ANTI-MOSQUITO PROPERTIES OF METHANOL EXTRACT OF *Clerodendrum polycephalum* Baker

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A Thesis in the Department of Zoology, Submitted to the Faculty of Science,

in partial fulfillment of the requirements for the Degree of

DOCTOR OF PHILOSOPHY

of the

UNIVERSITY OF IBADAN

FEBRUARY 2018

ABSTRACT

Mosquitoes are vectors of various diseases of public health importance and their control has been premised on the use of synthetic insecticides. However, these insecticides are laden with problems of high cost, environmental hazards and development of resistance in many species of mosquitoes. Alternatives to synthetic substances are the natural products. Plant species, *Clerodendrum inerme,* have been found to inhibit the growth of *Aedes aegypti* and *Culex quinquefasciatus* larvae. However, there is paucity of information on the anti-mosquito properties of the Nigerian species, *C. polycephalum* Baker. Therefore, the aim of the study was to investigate the anti-mosquito properties of the methanol extract of the leaves of *C. polycephalum*.

Fresh leaves (1.4 kg) of *C. polycephalum* were harvested and brought to the laboratory. The leaves were air-dried, powdered, extracted with methanol and concentrated to dryness using rotary evaporator. The crude methanol extract was subjected to phytochemical analysis according to standard method and separated into n-hexane, dichloromethane, ethyl acetate and ethanol fractions using vacuum liquid chromatography. The most active fraction (ethanol), was subjected to column chromatography. Gas Chromatography - Mass Spectroscopy (GC-MS) was used to analyse the subfraction which was ultra-violet active. The crude methanol extract and fractions (250–8000 ppm) were tested for larvicidal activity. The methanol extract was further tested for anti-oviposition, effects on growth and development on *Aedes aegypti*, *Anopheles gambiae* and *Culex quinquefasciatus* mosquitoes together with corresponding positive (lambda-cyhalothrin) and negative controls (dimethylsulfoxide) using World Health Organisation procedures. Toxic effects, determined by mortality, on representative non-target organisms was

evaluated on adult *Biomphalaria glabrata* (snail) and tadpoles of *Bufo regularis*. Ten each of the organisms were used per concentration. Data were analysed using descriptive statistics and student's t-test at $\alpha_{0.05}$.

Tannins, flavonoids, saponins and alkaloids were identified from the crude extract. Seventeen compounds were detected from ethanol subfraction, with quantities varying from 0.0 to 23.7%. Prominent compounds include 4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol (7.5%), 2-Hydroxy-5-methylbenzaldehyde (6.1%) and Ethyl iso-allocholate (23.7%). Larvicidal activities of crude methanol extract and fractions were in the order ethanol (100.0%) > methanol (96.6%) > n-hexane (7.5%) > DCM/ethyl-acetate (0.0%) at 2000ppm after 24h. Susceptibilities of mosquito larvae to methanol extract were also in the order *A. aegypti* > *A. gambiae* > *C. quinquefasciatus.* Mortality of the larvae was significantly different from the positive control. Anti-oviposition index increased (46.4-89.9) with extract concentrations. Morphological deformities of larvae and pupae were observed at the higher doses of 2000 ppm (*A. aegypti*), 5000 ppm (*A. gambiae*) and 7000 ppm (*C. quinquefasciatus*). Mosquito adult emergence inhibitions (24.0-100.0%) were recorded at 1100–5600 ppm concentration levels. The crude extract had no effect on the tadpoles at 250–1000 ppm, but the mortality of the snail at 250–2000ppm, varied from 10.0–95.0%.

Clerodendrum polycephalum leaves contained biological components capable of inhibiting the survival and development of mosquitoes and could be exploited in the control of mosquitoes.

Keywords: *Clerodendrum polycephalum* leaf extract, larvicidal effect, anti-oviposition, malaria control

Word count: 472

ACKNOWLEDGEMENT

"To God be the glory, great things He has done" says a popular Christian song. I am indeed grateful to God Almighty for making the vision of a Doctoral degree a possibility. The journey to this end has not been very smooth as one expected. In spite of all odds, I thank God I am alive today. I cannot thank God enough.

Many men and women contributed immensely to ensure that I got this task done. First, I want to thank my supervisor, Prof. A. B. Odaibo for his patience with me throughout the long journey. You made yourself available all the time to counsel and to direct the course of things. I promise not to disappoint you hereafter. Next to him was our Director in Drug Research and Production Unit, Obafemi Awolowo University, Ile-Ife, Prof. C. O. Adewunmi (though retired now) whose fatherly counsel all the time encouraged my heart. Your achievement in the academics is enough to motivate anyone around you at any time. I thank God, I met you.

I am indebted to the non-academic staff of the Department of Zoology, University of Ibadan. The individuals in charge of power-point set up during my seminar presentations, I am grateful. I thank past and present secretaries to the HOD for handling my documents appropriately anytime I approached them. I have passed through many postgraduate coordinators in the department during the programme. I am grateful to all of them, particularly, the one who completed the job, Dr.(Mrs.) Adedolapo Ayoade, for processing my abstract. I thank all lecturers in the department for their helpful contributions during my final seminar presentation. I want to specially thank the current HOD, Prof. Bakare, for organizing my defense and for his beautiful and meaningful contributions to make the write-up better.

My gratitude also goes to my friend and co-worker in the Unit, Dr (Mrs.) C. A. Elusiyan, a.k.a., Elubimbo, who worked tirelessly with me to make sure that the compounds were isolated

from my extract. I am also indebted to Prof. J. M. Agbedahunsi whose suggestions and methods of isolation eventually solved the problem.

I thank Dr (Mrs.) Dupe Ogunrombi and Dr. Segun Adeloye who took my sample to South Africa for analysis. Many of my colleagues at the Faculty of Pharmacy, O.A.U. were constantly on my neck. I thank you all for putting me on my toes all the time. Prominent in this regard are Prof. G. Alebiowu, Dr (Mrs.) A. Adepiti, Dr. M. Odediran, Dr.(Mrs.) C. M. Cyril-Olutayo a.k.a. M. O. J., Dr. B. J. Taiwo, Prof. I. O. Iwalewa, Dr. Gbolahan Subair, Dr. F. A. Fisusi among others. I thank my fellow Christian Brother, Dr. F. O. Olorunmola for his understanding and constant monitoring of progress made.

I appreciate every non-academic member of staff of Drug Research and Production Unit. Coming first on this list is Mr. Yakubu Akanni (now Late), the Herbalist, who helped in the collection of *Clerodendrum polycephalum*. I thank Dr. A. T. Oladele for helping to identify indigenous plants which were screened at the beginning of the research. I thank Mr. S. K. Omodara, Mr. A. Akinkunmi, and Samson for all the technical supports they rendered to me during the course of my research. My gratitude also go to Mrs. D. Ayemonisan, Mrs. E. Oderinu and Miss Dupe Grace Ogundiran for showing concern for my well-being. I will not forget Seun Agbaje, the former Youth Corper who also encouraged me to finish on time.

I thank all the members of Foursquare organisation for their prayers for good success and upliftment for me and my family. I thank my senior Pastor, Rev. (Prof.) Adebimpe Aderounmu for praying for my promotion. I appreciate the efforts of my District Overseer, Rev. Razak Akinwale, who monitored the events leading to the oral defense with fervent prayers. The entire members of Foursquare Gospel Church Ondo road are all appreciated for their prayers for me and for rallying round me to carry my burden.

My gratitude also goes to all my in-laws and members of the Adewoyin family who were concerned and also encouraged me.

Finally, I thank my darling wife, Elunike Joan Nkem Adewoyin for standing by me in all situations. You were there always to point important things out for me. You also made sure that I worked with sufficient strength by cooking delicious meal for me daily. Your words of encouragement to me when I was down helped to cross to the other side of my Jordan River. You are indeed the bone of my bone and the flesh of my flesh. If I happen to come back to this planet again I will choose you as my wife. To the boys, Ayanfeoluwa and Inioluwa, I say thank you for making the task easier for me in your own way.

CERTIFICATION

I certify that this work was carried out by Mr. F. B. Adewoyin in the Department of Zoology, University of Ibadan.

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TABLE OF CONTENT

LIST OF FIGURES

LIST OF PLATES

LIST OF TABLES

GLOSSARY/ABBRIEVIATIONS

TLC: Thin Layer Chromatography. It is a chromatographic technique used to separate the components of a mixture using a thin stationary phase supported by an inert backing.

GC-MS: Gas Chromatography- Mass Spectroscopy. Gas chromatography–mass spectrometry is an analytical method that combines the features of gas-chromatography and mass spectrometry to identify different substances within a test sample. Gas chromatography mass spectrometry is an instrumental technique, comprising a gas chromatograph (GC) coupled to a mass spectrometer (MS), by which complex mixtures of chemicals may be separated, identified and quantified.

Chromatography: Chromatography is an analytical technique commonly used for separating a mixture of chemical substances into its individual components, so that the individual components can be thoroughly analyzed.

Phytochemicals: Phytochemicals are chemical substances which contribute to the efficacy of medicinal plants, e. g. alkaloids, proteins etc

R_f: The Rf value is defined as the ratio of the distance moved by the solute (i.e. the dye or pigment under test) and the distance moved by the solvent (known as the Solvent front) along the paper, where both distances are measured from the common Origin or Application Baseline, that is the point where the sample is initially spotted on the paper.

Silical Gel: Silica gel is a granular, vitreous, porous form of silicon dioxide made synthetically from sodium silicate. Silica gel contains a nano-porous silica micro-structure, suspended inside a liquid. Most applications of silica gel require it to be dried, in which case it is called silica xerogel. They are usually in form of granules, and are used in the separation of mixture.

R_t : The time taken for a particular compound to travel through the column to the detector is known as its retention time. This time is measured from the time at which the sample is injected to the point at which the display shows a maximum peak height for that compound.

Vector: Vectors are organisms that transmit pathogens and parasites from one infected person (or animal) to another, causing serious diseases in human populations.

Epidemiology: The study of distribution of disease in human populations, against the background of their total environment

Ecology: This is a branch of science concerned with the interrelationship of organisms and their environments.

IGR: Insect growth regulators are substances which hinder the growth and development of an organism

PPM: A shortened form of parts per million

Ethno-botany: This is the scientific study of the traditional knowledge and customs of a people concerning plants and their medical, religious, and other uses.

Morphology: Morphology is a branch of biology dealing with the study of the form and structure of organisms and their specific structural features.

CHAPTER ONE

INTRODUCTION

Mosquitoes are the most important group of insects in terms of public health importance because they transmit a number of diseases such as malaria, filariasis, dengue, Japanese encephalitis, yellow fever, Zika virus, chikungunya etc. causing millions of deaths every year (Foster and Walker, 2002, Ganesan *et al.,* 2017, Murrell, 2017). A recent study suggested that mosquitoes can infect people with both Zika and chikungunya viruses in a single bite (Goertz *et al.,* 2017).

One of the approaches for the control of these diseases is the interruption of diseases transmission, by killing or preventing mosquitoes from biting human beings or by causing larval mortality on a large scale at the breeding sites of the vectors. Chemical control is an effective strategy used extensively and there are many kinds of compounds toxic to mosquitoes, including organophosphorus, organochlorine, carbamates, and pyrethroids (WHO, 2006). They are both effective and residual. Since the discovery of DDT, mosquito control approach has been completely based on synthetic organic insecticides (Chen, 1990). The continued use of synthetic chemical insecticide-based intervention has resulted in disruption of natural biological control system and led to resurgence in mosquito population (Ansari *et al.,* 2000). It has also led to the development of resistance, undesirable effects on non – target organisms and fostered environmental and human health concern.

