are 15th October 2015 – 14th October 2016.

This approval is subject to compliance with the following requirements:

a) Only approved documents (informed consents, study instruments, advertising materials etc) will be used.
b) All changes (amendments, deviations, violations etc) are submitted for review and approval by KNH/UoN ERC before implementation.
c) Death and life threatening problems and serious adverse events (SAEs) or unexpected adverse events whether related or unrelated to the study must be reported to the KNH/UoN ERC within 72 hours of notification.
d) Any changes, anticipated or otherwise that may increase the risks or affect safety or welfare of study participants and others or affect the integrity of the research must be reported to KNH/UoN ERC within 72 hours.
e) Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. (*Attach a comprehensive progress report to support the renewal*).
f) Clearance for export of biological specimens must be obtained from KNH/UoN-Ethics & Research Committee for each batch of shipment.
g) Submission of an *executive summary* report within 90 days upon completion of the study.
This information will form part of the data base that will be consulted in future when processing related research studies so as to minimize chances of study duplication and/or plagiarism.

For more details consult the KNH/UoN ERC website **http://www.erc.uonbi.ac.ke**

"Protect to Discover"

Yours sincerely,

PROF. M.L . CHINDIA
SECRETARY, KNH/UON-ERC

c.c. The Principal, College of Health Sciences, UoN
The Deputy Director CS, KNH
The Chairperson, KNH/UoN-ERC
The Assistant Director, Health Information Dept. KNH
Supervisors: Prof. James M. Mbaria, Dr. Joseph M. Nguta, Dr.Mbaabu Mathiu, Prof. Stephen G. Kiama

"Protect to Discover"

UNIVERSITY OF NAIROBI
FACULTY OF VETERINARY MEDICINE
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Tel: 4449004/4442014/ 6
Ext. 2300
Direct Line. 4448648

Mr Joseph Kisivo Musau
Dept of Public Health, Pharmacology & Toxicology

2/05/2013

Dear Mr Musau,

RE: Approval of proposal by FVM Biosafety, Animal use and Ethics committee
Development of a Biopesticide for use against mosquitoes from selected plants in Msambweni district, Kenya
By Joseph Kisivo Musau (our ref:1330413)

We have reviewed your PhD proposal, particularly section 3.4.2 that involves use of rabbits for dermal tests. We are satisfied that the proposed treatment and care of the animals meets acceptable standards for animal welfare. Furthermore, the numbers proposed are reasonable

We hereby give approval for you to proceed with the experiments as outlined in part 3.4.2 of the proposal you submitted to the committee.

Yours sincerely

Rodi O. Ojoo BVM M.Sc Ph.D
Chairman,
Biosafety, Animal Use and Ethics Committee
Faculty of Veterinary Medicine.

cc. Prof Mbaria - PHPT

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