The use of environmental-friendly and biodegradable natural insecticides of plant origin has received importance for diseases vector control over a decade ago (Singh *et al.,* 2007). Herbal products with proven potentials as insecticides or repellents can play an important role in the interruption of the transmission of mosquito – borne diseases at the individual and community levels. Anabasine from

Anabasis aphylia, rotenone from *Derris eliptical* and pyrethrums from *Chrysanthemum cinererifolium* flowers have been used as natural insecticides even before the discovery of synthetic organic insecticides (Duke, 1990). Other plants have been tested and shown to be effective against a wide range of insects, including mosquitoes. These are the seed and leaves of the neem treem, *Azadirachta indica* (Schumutterer, 1990), *Rhazya stricta* (Elhag *et al.*, 1996) and cloves, *Syzygium aromaticum* (Caledrone *et al.*, 1991). On a general note, the most promising botanical groups that insecticidal in nature are Meliaceae, Rutaceae, Asteraceae, Annonaceae, Labiatae, Aristolochiaceae and Malvaceae (Regnault-Riger, 1997).

Plant products can be obtained either from the whole plant or from a specific part by extraction in different types of solvents such as aqueous, methanol, chloroform, n-hexane and so on, depending on the polarity of the phytochemicals. Studies carried out so far have shown that some phytochemicals act as general toxicant (insecticides/larvicides) both against adult as well as larval stages of mosquitoes, while others interfere with growth and development (growth inhibitors) or with reproduction (chemosterilant) or produce olfactory stimuli thus acting as repellent or attractant.

According to O'Neil (1998), plants suffer from a range of parasites, including protozoans, nematodes and fungi. They show a variety of protective adaptations, such as thick or spiky epidermal layers or bitter chemicals to discourage herbivores, but some plants produce chemicals (phytoalexins) specifically in response to attack by microorganisms and parasites. The term phytoalexin is derived from Greek and means 'warding off in plants'. The phytoalexin concept was originally proposed to account for the active response of certain plants to infection, and their acquisition of resistance after exposure to an infecting organism.

The first phytoalexins isolated were both pterocarpan structures: phaseolin from beans (*Phaseolus vulgaris*) and psatin from peas (*Pisurn sativum* Linn.) following inoculation of each plant with fungal spores of *Monilinia fructiocola*. Subsequently, several hundred compounds from plants have been identified as phytoalexins. The phytoalexins response can be elicited by variety of agents including viruses, bacteria and nematodes. These phytoalexins possess vital biological activities.

1.1 Aim and Objectives

The main aim of this research is to develop capacity for the control of mosquito using the extract of *Clerodendrum polycephalum*.

Specific Objectives include:

i. To gather information on locally available medicinal insecticidal plants which can be used against mosquitoes
ii. To determine the larvicidal and pupacidal activities of the crude extract of *C. polycephalum* against three species of mosquitoes: *Anopheles gambiae. Aedes aegypti* and *Culex quinquefasciatus.*
iii. To investigate the effects of sub lethal concentrations on the growth and development of the three species of mosquitoes
iv. To evaluate the effects of the crude extract on oviposition preference and egg hatching of *A. aegypti* mosquitoes
v. To carry out activity-directed fractionation of the crude extract of *C. polycephalum* on the larvae of *Aedes aegypti*
vi. To study the effects of the extract of *C. polycephalum* on non-target organisms and
vii. To identify the active compounds in the most active fraction.

CHAPTER TWO

LITERATURE REVIEW

2.1 History of Medicinal Plants

The use of plants as medicines dates as far back as the origin of humankind and perhaps even earlier, as animals are known to seek out and consume certain plants when ill. People have relied primarily on plants for nourishment from time immemorial. Through trial and error, they discovered that some plants are good for food, that some are poisonous and that some produce bodily changes such as increased perspiration, bowel movement, urination, relief of pain, hallucination and healing (Akpata, 1979). Over the millennia, these observations were passed orally from generation to generation, with each generation adding to and refining the body of knowledge. Every culture the world over has in this manner developed a body of herbal knowledge as part of its tradition (Kelly, 2009).

The first written record of herbs used as medicines was made over five thousand years ago by the Sumerians in ancient Mesopotamia (present day Iraq). The roots of Chinese medicine, which is based largely on herbalism, also date back approximately 5,000 years. The roots of Indian medicine were set forth in the sacred writings called the Vedas, which date back as far as the 2nd century BC. The Indian system of medicine was called the Ayurveda. Ayurveda is still practiced today, and many authentic, traditional formulations are available outside India. The Greek physician Hippocrates (c. 460 - 377 BC), who is often referred to as the "Father of modern medicine" was a herbalist. He is credited with greatly advancing the systematic study of clinical medicine, having written, "Let your foods be your medicines, and your medicines your food" (Leroi-Gourhan, 1975; Waller, 1998).

During the Middle Ages, the knowledge of medicinal plants was further provided by monks in Europe who studied and grew medicinal plants and translated the Arabic works on herbalism. From native Africans came the herb pygeum (*Prunus africana*), which has proven to be beneficial for the prostate gland. From the Australian Aborigines came Ten Tree oil - from the leaves of the melaleuca tree - which was used by British soldiers during World War II as an antiseptic for wounds. In ancient times, herbalism was mixed with magic and superstition. Today, through modern methods, we can determine what superstition is and what fact is. Many traditionally- used herbs have been put to the scientific tests and many have proven to possess remarkable curative powers.

Herbs are staging a comeback and herbal 'renaissance' is happening all over the globe. Herbal products today symbolise safety in contrast to the synthetics that are regarded as unsafe to human and environment (Kumar, *et al.*, 2012). While herbs had been priced for their medicinal, flavouring and aromatic qualities for centuries, the synthetic products of the modern age surpassed their importance, for a while. However, the dependence on synthetics is over and people are returning to the naturals with high hope. Over three-quarters of the world population relies mainly on plants extracts for health care (Joy *et al.*, 1998). More than 30% of the entire plant species, at one time or the other, were used for medicinal purposes.

It has been estimated that in developed countries such as United States, plant drugs constitute as much as 25% of the total drugs, while in fast developing countries such as China and India, the contribution is as much as 80%. Thus, the economic importance of medicinal plants is much more to countries such as India than to the rest of the world. China and India countries provide two third of the plants used in current system of medicine (Lonnelle, 1977).

The use of plant extracts and powdered plant parts as insecticides goes back at least as far as the Roman Empire. For instance, there are reports that in 400 B.C. during Persian King Xerxes' reign, the delousing procedure for children was with a powder obtained from the dry flowers of a plant known as pyrethrum (*Tanacetum cinerariaefolium*, Compositae). There are several sources for pyrethrum. Three of the most important species are Dalmatian insect flowers from *Chrysanthemum cinerariaefolium*, Persian insect flowers from *C. coccineum* and Caucasian insect flowers from *C. marshallii*. The Dalmatian species is favoured. It is a slender, glaucous, pubescent perennial, 18-30 inches in height with pinnate leaves and small daisy-like flowers. It is a native of Dalmatia where it has been cultivated for centuries. Japan used to be the leading producer of pyrethrum flowers and they constituted one of its most valuable exports. Great care was exercised in gathering, drying and packing the crop. Later the species was being cultivated in California and other parts of the United States, Kenya, Italy, Australia, Brazil, Peru and Ecuador (Smith and Secoy, 1976). Today pyrethrum is used in some mosquito coils which are burned as incense.

The first botanical insecticide dates back to the Seventeen Century when it was shown that nicotine, obtained from tobacco leaves, would kill plum beetles. Around 1850, a new plant insecticide known as rotenone was introduced. It is obtained from the roots of plants called timbó. The use of these climbers and creepers of the Leguminosae as fish poisons was noted by De Rochefort in 1665 and Aublet in 1775 (Hill, 1952). Later on plants with irritating properties like incense and sabadilla were used: extracts from the latter plant were used as decongestants. These plants did not kill insects directly but it was said that they "scared them off."

2.2 Medicinal Plants Used in This Study

Plants were selected based on their inclusion among common medicinal plant that are toxic to parasites. Those that are indigenous to us in south west Nigeria were picked. In addition, a Herbalist was also interviewed to collect information on the medicinal plants. The ten plants which were screened for larvicidal activities are described below.

2.2.1 The genus Clerodendrum

The genus *Clerodendrum* Baker (family: Lamiaceae) is very widely distributed in tropical and subtropical regions of the world and is comprised of small trees, shrubs and herbs. Over than five hundred species of the genus have been identified and are widely distributed in Asia, Australia, Africa and America. The genus is taxonomically characterized by its entire or toothed, oppositely arranged leaves, terete stems, terminally or axillary cymose inflorescence, hypogynous bisexual flowers, persistent calyx, cylindrical corolla tube with spreading 5- lobed at the top, exerted stamens, short bifided stigma, imperfectly 4-celled ovary, exalbumenous seeds and endocarp separating into 4 stony pyrenes (Kirtikar and Basu, 1995; Steane, *et al*., 1999).

Ethno-medical importance of various species of the genus *Clerodendrum* has been reported in various indigenous systems of medicines and as folk medicines. Many plants in the genus are being used as medicines specifically in Indian, Chinese, Thailand, Korean, Japanese systems of medicine for the treatment of various life-threatening diseases such as syphilis, typhoid, cancer, jaundice and hypertension. Few species of the genus like *Clerodendrum inerme* (L) Gaertn., *C. thomsoniae* Balf.f. , *C. indicum* (L.) Kuntze, and *C. speciosum* L. are ornamental and are being cultivated for

aesthetic purposes. The powder/paste form and the various extracts of root, stem and leaves are reported to be used as medicine for the treatment of asthma, pyreticosis, cataract, malaria and diseases of blood, skin and lung. To prove these ethno-medical claims, some of these species are being extensively studied for their biological activities using various animals' models (Shrivastva and Patel, 2007). Thus anti-inflammatory, anti-oxidant, antimalarial, anti-microbial properties of the plants have been investigated and reported in literatures. Other major biological activities reported for this genus are antihypertensive, antitumor, antidiabetic, antidiabetic, antihyperlipidemic, larvicidal and antidiarrhoel activities. One Indian species, *C. inerme* inhibited the growth of larvae of *Aedes aegypti*, *Culex quinquefasciatus* and *Cx. pipiens* at 80 and 100 ppm concentration of petroleum ether and ether extracts (Kalyanasundaram and Das, 1985).

Isolation and identification studies of chemical constituents have been studied. This has also been correlated with the biological activities of the genus. The major chemical components reported from the genus are phenolics, steroids, di- and triterpenes, flavonoids, volatile oils, etc. Specifically, they include Hispudilin, 5-0-ethylclerodendricin, Iridiod diglucoside, Colebrin, Clerodermic acid, Bungein, Apigenin. Jionoside D, 2 (R) Prunasin and Clerosterol (Shrivastava and Patel, 2007).

2.2.2 Scientific classification of *Clerodendrum polycephalum*

Kingdom: Plantae

Phylum: Tracheophyta

Class: Magnoliopsida

Order: Lamiales

Family: Lamiaceae

Genus: *Clerodendrum*

Species: *polycephalum*

Clerodendrum polycephalum Baker

2.2.3 *Clerodendrum polycephalum* Baker (Lamiaceae)

The description of the plant has been given by Hepper (1963). It is a climbing or erect shrub up to 15ft high found in savanna and closed jungle, in Guinea to South Nigeria, and in Last Cameroun. The leaves and branches are yellow or brown pilose. The flowers are usually small, white, and arranged in headlike clusters (Plate 2.1). The infusion of the leaves is used as antidote for snake poison (Dalziel, 1937). The decoction of the leaves is also used as antimalarial in certain parts of south west Nigeria. Leaf-sap is used in Ivory Coast to wash the face of persons subject to fainting, giddiness and attacks of epilepsy (Burkill, 1985). Recently, Deji-Agboola, *et al.*, (2009) reported the antimicrobial activity of the leaf extract against some clinical isolates against the background that it is being used to treat infected wound. The extracts exhibited broad-spectrum antimicrobial activities, which was more in ethanol extract than aqueous extract. Adewoyin *et al.* (2016) also reported the antimalarial property of the methanol leaf extract which was effective against *Plasmodium berghei berghei* in mice. No specific compound has been isolated from the plant yet.

2.2.4 *Phyllanthus muellerianus* (Kuntze) Exell (Euphorbiaceae)

Phyllanthus muellerianus is a monoecious, glabrous, straggling or climbing shrub or small tree up to 12m tall (Plate 2.2). Its branches are spreading or pendulous, with main branches stout, angular, redish tinged branchlets 15-25 cm long with several short axillary shoots. The leaves are alternate, distichous along lateral, simple and glabrous twigs. The flowers are unisexual. The stem seldom becomes large. In

Bendel State of Nigeria it is reported as a weed of rice-fields, plainly by lack of timely cultivation. (Daziel, 1937).

Plate 2.1: *Clerodendrum polycephalum* (arrowed) with ripe white flower

Source: Photograph taken at Ooni-Ilare Street, Ile-Ife on 15/8/2009.

Plate 2.2. *Phyllanthus muellerianus*

Source: Photograph taken at farm gate of OAU, Ife, on 24/2/2018

Clear potable water may be obtained from the cut stem and this sap is used in Sierra Leone to relieve ophthalmia, and in Nigeria, for pain in the eyes, or removal of a foreign body. The bark is sometimes added to palm wine to render it strongly intoxicating, which amongst some tribes of the Cameroons may give rise to a sort of frenzy. In Congo Brazzaville, dried bark-powder is taken for colds and sinusitis. The roots are widely used for intestinal troubles.

Leaves from *Phyllanthus muellerianus* (Kuntze) Exell are traditionally used for treating wound healing in Western Africa. The activity showed by stem bark extracts against streptococci and *Clostridium sporogenes* ATCC 3584 and stimulation of proliferation of human keratinocytes and dermal fibroblasts by the aqueous extracts of dried leaves, seemed to have justified the traditional and clinical use of such extracts for wound healing (Brusotti *et al*., 2011, Agyare *et al*., 2011). Methanolic extract of stem of *P. muellerianus* was found to exhibit cytotoxicity with a positive lethality of LD_{50} 4.867µg/ml, low phytotoxicity at 100µg/ml and significant phytotoxicity at 1000 µg/ml. It showed no anti-leishmanial activity (Onocha and Ali, 2010). Saleem *et al.*, (2009) reported the isolation of five compounds, bis (2-ethyloctyl)phthalate, bis(2- ethylicosyl)phthalate, 3 -friedelanone, β-sitosterol, and methyl gallate.

2.2.5 *Aspilia africana* C. D. Adams (Asteracea)

The plant is a tropical shrub which grows wildly at roadsides in Nigeria and popularly used as fodder in most villages. It is also a common secondary formation

species in cultivated farms and fallow lands, occurring in West Africa. It is lignous at the base and can grow up to 1.5 - 2 m high. The fruits are quadrangular akenes and the leaves are opposite and hairy. The flowers are composed of floret of a yellow glaring and dazzling colour (Adjanohoun *et al*., 1996) (Plate 2.3). It is very polymorphic and occurs throughout the region on wasteland of the savannah forest. It is also widely

Plate 2.3. *Aspilia africana* leaves

Source: http://tropical.theferns.info/viewtropical.php?id=Melanthera+scandens

(22/2/2018)

distributed across tropical Africa (Dalziel, 1973). It is commonly known as "yurinyun" by the Yorubas, "Orangila" by Igbos, "Tozakin" by Hausas and "Edemedong" by Efiks,

while in Ekpeye it is called "Uwhoridhoatu" and "Eutung" of Cross Rivers State of Nigeria call it Ottabi (Iwu, 1993).

Aspilia africana is widely used in ethno medical practice in Africa for its ability to stop bleeding, as well as promote rapid healing of wounds and sores and the management of problems related to cardiovascular diseases (Dimo *et al*., 2002). It has also been established that *A. africana* has an anticoagulant activity when applied to wounds (Hanna and Niemetz, 1987). Infusion of a liquid made from the leaves is topically taken by children and can also be mixed with clay as a medicine for stomach trouble (Okwu and Josiah, 2006). Eweka, (2008) reported that in some communities in Nigeria women boil and filter the leaves of *A. africana*, which they drink to prevent conception. It has been reported that the plant is effective against malarial (*P. falciparum*) infection (Okokon *et al*., 2006). It has been classified among substances with a low potential for toxicity, with an LD_{50} averaging 6.6g/kg body weight (Taziebou *et al*., 2007). The methanolic and aqueous extracts of the leaves of *A. africana* have exhibited differential anti-infective activities on both Gram-positive and Gram-negative bacteria species (Macfoy and Cline, 1990; Adeniyi and Odufowora, 2000).

2.2.6 *Cissampelos owariensis* P. Beauv (Menispermaceae)

Cissampelos owariensis (P. Beauv) is a climber with twinning spindly hairy leaves and bears small flowers with green leaves (Plate 2.4). The English name is referred to as velvet leaf and it is known across West Africa by different names. The leaves are used widely for their healing properties; alone they are applied externally to abscess or in some form of preparation to abscess, scabies and sores (Watt and Breyer-

Plate 2.4. *Cissampelos owariensis* (P. Beauv) leaves

Source:http://www.thepharmajournal.com/archives/?year=2015&vol=3&issue=11&part=B&ArticleId=481 (22/2/2018)

brandwijk, 1962; Bouquet and Debray, 1974). When the leaves are crushed up, macerated or cooked, it can be taken internally for diarrhoea, to promote menstrual flow and for painful or irregular menses (Walker, 1953; Bouquet, 1969). The root aids fertility, assist in difficult pregnancy and prevent threatened miscarriage. When chewed with Tiger nuts, the rhizomes of *Cyperus esculentus* Linn. are said to be aphrodisiac (Dalziel, 1937).

The crude methanol extract was found to be active against the test organisms except *S. pyogenes* and *P. aeruginosa*. The crude extract as well as the polar neutral fraction and the aqueous residue also showed very promising activity against three strains of *Mycobacterium tuberculosis* pathogens that are of commercial significance, at about 3000 µg/ml (Akande *et al.*, 2013). Two compounds, namely 2Hcyclopropa[a]naphthalene-2, 5-dione, 1, 1a, 3, 4, 6, 7, 7a, 7b-octahydro-1, 1, 7a, 7b-tetramethyl, and 1, 2-benzenedicarboxylic acid, di-octyl ester had been isolated and identified from it (Erhirhie *et al.*, 2015).

2.2.7 *Peltophorum pterocarpum* Backer ex Heyne (Fabaceae)

Peltophorum pterocarpum (otherwise known as Yellow flame) is a deciduous tree usually reaching a height of 15 -24 m, although it may attain 50 m and a diameter

of 50 -100 cm (Plate 2.5). The bark is smooth and grey while the crown is dense and spreading. It has large leaves, 30-60 cm long, with 8-10 pairs of pinnae each bearing 10-20 pairs of oblong leaflets 0.8-2.5 cm long with oblique bases. The flowers are orange-yellow, each about 2.5 cm in diameter, fragrant, particularly at night; inflorescence brown-tomentose, panicles terminal with rust-coloured buds. The fruits have 1-4 seeded pods, which are flat, thin, winged, 5-10 cm long, dark red when ripe, then turning black. *P. pterocarpum* has a deep root system. It has the ability to fix nitrogen in the soil (Orwa *et al*., 2009).

Plate 2.5. *Peltophorum pterocarpum* Backer ex Heyne

Source: http://www.frim.gov.my/v1/images/colourfrim/Jemerlang/Picture4.JPG.

(22/2/2018)

It is suitable for use as a fodder. In India, it is a source of pollen for the dammer bee (*Trigona iridipennis*). The tree is used as fuel wood. The sapwood is greyish-white, turning grey-brown on aging. The heartwood is light reddish-brown or black, moderately hard, moderately heavy, and somewhat lustrous, with a straight to interlocking grain. The wood is used locally for light construction purposes, cabinet making, sawn or hewn building timbers, wood ware, woodcarving and marquetry. The bark of *P. pterocarpum* has been an important component of the dark or black 'soga' dye in Java, used for batik work. It is also used for tanning leather, and preserving and dyeing fishing nets. In Indonesia, the bark is used for fermenting palm wine. In traditional medicine, it is used as an astringent to cure or relieve intestinal disorders after pain at childbirth, sprains, bruises and swelling or as a lotion for eye troubles, muscular pains and sores. It is also used for gargles and tooth powders.

Phytochemicals that have been isolated include aliphatic alcohol, fatty acids, amino acids, terpenoids, phenolics, flavonoids, alkaloids, steroids. A total of eighty-three phytochemicals has been reported so far from this plant (Nathan *et al*., 2012). Besides, some investigations regarding class of chemical constituents present in different extract of this plant have been studied (Jam *et al*., 2012).

2.2.8 *Canavalia ensiformis* Linnaeus D. C. (Fabaceae)

It is a garden plant that can reach up to 2.30 meters, provided it gets enough nutrients, rich soil, sun and warmth. The stems can support themselves by twining around other plants (Plate 2.6). It grows, therefore, in rich soil, or use extra nutrients, in a sunny warm place. It has deep roots, which makes it drought resistant. The plant can spread via long runners. The flowers are pink-purple in colour. The pods are up to 36 centimetres (14 inches) long with large white seeds. The beans are mildly toxic, and copious consumption should be avoided. Animals affected by eating too much of the

Figure 2.6. *Canavalia ensiformis* with leaves and matured fruit

Source: https://www.amazon.in/Seedstores-Canavalia-Gladiata-Valavara-Valamara/dp/B00LI47NTS (21/4/2017)

plant or meal reach a temperature of 30^{o}C, have a clear nasal discharge, and exhibit lameness and prostration. Mucus membranes of such animals become muddy in appearance and clear urine is passed more frequently than usual. The whole plant is

used for fodder, although it cannot be used in fodder mixtures containing urea, since it contains large quantities of the enzyme urease, which liberates harmful ammonia from urea (Leon *et al.*, 1991).

The mature bean contains potentially harmful saponins, cyanogenic glycosides, terpenoids, alkaloids and tannic acid (Udedibie and Carlini, 1998) and therefore must be cooked before eating. There is also a pharmaceutical interest in the use of *C. ensiformis* as a source for the anti-cancer agents' trigonelline and canavanine (Morris, 1999). Jack bean seed has been promoted in developing nations as a potential source of affordable and abundant protein. It has 29.0% protein content (Adebowale and Lawal, 2004). Chemical analysis of the seed revealed 91.1 percent dry matter of which 33.9 percent is crude protein, 2.2 percent fat, I 1 .2 percent crude fibre. 49.6 percent nitrogen-free extract and 3.1 percent ash.

2.2.9 *Paulinia pinnata* L. (Sapindaceae)

It is a stout and strong woody vine that forms mats in forest openings and ascends rocks and trees by means of forked tendrils to access full sunlight. It may reach 10m of extension and 12 cm or more of stem diameter (Plate 2.7). The young, green stems are angularly striated; the large, lower stems have a rounded triangular cross section. The plant is easily recognized from the leaves that have five serrated leaflets with prominent veins and a winged rachis and petiole. The fruits are red or dark pink when ripe (Liogier, 1994). In East Africa leaves are used against snake bites, rabies, mental problems, blindness and eye troubles, together with the roots, against gonorrhoea, paralysis, wounds, threatened abortion, malaria, ancylostomiasis, and to

Plate 2.7. *Paulinia pinnata* L

Source: http://tropical.theferns.info/viewtropical.php?id=Paullinia+pinnata (22/2/2018)

expel the placenta. Roots are applied against eczema, as a tonic and as a styptic medicine. The whole plant is applied for bad skin conditions, for wounds and

microbial infections. The root decoction is drunk in the case of nausea and vomiting (Chabra *et al*., 1991).

Methanolic leave and root extracts are rich of phenolic compounds (Zamble *et al*., 2006). Phytochemical investigation of the air-dried leaves resulted in the iolation of two new flavone glycosides (1) Diosmetin -7-O-(2”-O-β-D-apiofuranosyl-6”-acetyl-β-D-glucopyranoside), pale yellow amorphous powder, melting point 221-223OC, (2) Tricetin-4-O-methyl-7-O-(2-O-β-D-apiofuranosyl-6-acetyl-β-D-glucopyranosid), also a pale-yellow powder, melting point 230-232 °C. Furthermore, triterpene, saponines and cardiotonic catechol tannins are present (Abourashed *et al*., 1999).

Zamble *et al*. (2006) reported that *Paullinia pinnata* extracts are rich in polyphenols and that they promote vascular relaxation via endothelium-dependent mechanisms. They suggested that the arterial relaxation induced by both extracts could be mainly linked to their capacities to inhibit nitric oxide oxidation through their antioxidant properties. The in vitro antioxidant activities of the methanol extract of *Paullinia pinnata* leaves were evaluated by Jimoh *et al*. (2007) using different testing systems. The results showed that *Paullinia pinnata* possessed strong scavenging activity and moderate reducing power. The total phenol, flavonoid, and proanthocyanidin contents of the extracts were very close to those reported for most medicinal plants and showed good correlation with its antioxidant activities. These properties are probably part of the reasons why *Paullinia pinnata* is effective in traditional medicine.

2.2.10 *Lycopersicon esculentum* Mill (Solanaceae)

Lycopersicon esculentum Mill belongs to the family Solanaceae. It is a small annual herb which grows up to 1 meter in height (Plate 2.8). The leaves are compound, imparipinnate, and curled, while the leaflets are acute or acuminate. The flowers are yellow, in axillary cymes. Its fruits are globose, pale green when young, and turn red on ripening. It contains many small seeds embedded in fleshy placentum. There are 7,500 tomato varieties grown for various purposes and they are divided into various categories based on their shape and sizes (Eck *et al*., 2006).

The tomato plant is popular throughout the world because of its edible fruits. Either raw or cooked, it can be used as a savoury vegetable or flavouring in cooked foods, or can be eaten raw as a dessert fruit. It is much used in salads and as flavouring in soups and other cooked foods. A juice made from the fruit is often sold in health food

shops. The fruit can also be dried and ground into a powder that can be used as a flavoring and thickening agent in soups, breads and pancakes.

Apart from this, the plant has many medicinal values. Traditionally in western part of Nigeria, the aerial part of the plant is used to fight skin rashes by making it into soap for regular bathing. In some other parts of the world, the strong aroma of this plant is said to repel insects from nearby plants. The pulped fruit is said to be beneficial to people with oily skin while the sliced fruits are used as quick and easy first aid treatment

for burns, scalds and sunburn. A decoction of the root can be ingested in the treatment of toothache. The fruit skin is a good source of lycopene, a substance which protects people from heart attacks. Lycopene is a bright red carotene and carotenoid pigment and phytochemicals found not only in tomatoes but also in other red fruits and vegetables, such as red carrot, watermelons and papayas. Tomatoes and tomato

Fig. 2.8. *Lycopersicon esculentum* showing the leaves and fruits

Source: https://tangsphoto.photoshelter.com/image/I0000XqkB9I61JkE (22/2/2018)

sauces and puree are said to help lower urinary tract symptoms (BPH) and may have anticancer properties. There are several other traditional medicinal uses of the plant which vary from country to country. The leaves are used in many countries to arrest excessive bleeding and induce vomiting in children (Basu and Imrhan 2007; PolIvková *et al*., 2010).

Compounds that retard the larval growth of the tomato fruitwonn, *Heliothis zea* (Boddie) have been isolated and identified from tomato leaves. The major allelochemics are tomatine, chlorogenic acid, rutin, and caffeyl derivative of an aldaric acid (Elliger *et al*., 1981). Three steroidal alkaloid glycosides, lycoperosides F-H, were isolated from tomato fruits along with lycoperosides A-D, esculeoside A, and rutin. In all, at least a hundred compounds have been identified and isolated from the plant, including aerial parts, root and the fruits (Yahara *et al*., 2004).

The reported antibacterial activities of the ethanol and water extracts of the aerial and root parts of *L. esculentum* revealed no activity against *Escherichia coli* and *Staphylococcus aureus* (Duffcy and Isman, 1981). Acetone extract of the dried leaf was also inactive against *Macrosiphium solanfolii* and *Orzyaephilus surinamensis*. Phenolic fraction of the trichomes in the ration at a dose of 0.1% was active on *Heliothis zea* (Elliger, *et al*., 1981). Several other pharmacological activities have been reported.

2.2.11 *Euphorbia hirta* L. (Euphorbiaceae)

Euphorbia hirta belongs to the plant family *Euphorbiaceae* and genus Euphorbia. It is a slender- stemmed, annual hairy plant with many branches from the base to top, spreading up to 50 cm in height, reddish or purplish in color (Plate 2.9). Leaves are opposite, elliptic - oblong to oblong- lanceolate, acute or subacute, dark

green above, pale beneath, 1- 2.5 cm long, blotched with purple in the middle, and toothed at the edge. The fruits are yellow, three- celled, hairy, keeled capsules, 1-2 mm

Fig. 2.9. *Euphorbia hirta* whole plant

Source: Photograph taken at glasshouse, Olurin area, Ondo Road, Ile-Ife (28/2/2018)

in diameter, containing three brown, four-sided, angular, wrinkled seeds (Williamson, 2002; Prajapati *et al*., 2003 and Kirtikar *et al*., 2003).

Euphorbia hirta is used in the treatment of gastrointestinal disorders (diarrhea, dysentery, intestinal parasitosis, etc.). The latex of the plant is used to cure some wounds. The stalks and leaves are used to prepare a drink flavoring the milk of young mothers. It is also popular remedy for coughs, coryza, hay fever, bronchial infections and respiratory disorders. In traditional Cambodian medicine, it is given to expel worms, bowel complaints and as a paste for gonorrhoea and other venereal diseases (Holm *et al*., 1991). A tincture is suitable for spasmodic dyspnoea due to asthma, bronchitis, emphysema and pulmonary, cardiac disorders, and in conjunctivitis. Hypotensive and tonic properties are also reported in *E. hirta*. The aqueous extract exhibits anxiolytic, analgesic, antipyretic, and anti-inflammatory activities. The stem sap is used in the treatment of eyelid styles and a leaf poultice is used on swelling and boils.

Extracts of *E. hirta* have been found to show anticancer activity. The latex of *E. hirta* is applied on lower eyelids, to cure eye sores. The root exudate exhibits nematicidal activity against juveniles of *Meloidogyne incognita*. Decoction of dry herbs is used for skin diseases. Decoction of fresh herbs is used as gargle for the treatment of thrush. Root decoction is also beneficial for nursing mothers deficient in milk. Roots are also used for snake bites

Several compounds have been identified and isolated from the plant which include afzelin, quercitrin, myricitrin, rutin, gallic acid, quercitin, euphorbin-A and

ephorbin-B, euphorbin-C, euphorbin-D, β-amyrin, 24-methylenecycloartenol, β-sitosterol, heptacosane, n-nonacosane (Rastogi and Mehrotra, 2002), shikmic acid, tinyatoxin, choline, camphol, and quercitol derivatives containing rhamnose, and chtolphenolic acid (Sood, 2005).

2.2.12 *Combretum paniculata* Vent. (Combretaceae)

Combretum paniculatum is also known as the burning bush or forest flame-creeper. It is usually an evergreen climber or a scrambling shrub, growing up to 15m in length, in the absence of support (Plate 2.10). The flowers are red during the dry season. The stem are used to make a rope in Tanzania, to weave into winnowing basket in Kenyan and stem and roots to tie up beehives (Burkill, 1985). The plant has been used widely in ethnomedicine in the treatment of chronic diarrhoea, dysentery, flatulence, vomiting, enlarged spleen and liver (Chenge *et al*., 2003). The seeds are used in treating toothache (Oboh *et al*., 2008). The leaves and the aqueous extract of the inflorescence have been reported to have activities against carcinomous tumours (Burkill, 1985). Antimicrobial compounds such as cholest-5-en-3-ol, gallocatechin and apigenin, found

in the plant, have been reported by Samdumu (2007). The anti-HIV activity of the plant has also been reported (Asres *et al*., 2001).

2.2.13 *Nicotiana tabacum* L. (Solanaceae)

*Nicotiana tabacum*L is a perennial herbaceous plant. It is found only in cultivation. It grows to heights between 1 to 2 metres. *N. tabacum* Linné is a robust annual little branched herb up to 2.5 m (8.2 ft) high with large green leaves and long trumpet shaped white-pinkish flowers (Plate 2.11). All parts are sticky, covered with short viscidglandular hairs, which exude a yellow secretion containing nicotine (Rawat and Mali, 2013). *N. tabacum* is a native of tropical and subtropical America

but it is now commercially cultivated worldwide. Other varieties are cultivated as ornamental plants or grow as a weed.

Fig. 2.10. *Combretum paniculata*

Source: https://en.wikipedia.org/wiki/Combretum_apiculatum (22/2/2018)

Plate 2.11. *Nicotiana tabacum* L.

Source: Photograph taken at Ondo Road, Ile-Ife on 24/2/2017

Tobacco, *Nicotiana tabacum*, originated in South America. Tobacco is an agricultural product processed from the leaves of the plant. It can be consumed as a smoke, a snuff or a chew, used as a pesticide and, in the form of nicotine tartrate, used in some medicines. Upon consumption or inhalation, the nicotine influences the intelect, stimulates the imagination and improves the endurance (pain and strenght) of the consumer. It is probably the most widely cultivated non-food crop in the world (Villegier *et al*., 2003).

Tobacco leaf contains several pyridine alkaloids, mainly nicotine. Other alkaloids present include nicoteine, nicotimine, anabaine anatalline and nornicotine. It also contains a high percentage of organic acids. The leaves also contain glucosides, tahacinin, tahacilin and isoquercitrin, 1-quinic, chlorogenic, caffeic and oxalic acids. In addition, they also contain terpenic and carcinogenic substances (Fowles, 2003). Anatabine and nornicotine have been isolated from roots. Quercetin-3,3-dimethyl ether and quercetin3-Me ether have been isolated from flowers. Three new gibberellins - nicotiana α, β and γ along with gibberllins A and A3 have been isolated from shoot apices and flower buds. Seed contains cycloartanol, cycloartenol 24-daturadiol and solavetivone. Cholesterol, cholest-7- enol, 24-methylenecholesterol, campesterol, stigmasterol, sitosterol, 28-isofucosterol, lanosterol, 31- norlanosterol, lanost-8-enol, obtusifoliol, 31-norcycloartenol, cycloeucalenol, granisterol, citrostadienol, β-amyrin, lupeol, cycloartanol and 24-methylenecycloartanol have also been reported in seed oil (Leete, 1983).

2.3 Ecology and Biology of Mosquitoes

Mosquitoes are members of a family of nematocerid flies. The Culicidae is derived from the Latin culex, genitive culicis meaning midge or gnat. The word *Mosquito* is from the Spanish and Portuguese for little fly. Mosquitoes differ from all other members of the Nematocera by having a longscaled proboscis (labium and stylets), always longer than the thorax, which projects forward together with the maxillary palps (Figure 2.1). The latter are as long as or longer than the proboscis in males of most species and females of the genus *Anopheles*. The head, thorax, and abdomen are covered with scales and setae: the extent of coverage is genus specific. The legs, wing margins, and wing veins are typically clothed with scales. The closest resemblance of the body shape is found within the families of slender, long-legged crane flies (Tipulidae) and nonbiting midges (Chironomidae), the latter often being mistaken for mosquitoes, especially around artificial lights at night. Notably, none of these families have mouthparts for piercing and sucking (Becker *et al.*, 20 10).

Scientific classification of mosquitoes is as follows:

Kingdom: Animalia

Phylum: Arthropoda

Class: Insecta

Order: Diptera

Suborder: Nematocera

Infraorder: Culicomorpha

Superfamily: Culicoidea

Family: Culicidae

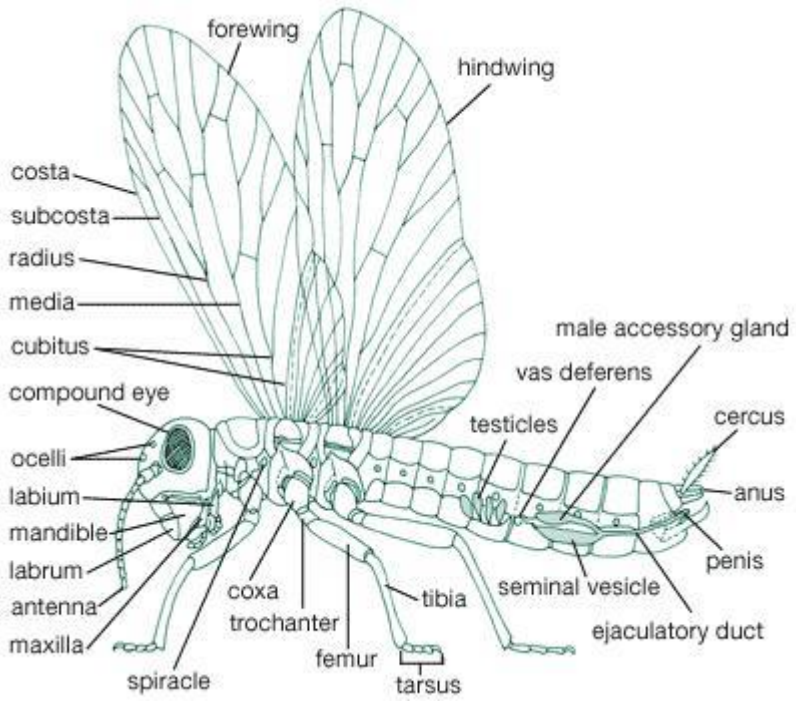


Figure 2.1. Morphological structure of a male adult mosquito

Source: https://media1.britannica.com/eb-media/20/51120-004-FC35E11C.jpg(16/2/2018)

Mosquitoes thrive in a variety of environments. There is hardly any aquatic habitat anywhere in the world that does not permit mosquitoes to breed. They colonise the temporary, permanent and highly polluted as well as clean, large and small water bodies; even the smallest accumulations such as water-filled buckets, flower vases, tyres, hoof prints and leaf axes are potential habitat.

In temporarily flooded areas, along rivers or lakes with water fluctuations, floodwater mosquitoes such as *Aedes vexans* or *Ochierotatus sticticus* develop in large numbers and with a flight range of several miles, become a tremendous nuisance even in places located far away from their breeding sites (Becker and Ludwig 1981; Schafer *et al*., 1997). In swampy woodlands, snow-melt mosquitoes such as *Oc. cantans, Oc. Communis, Oc. punctor* encounter ideal conditions for development in pools that are formed after the snow melts or after heavy rainfall. In floodplains along coastal areas, the halophilous species such as *Oc. taeniorhynchus, Oc. sollicitans, Oc. vigilax, Oc. caspius, Oc. detritus*, develop in huge numbers in brackish or salt water habitats.

Larvae of the *Anopheles* can be found in association with other mosquito species in fresh- or salt-water marshes, mangrove swamps, rice fields, grassy ditches, edges of streams as well as in small temporary water collections. Many species reside in habitats either with or without vegetation. Tree-holes are the habitat of arboreal species such as *Oc. geniculatus, Ae. cretinus, Anopheles plumbeus* and *Orthopodomyia pulcripalpis.* Species like *Cx. p. pipiens, Ae. Aegypti, Ae. albopictus,*

or Oc. j. japonicus can even breed in a variety of small water containers such as rainwater drums, tyres, cemetery vases, small clay pots, etc.

Mosquitoes have the capacity to adapt to various climatic or changing environmental conditions. For instance, *Ae. albopictus* has the ability to adapt to moderate climatic conditions. Their eggs are resistant to desiccation and survive for more than a year. The species also can as well breed in artificial breeding sites such as tyres and flower pots. This has contributed to its spread globally via international trade in plants like *Dracaena* spp. ("lucky bamboo") and tyres. Within hours or days, they can be transported from one country or continent to another by cars, aircrafts or trans-oceanic containers (Madon *et al*., 2002).

Like all Diptera, mosquitoes exhibit complete metamorphosis. All mosquitoes need aquatic habitats for their development, although *Aedes/Ochierotatus* spp. can lay their eggs in moist soil. After hatching they pass through four larval instars and a pupal stage when the transformation into an adult takes place. Most species are unautogenous: following copulation, the females have to take a blood-meal to complete the egg development.

2.3.1 Oviposition: Female mosquitoes lay between 50 and 500 eggs, 2-4 days (or longer in cool temperate climates) after the blood-meal. In general, the mosquitoes can be divided into two groups depending on their egg-laying behaviour (Barr, 1958) and whether or not the embryos enter into a period of dormancy (externally triggered resting period) or diapause (genetically determined resting period).

In the first group, females deposit their eggs onto the water surface either singly (*Anopheles*) or in batches (e.g. *Culex, Uranotaenia, Coquillettidia, Orthopodomyia* and subgenus *Culiseta*). The *Culex* females lay their eggs in rafts comprising several hundred eggs locked together in a boat-shaped structure. During

oviposition, the females stand on the water surface with the hind-legs in a V-shaped position. The eggs are released through the genital opening and grouped together between the hind-legs, forming a raft where the eggs stand vertically on their anterior poles attached together by chorionic protrusions (Clements, 1992). Immediately following oviposition, the eggs are soft and white, but they sclerotize and darken within 1-2h. Anophelines lay single eggs while standing on the water surface or hovering above it. The eggs of this subfamily are adapted for floating and can easily be dysfunctioned by desiccation. The embryos of the first group do not enter dormancy or diapause and hatch when the embryonic development is completed.

Factors such as water quality, incidence of light, existing eggs, available food, and local vegetation are decisive factors in selecting a favourable breeding site. For *Cx. p. pziens*, it is known that the content of organic material in the water plays an important role in attracting the females about to lay eggs. Apparently, gaseous substances such as ammonia, methane, or carbon dioxide, which are released when organic material decomposes, create an effect of attracting the females of *Cx. p. pipiens* (Becker, 1989). They recognise that such a site has adequate food and that favourable conditions prevail for the development of their brood.

The second group lays eggs which do not hatch immediately after oviposition. The egg laying behaviour of the floodwater mosquitoes (e.g. *Ae. vexans*), and the genus Culiseta which lay their eggs singly into the moist soil which is subsequently floaded are worthy examples.

After eggs have been laid, embryonic development starts almost immediately. It takes about 2-7 days or more for embryo to fully develop depending on the temperature. At a temperature of 30°C, the *Cx. p. pipiens* larvae hatch 1 day after the

eggs have been laid, at 20°C and 10°C it takes 3 and 10 days respectively, and at 4°C, the embryonic development of the *Cx. p. pipiens* cannot be completed.

2.3.2 Larvae: The legless (apodous) larval body is divided into three distinct parts: (a) the head with mouth-parts, eyes and antennae; (b) the broader thorax and (c) the abdomen which is composed of seven almost identical segments and three modified posterior segments. These posterior segments bear four anal papillae to regulate the electrolyte levels. At the abdominal segment VIII, a siphon in culicines, or only spiracular lobes in anophelines, are developed where the tracheal trunks open at spiracles for the intake of oxygen. Usually the culicine larvae hang head downwards from the water surface. Anopheline larvae lie horizontally at the water surface (Harbach, 1977).

Their body is held horizontally by specialized setae (palmate setae), the notched organ located at the anterior margin of the prothorax and the spiracular lobes which are flush with the dorsal surface of the larval body and have direct contact with the air. The spiracular apparatus at the distal end of the siphon contains hooks and a saw-like blade with teeth to pierce the plant tissues. These larvae have a more sessile habit, hanging head downwards whilst attached to the plant tissues and filtering the water column for food. Larva food consists of microorganisms, algae, protozoa, invertibrates and detritus. The size of food particles is usually less than 50μm (Eisenberg *et al.*, 2000).

Larvae moult four times resulting in four instars at intervals, before reaching the pupal stage. At each moult, the head capsule is increased to the fullsize characteristic of the next instar, whereas the body grows continuously. Thus, the size of the head capsule is a fairly good morphometric indicator for the larval instar. Each moult is coordinated by the relative concentrations and interactions of juvenile

hormone and ecdysone, a molting hormone. The development of larvae is temperature dependent. There are great differences in the optimum temperature for the development of different mosquito species. Snow water mosquitoes can complete their development between 10-25°C, while floodwater mosquitoes can successfully develop in wide range of temperature (10-30°C) (Becker *et al*., 2010).

2.3.3. Pupae: With the fourth moulth, the pupa appears. The pupae are also aquatic, the pupal stages usually lasting about 2 days, however, this period may be reduced or extended, at higher or lower temperatures respectively. During the pupal stage, the process of metamorphosis takes place. Some larval organs are histolysed, whilst the body of the adult is formed through the development of imaginal disks (cells or groups of cells that remained quiescent in the larval body until the pupal stage). Characteristically, the head and thorax of the pupae are fused into a prominent cephalothorax which carries anterolaterally two respiratory trumpets, which are connected to the mesothoracic spiracles of the developing adults to provide oxygen. The abdomen terminates with two paddles and is kept flexed under the cephalothorax. When at rest, the pupae float motionless at the water surface, with the tip of the trumpets and the palmate setae of the first abdominal segment in contact with the water surface. The hydrophobic rims of the trumpets protrude through the water surface for respiration. An air bubble between the appendages of the cephalothorax makes the pupa positively buoyant. Mosquito pupae are quite mobile (unlike the pupae of most other insects). When the pupa is disturbed, it dives by straightening the abdomen and spreading the paddles rapidly flexing the abdomen which has retained the larval musculature. In contrast to larvae which have to swim actively to the water surface, the pupa floats passively back to the surface after diving. Unlike larvae, pupae do not feed (Rey, *et al.*,1999; Spielman *et al*., 2001).

2.3.4. Adult: The final stage of metamorphosis is completed when gas is forced between the pupal and the pharate adult cuticle, and into its midgut. The pupa straightens the abdomen into a horizontal position, and by swallowing air it further increases the internal pressure. The cephalothoracic cuticle of the pupa then splits along the ecdysial line and the adult slowly emerges from the pupal skin. The emerging adult moves cautiously to avoid falling onto the water surface, whilst its appendages still remain partly in the exuvia. In this phase, the emerging individual is highly susceptible to strong winds and predators. After emergence, the adult increases the haemolymph pressure which causes the legs and wings to stretch. It then immediately ejects droplets of fluid to empty the gut, while air is dispelled from the gut some hours later. Within a few minutes, it is able to fly when the soft cuticle has sclerotized.

There is also a difference between male and female sexual maturity at the time of emergence. The males in the population usually emerge 1-2 days before the females in order to achieve sexual maturity at the same time as the emerging females. Since the pupal stage of the two sexes appears to be about the same length, the shortening in development of males takes place primarily in the larval stage. Consequently, the male pupae and adults of a population are smaller in size than the corresponding females. Following emergence, the adults are ready to begin their life cycle of mating, feeding and oviposition again.

Mating takes place when females enter swarms of flying males. When a female enters a swarm, it will be seized immediately by a male. Usually the male and female copulate face to face when flying outside the swarm. It takes less than half a minute for the male to deposit the spermatozoan in the bursa copulatrix of the female, the sperm then moves to the spermathecae. The females store sufficient sperm to

fertilise several batches of eggs without further copulation. Swarming (eurygamy) is not necessary for all species, and some species may mate without it (stenogamy). After insemination, the search for a host to obtain a blood meal is the next stage in the reproductive life of the female.

Identification of adult mosquitoes is simply by resting position: Culicinae stay parallel to the resting surface, while Anopheline stand at an angle to the surface (Becker *et al*., 2010). The general life cycle of mosquitoes is shown in Figure 2.1.

2.4 Mosquitoes Behaviour in the Transmission of Diseases

The two important subfamilies of mosquitoes are Anophelinae (which includes the genus *Anopheles*), and Culicinae (which includes the genera *Aedes*, *Culex*, *Mansonia* and *Haemagogus*, the mosquito vectors for arboviruses). Each subfamily has hundreds of species within it, although only a few dozen bite humans and therefore are capable of serving as disease vectors. Mosquitoes are found throughout the world except in places that are permanently frozen. There are about 3,500 species, of which nearly three-quarters are native to the humid tropics and subtropics. The largest populations of individual species occur in the Arctic tundra, where colossal numbers emerge in a single brood each summer from snowmelt pools that overlie the permafrost.

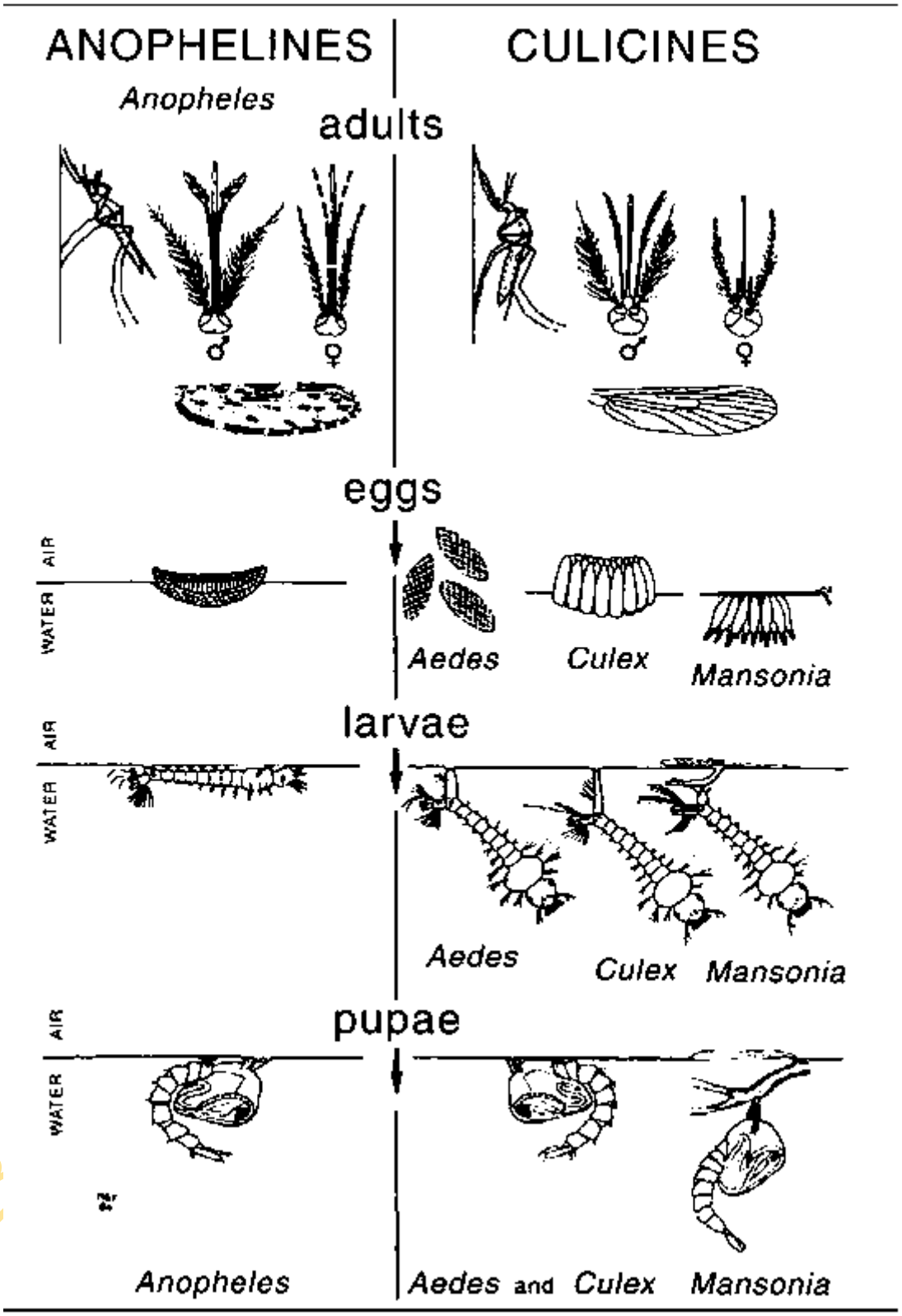


Figure 2.2. The life-cycles of *Anopheles*, *Aedes* and *Culex* mosquitoes.
Source: Warrel and Gilles (2002)

In nearly all mosquito species, the female obtains the protein she needs for the developments of her eggs by feeding on vertebrate blood. Some species are highly selective, restricting themselves to one or at most a few closely related host species. Others have a less clearly defined host preference and may alternate among birds, mammals, and even reptiles. Mosquitoes have a complex salivary secretions which facilitate feeding. It is the direct injection of this fluid into the capillaries that enables several life forms - viruses, protozoa, and nematode worms - to exploit mosquitoes as a means of transfer between vertebrate hosts. Generally, mosquitoes that are important in the transmission of diseases to man are those that:

i. are relatively host specific in their interaction with man for the purpose of blood meal;

ii. live long enough to permit the disease organisms to develop to the infective stage

iii. feed repeatedly and

iv. are commonly present (Goma, 1966).

2.5 Diseases Transmitted by Mosquitoes

Mosquitoes are vectors of three types of organisms pathogenic to man namely: (i.) the plasmodium, causative organisms of the human malaria, belonging to the phylum protozoa; (ii.) the viruses of yellow fever, dengue, zika, the encephalitides, and other diseases of man; and (iii) filarial worms of the genus *Wuchereria*, the causative organisms of the Bancrofti and Brugia's filariasis.

2.5.1 Epidemiology of Malaria

Human malaria is a disease of wide distribution caused by sporozoa of the genus *Plasmodium*. The identification of the species depends on the morphology and the staining characteristics of the parasites and associated changes in the containing

cells. The most common and important infections are those caused by *P. falciparum* and *P. vivax*. Mixed infections do occur. The arthropod hosts are females of Anopheles mosquitoes and the predominant malaria vectors are *A. gambiae, A. funestus, A. darling* and *A. punctulatus* (Charlwood *et al.*, 1997).

Falciparum malaria causes an acute illness with initially non-specific symptoms including fever, headache, malaise, mild jaundice, hyperventilation, hepatosplenomegaly, myalgia etc (Lagerberg, 2008). The fever peaks occur at the time of erythrocyte rupture with the release of merozoites and malaria toxins [e.g. glycosylphosphatidylinositol (GPI)]. These toxins induce the secretion of pro-inflammatory cytokines by the macrophages and parasite antigens stimulate T-cells to directly secrete or induce cytokine production by other cells (Miller *et al*., 1998; Mackintosh *et al.*, 2004). The tertian episodes of fever and erythrocyte destruction often lead to severe aneamia and other complications specific to *P. falciparum* infection, such as cerebral malaria, anaemia, hypoglyceamia, renal failure and noncardiac pulmonary oedema. In the non-immune patient, these complications may occur in isolation or in combination, resulting in an often-complex clinical syndrome (Miller *et al.*, 1994). However, the clinical presentation of severe disease in the previously exposed African child differs and renal failure and noncardiac pulmonary oedema do not occur (Miller *et al.*, 1994).

Severe malaria is one of the potentially fatal complications of *Plasmodium falciparum* infection. It was previously regarded as either severe anaemia (due to erythrocyte destruction) or cerebral malaria (due to small blood vessel obstruction of the brain), but nowadays it is recognised to be a complex multi-system disorder with many similarities to sepsis syndromes (Mackintosh *et al.*, 2004)

Pregnant women are particularly vulnerable to malaria and are more likely to become infected than non-pregnant women with *P. falciparum*, resulting in severe disease. This is partially due to the transient depression of cell-mediated immunity that occurs during pregnancy.

In 2012, WHO reported that malaria occured in 99 countries of the world. It threatens the lives and livelihoods of more than 500 million Africans and exerts such a huge public health burden that it has been incriminated in the continued underdevelopment of the continent as a whole (Snow *et al.*, 1999; Sachs and Malaney, 2002). An estimated 3.3 billion persons were at risk of malaria in 2010. Approximately 86% of malaria deaths occur in children under 5 years of age and the majority of are in sub-Saharan Africa. The estimated incidence of malaria decreased by 17% globally from 2000 to 2010 and the malaria mortality rate decreased by 26% following increases in prevention and treatment. The annual report of the World Health Organization (WHO, 2012) shows clear progress in the fight against malaria and a decline in estimated malaria cases and deaths. The 2010 data provides the following snapshot: Malaria mortality rates have fallen by more than 25% since 2000, with the largest percentage reductions seen in the European (99%), American (55%) and Western Pacific (42%) and African Regions (33%). Out of 99 countries with ongoing malaria transmission, 43 recorded decreases of more than 50% in the number of malaria cases between 2000 and 2010. Another 8 countries recorded decreases of more than 25%. These improvements have come about through increased distribution and use of insecticide-treated bednets, artemisinin-combination therapy, and preventive treatments for pregnant women and children (WHO, 2012).

In eastern and southern Africa, the proportion of deaths caused by malaria has increased from 18% in the 1980s to 37% in the 1 990s. Endemic malaria cripples

economies and is estimated to slow economic growth by approximately 1.3% per year (Sachs and Malaney, 2002). Malaria has been identified as a key contributor to weak economic growth and investment in Africa because it experiences the most intense malaria transmission in the world (Beier, *et al*., 1999; Hay, 2000). It is commonplace in tropical Africa for more than half the population to be infected with *Plasmodium falciparum*, by far the most dangerous of the four parasite species that infect humans. Not only does malaria place a huge burden directly upon the health care systems of African nations, it has also been shown that malaria control can have huge macroeconomic impacts and greatly facilitate economic development at national level (Utzinger *et al.*, 2002; Spielman *et al.*, 2002). Although some countries in southern Africa are successfully applying integrated malaria control (Martin *et al.*, 2002), such programs currently cover only a small proportion of those at risk on the continent.

In Nigeria, malaria is a major public health accounting for more cases and deaths than any other country in the world. Malaria is a risk for 97% of Nigeria's population. The remaining 3% of the population live in the malaria free highlands. There are an estimated 100 million malaria cases with over 300,000 deaths per year in Nigeria. This compares with 215,000 deaths per year in Nigeria from HIV/AIDS. Malaria contributes to an estimated 11% of maternal mortality. It also accounts for 60% of outpatient visits and 30% of hospitalizations among children under five years of age in Nigeria. Malaria has the greatest prevalence, close to 50%, in children age 6-59 months in the South West, North Central, and North West regions. Malaria has the least prevalence, 27.6 percent, in children age 6 to 59 months in the South-East region (US Embasy in Nigeria, 2011).

2.5.2 Epidemiology of Yellow Fever

Yellow fever in man is an acute infectious disease of short duration characterized by sudden onset, fever, headache, backache, prostration, nausea and vomiting. In fatal cases death usually occurs between the fifth and eight day of illness. If the patient survives and recovers, one attack confers lifelong immunity (Goma, 1966). Yellow fever exists in two distinct forms, the urban and jungle, occurring in two entirely different environments. Jungle yellow fever is a disease of forest animals especially monkeys by *Ae. africanus* which breeds in tree holes at high level. These tree monkeys frequently leave the canopy and come to ground level to raid plantation for food. In this way, another day-biting mosquito *Ae. simpsoni* pick the infection from the monkey and transmit to man. Various species of haemagogus, especially in Central and South America are also involved in their transmission (Gordon and Lavoipierre, 1978).

Urban yellow fever is the form that occurs in the urban and rural area where human beings are congregated. It is transmitted from man to man almost entirely by the domestic mosquito *Ae. aegypti*. The animals involved serve as reservoir hosts. Yellow fever has been one of the great plagues of the world, its presence in West Africa was one reason why the region was called the white man's grave. The mortality rate varies from 5-30%.

2.5.3 Epidemiology of Dengue Fever

Dengue fever is a severe, flu-like illness that affects infants, young children and adults, but seldom causes death. The clinical features of dengue fever vary according to the age of the patient. Infants and young children may have a nonspecific febrile illness with rash. Older children and adults may have either a mild febrile

syndrome or the classical incapacitating disease with abrupt onset and high fever, severe headache, pain behind the eyes, muscle and joint pains, and rash. (Gubler, 1998).

Dengue viruses are transmitted to humans through the bites of infective female *Aedes* mosquitoes. Mosquitoes generally acquire the virus while feeding on the blood of an infected person. After virus incubation for 8-10 days, an infected mosquito is capable, during probing and blood feeding, of transmitting the virus, to susceptible individuals for the rest of its life. Infected female mosquitoes may also transmit the virus to their offspring by transovarial transmission, but the role of this in sustaining transmission of virus to human has not yet been delineated.

Human are the main amplifying host of the virus, although studies have shown that in some parts of the world monkeys may become infected and perhaps serve as a source of virus for uninfected mosquitoes. The virus circulates in the blood of infected humans for two to seven days, at approximately the same time as they have fever; *Aedes* mosquitoes may acquire the virus when they feed on an individual during this period (Scott *et al.*,1997). In fatal cases, the neck becomes stiff; there is lethargy, stupor, coma and sometimes convulsion or paralysis.

2.5.4 Epidemiology of Encephalitides

Clinical manifestations of encephalitis vary considerably, depending on the type of form and its severity. Severe cases are marked by fever, headache, nausea and vomiting, muscular weakness and twitching, malaise and mental disorientations. In fatal cases, the neck becomes stiff; there is lethargy, stupor, coma and sometimes convulsion or paralysis.

Six types of encephalitis are known. These include the following: Japanese B Encephalitis (JBE); Venezuelan Equine Encephalitis (VEE); Eastern Equine Encephalitis (EEE); Western Equine Encephalitis (WEE); St. Louis Equine Encephalitis (SLE); and Murray Valley Encephalitis (MVE). JBE is endemic in the Far East from Korea to India and in many islands of Western Pacific. VEE is a disease whose range is from northern South America and Trinidad to Mexico. EEE occurs along the Atlantic seaboard from Canada. SLE occurs in most of the United States with the exception of the north-eastern states. Lastly, MVE occurs in all the states of Australia and New Guinea.

2.5.5 Epidemiology of Lymphatic Filariasis

Filariasis is a group of parasitic infections caused by thread-like nematodes that belong to the roundworm superfamily Filarioidea. Eight species of filariae parasitise man and can be grouped into three depending on the body area they occupy. Thus, there are lymphatic filariae which include *Wuchereria bancrofti, Brugia malayi* and *B. timori*. In chronic cases, the host may develop elephantiasis, a condition where parts of the body swell to massive proportion. The second involves the subcutaneous tissue and caused by the worms *Loa loa, Onchocerca volvulus, Mansonella streptocerca,* and *Dracunculus medinensis* (guinea worm). The third group infect the serous cavity of the abdomen and they are caused *by Mansonella perstans* and *M. ozzardi*.

Lymphatic filariae are transmitted by many species of mosquitoes, belonging to the genera *Culex, Aedes, Anopheles* and *Mansonioides*. The microscopic worms pass from the mosquito through the skin, and travel to the lymph vessels. In the lymph vessels, they grow into adults. An adult worm lives for about 5-7 years. The adult

worms mate and release millions of microscopic worms, called microfilariae (which are about 200 – 300 µm), into the blood. Microfilariae of both *W. bancrofti* and *B. malayi* appear in the peripheral blood at distinct times of the day (periodicity). It is estimated that more than 120 million people worldwide are infected. More than 90 percent of these infections are due to *W. bancrofti*, while the remainder are due largely to *B. malayi*. Estimates suggest that more than 40 million infected individuals are seriously incapacitated and disfigured by lymphatic filariasis (Lucas and Gilles, 1986).

2.6 Factors That Influence Disease Transmission by Mosquitoes

Transmission is the mechanism by which parasite species achieve the infection of new host (Otubanjo, 2007). Generally, distribution of vector-borne diseases is determined by complex demographic, environmental and social factors. Understanding how the environment effects life history and population dynamics of mosquitoes is both ecologically and medically important, as these traits can impact disease transmission (Dye, 1986). Interactions of mosquitoes with abiotic and biotic factors of the environment can affect life history traits including development, adult size, fecundity, lifespan, and gonotrophic cycles, and have population level consequences including survival and population growth.

2.6.1 Climatic factors: The ecology, development, behaviour, and survival of mosquitoes and the transmission dynamics of the diseases they transmit are strongly influenced by climatic factors. Temperature, rainfall, and humidity are especially important, but others, such as wind and the duration of daylight, can also be significant. The same factors also play a crucial role in the survival and transmission rate of mosquito-borne pathogens. As heterogenous organism, in particular,

temperature affects their rate of multiplication in the insect. In turn, this affects the rate at which the salivary secretions become infected, and thus the likelihood of successful transmission to another host, of course, if the development time of the pathogen exceeds the life span of the insect, transmission cannot occur. It is the complex interplay of these factors that determines the overall effect of climate on the local prevalence of mosquito borne diseases (Cook, 1996).

Seasonality is a key component of climate. Summer temperatures in many temperate regions are at least as high as in the wannest seasons of much of the tropics. The crucial difference is that tropics do not have cold winters. Tropical crops such as rice and groundnuts can be cultivated in temperate regions if they are planted in springtime. Similarly, if tropical mosquito-borne pathogens are introduced in the right season, they can be transmitted if suitable vectors are present; in most cases, they are eliminated when winter sets in.

Mosquito native to temperate regions have had to evolve strategies to survive the winter. In the tropics, comparable adaptations are necessary for surviving in unfavourable dry periods, which can last for several years. In both cases, such adaptations impose seasonality on transmission. For example, before eradication, the transmission season for *Plasmodium falciparum* in Italy was July-September (Bruce-Chwatt, 1980). These 3 months constitute the malaria season in Mali, where the disease is still endemic (Craig *et al*., 1999).

The physical environment is an important modifier of local climate (Geiger, 1980). In dense forest, daily mean temperatures at ground level can be as much as 10°C lower than in adjacent open areas (Ricklefs, 1999). The temperature of an indoor or outdoor environment is however a function of design, construction materials, and ventilation. Mosquitoes use a variety of strategies to exploit the timing

and location of such microclimates to maximum advantage. For example, in *Anopheles gambiae*, a physiologic "clock" ensures that whatever the rate of metamorphosis in the pupal stage, the adults always emerge from the water at sundown or in the early hours of the night (Omer and Cloudsley-Thompson, 1970). In the laboratory, if *An. gambiae* pupae are maintained in constant light, the duration of the pupal stage is a direct function of temperature; it lasts about 2 days at 22°C but only 1 day at 32°C. However, in a light regime of l2hr light and l2hr dark (LD 12:12), the timing is modified to minimize emergence in daylight (Reiter, 1975, 1978). Similar circadian rhythms ensure that other behaviours such as feeding, resting, and oviposition are restricted to optimum times, regardless of ambient temperature (Jones and Reiter, 1975). Thus, *An. gambiae* can survive in the Sudan (mean monthly temperatures of 42°C; actual outdoor temperature can be over 55°C) by emerging after sunset, hiding in the thatch of building in the daytime, feeding after midnight, and ovipositing at dawn or dusk (Omer and Cloudsley-Thompson, 1970).

2.6.2 Human Factors: Human activities are also crucial to transmission. Forest clearance eliminates species that breed in water in tree holes (e.g., the forest Aedes species that transmit yellow fever) but provides favourable conditions for those that prefer temporary ground poois exposed to full sunlight (e.g., many of the Anopheles species that transmit malaria). Drainage of wetlands eliminates the marshy pools exploited by many species but can provide the open channels preferred by others (e.g., some important European vectors of malaria and *Culex tarsalis*, a vector of St. Louis encephalitis). Agricultural fertilizers can promote the growth of algae and other larval nutrients, whereas herbicides may eliminate them altogether, cisterns, pit latrines, sewage-polluted ditches, storm drains, and blocked gutters can support large population of *Cx. quinquefasciatus*, an important vector of *Bancroftian filariasis*.

Wells are often a significant source of malaria vectors. Water storage jars and drums, discarded rubber tires, buckets, pots, and other man-made containers are all favourable breeding habitat of *Ae. Aegypti*.

Additional factors arise from behaviour and cultural traits. Daily activity patterns - work, rest, and recreation - the location of homes in relation to mosquito breeding sites, the design of buildings, the materials used to build them, the use of screens and bed nets, and many other factors can be significant. Finally, chemotherapy, vaccination, and mosquito control have played major roles in reducing transmission in many parts of the world (Reiter, 2001).

2.7 Control of Mosquitoes

Insect vector control has proved an effective method of reducing the transmission of disease-causing organisms to human populations in many tropical countries. For the purposes of vector control, it is important to:

- understand the life-cycle and natural history of mosquitoes;
- be able to recognize different types of mosquito;
- understand mosquito behaviour; and
- know the susceptibility of mosquitoes to insecticides (WHO, 2005a).

A variety of methods have been employed for suppressing mosquito vector populations (Curtis and Townson, 1998). Mosquito control involves three approaches: None chemical control, Biological control and chemical control.

2.7.1 Non-Chemical Control

Source reduction i.e. eliminating or altering the water so that the mosquitoes cannot breed or complete their life cycle is the first choice for control. It is simply the use of mechanical method to eliminate standing water. Source reduction involves

filling, deepening draining, ditching, managing water levels, maintaining shorelines, managing aquatic and inundated vegetation, and others.

In 1979, WHO put forth the statement that the major approaches of environmental management comprise: (1) environmental modification, which aims to create a permanent or long-lasting effect on land, water, or vegetation to reduce vector habitats; (2) environmental manipulation, which produces temporary unfavourable conditions for the vector; and (3) modification or manipulation of human habitation or behaviour, which reduces man-vector contact (WHO, 1982). These somewhat cumbersome articulations become more transparent if one thinks of environmental management interventions as the installation, cleaning, and maintenance of drains, vegetation management and river boundary modifications to promote flowing water, intermittent irrigation, and systematic elimination of standing pools of water. The specificities must, of necessity, vary with local ecosystem structure; therefore, there is no single uniform environmental management recipe that is appropriate in all settings.

Regarding malaria, environmental management can be coarsely grouped into four distinct eco-epidemiological settings: (1) malaria of deep forests, forest fringe, and hills; (2) rural malaria attributable to water resources development and management (e.g., irrigation and large dams); (3) rural malaria attributable to wetlands, rivers, streams, coasts, and non-agricultural man-made habitats; and (4) urban and peri-urban malaria (Keiser, 2005). Typical features of these eco-epidemiological settings and possible environmental manipulation and modification approaches, including changes in the human habitations. While these methods may prove to be more extensive and more expensive than some other controls, in most cases, they need be done only once.

Source reduction controls the immature stages-eggs, larvae, and pupae. Because these stages are concentrated in discreet bodies of water, they are much easier to control than are dispersed adult mosquitoes. Two water management tactics are ditching and ponding. Ditching controls mosquitoes in two ways. In some cases, water drains out of the breeding sites while in others it allows fish access to the isolated pools where they prey upon the larvae and pupae.

Ecologic control of malaria by managing the availability of water and vertebrate host resources for mosquitoes is receiving renewed attention, but has seen little application in Africa for more than half a century. Integrated packages of multiple malaria control interventions, relying heavily on environmental management, proved particularly successful in the copper mining regions of Zambia, even before the advent of DDT or chloroquine. Environmental management has also been successfully applied in African cities, notably Dar es Salaam in Tanzania, and may have an important role to play in protecting the rapidly growing urban population of Africa from malaria.

Apart from source reduction method, personal protection through the use of mosquito bed-nets, screening of houses, use of suitable clothing and repellents as well as avoiding areas with high infestation can also be effective in control.

2.7.2 Biological Control

WHO (1982), has defined biological control to mean "the control of pests, including the vectors of human diseases by the direct or indirect use of natural enemies with or without their metabolites". Biological control involves the introduction of organisms that prey upon dengue mosquitoes in order to reduce the size of the vector propagation. Examples include invertebrate copepods introduced into water storage tanks in Vietnam, and larvivorous (larvae-eating) fish. The biocide

BTI is a biologically derived insecticide that has been widely adopted in recent years. Biological control methods do not cause any chemical contamination of the environment and are often species specific so they do not adversely affect the rest of the ecosystem. However, the costs of rearing these organisms can be high and their use is often specific to a limited number of suitable locations. The promising agents include fungi, bacteria viruses, nematodes and larvivorous fish. Unlike insecticides they do not pollute water and furthermore, they can be maintained by unsophisticated manpower.

2.7.3 Chemical Control

The term insecticide is used for the chemicals which kill the insects after coming in contact (contact insecticides) with the body of insects or after taking along with food (stomach insecticides). Generally, insecticides can be grouped into growth regulators, larviciding, adulticiding, insect repellents, genetic control and botanical insecticides.

2.7.3.1 Growth regulators

Some of the earlier compounds investigated were chemically related to the natural juvenile hormones (JHs) of insects and these were therefore designated as JH analogues or mimics, commonly known as juvenoids (Slama *et al.*, 1974). There are other compounds that are not chemically related to insect juvenile hormones but produce similar effects by inhibiting cuticle formation (Van-Eck, I 979; Grosscurt and Tipker 1980; Itoh, 198l; Mulla, l99l). These compounds are also designated as IGRs. Compounds having insect growth regulating properties are found in the classes of terpenoids, benzamides, carbamates, triazines, benzoylureas, and other classes of chemicals. Insect growth regulators such as methoprene do not kill the larvae but prevent them from developing into adult. Timing of application is important since

only mature larvae are affected. Larvae that have already pupated will continue to develop into biting mosquitoes.

Ho *et al.* (1990) investigated the effectiveness of eight insect growth regulators (IGRs) (chlorfluazuron, diflubenzuron, EL-494, flufenoxuron, teflubenzuron, juglone, plumbagin and methoprene) against five mosquito vectors (*Armigeres subalbatus, Aedes albopictus, Aedes aegypti, Culex tritaeniorhynchus, and Culex quinquefasciatus*) in the laboratory. Administration of diflubenzuron (1-5 ppm), flufenoxuron (0.025 ppm), and teflubenzuron (1-5 ppm) reduced *Culex quinquefasciatus* larvae in ditches by 40-90%. The administration of diflubenzuron (0.5 ppm) to containers reduced 97% of the *Aedes albopictus* larvae.

2.7.3.2 Larviciding

Petroleum oils or specialized mineral oils can be applied to the water. The oil forms a thin film over the surface which suffocates egg, larvae, and pupae. In the presence of wind, waves, or rain, the oil film breaks up and is less effective. Some oils are toxic to fish, other organisms, and aquatic plants. Various insecticides can be applied to the water as dust, granular, wettable powers, or emulsion.

2.7.3.3 Adulticiding

Adulticiding is a pace spraying for adult mosquitoes with insecticides. It is generally the last resort in an integrated mosquito control programme, since spraying of adult mosquitoes provides only temporary relief. According to WHO, (2017) on the control of dengue, adulticides are applied either as residual surface treatments or as space treatments.

2.7.3.4 Insect repellents

Insect repellent is a substance applied to skin, clothing, or other surfaces which discourages insects, or arthropods in general, from landing or climbing on the

surface. Many commercial insect repellents contain 5 to 25% DEET (N,N-diethyl-*m*-toluamide) which when applied to skin or clothing provides protection from biting. Other common repellents include Birch, Neem oil, Citronella oil, Icaridin, also known as picaridin, Bayrepel®, and KBR 3023 (America Chemical Society, 2001). Environmental Protection Agency (2016), also listed repellents to include (i) Clip-on products that have a pad with the repellent and a fan or other mechanism that disperses the repellent near the body. (ii) Spatial repellents that use a heating mechanism to disperse repellent in an outdoor area. Examples of dispersal mechanisms for spatial repellents include Lanterns, Torches, Table-top diffusers, Candles, Coils.

2.7.4 Genetic Control

The term genetic control covers all methods by which a mechanism for part or vector control is introduced into a wild population through mating. These include (a) the sterile insert release methods or sterile insect technique (SIT), in which males are sterilised by irradiation or other means and released to mate with wild females, using them to lay sterile eggs; and (b) introduction of genetic factors into wild populations that render pests harmless to humans.

The Sterile Insect Technique (SIT) is a species-specific and environmentally benign method for insect population control. SIT is based on mass rearing, radiation mediated sterilization, and release of a large number of male insects into a given target area. Any successful mating with the sterile insect will result in no offspring. If enough sterile insects are released, the population will decline. Reduction or elimination of vector populations will tend to reduce or eliminate transmission of vector-borne diseases and has been an effective method of disease control in many

regions. One of the major advantages of SIT over other techniques, such as insecticides, larvicides, and breeding site removal is that the males are very good at seeking out females of the same species and the technique becomes more effective as the population is reduced (Alphey *et al*., 2007; Wilke *et al*., 2009). The Sterile Insect Technique is amongst the most non-disruptive pest control methods. Unlike some other biologically-based methods, it is species specific, does not release exotic agents into new environments and does not even introduce new genetic material into existing populations as the released organisms are not self-replicating (Alphey *et al*., 2007).

2.7.5 Botanical Insecticides

At present, there are four major types of botanical products used for insect control (pyrethrum, rotenone, neem, and essential oils), along with three others in limited use (ryania, nicotine, and sapodilla). Pyrethrum refers to the oleoresin extracted from the dried flowers of the pyrethrum daisy *Tanacetum cinerarefolium* (Asteraceae). Technical grade pyrethrum, the resin used in formulating commercial insecticide, typically contains from 20% to 25% pyrethrums (Casanova *et al*., 2002). Rotenone, a pentacyclic isoflavone obtained from *Derris*, *Lonchocarpus* and *Tephrosia* spp., has also piscidal activity, whereas nicotine and even more anabasine present in *Nicotiana tabacum*, prevent aphids from sucking (antifeedant).

Botanicals are basically secondary metabolites that serve as a means of defence mechanism of the plants to withstand the continuous selection pressure from herbivore predators and other environmental factors. Several groups of phytochemicals such as alkaloids, steroids, terpenoids, essential oils and phenolics from different plants have been reported previously for their insecticidal activities (Shaalan *et al.*, 2005). Insecticidal effects of plant extracts vary not only according to plant species, mosquito species, geographical varieties and parts used, but also due to

traction methodology adopted and the polarity of the solvents used during extraction (Ghosh *et al* ., 2012). A wide selection of plants from herbs, shrubs and large trees was used for extraction of mosquito toxins.

Polar solvent will extract polar molecules and non-polar solvents extract nonpolar molecules. This can be achieved by using mainly solvent systems ranging from hexane/petroleum ether, the most non-polar (polarity index of 0.1 that mainly extracts essential oil) to that of water, the most polar (polarity index of 10.2) that extracts biochemical with higher molecular weights such as proteins, glycans, etc. Chloroform or ethyl acetate are moderately polar (polarity index of 4.1) that mainly extracts steroids, alkaloids, etc. It has been found that in many studies that solvent with minimum polarity have been used such as hexane or petroleum ether or that with maximum polarity such as aqueous steam distillation. However, those biochemical that were extracted using moderately polar solvents were also seen to give good results as reported by a few bio-assay. Thus, different solvent types can significantly affect the potency of extracted plant compounds and there is difference in the chemoprofile of the plant species. Phytochemicals were extracted either from the whole body of little herbs or from various parts like fruits, leaves, stems, barks, roots, etc., of larger plants or trees. In all cases where the most toxic substances were concentrated upon, found and extracted for mosquito control.

Plant products especially the secondary metabolites can be used, either as insecticides for killing larvae or adult mosquitoes, as repellents for protection against mosquito bites or as oviposition deterrents, depending on the type of activity they possess (Sukumar *et al.*, 1991). The control of mosquitoes at larval stage is considered as an efficient way in the integrated vector management (Rutledge *et al*., 2003). Furthermore, altering the behavior of mosquitoes for oviposition may also reduce the

breeding of mosquitoes. Some plant metabolites may also inhibit the egg laying of female mosquito which can be used as oviposition deterrents in integrated vector management. Many studies have been carried out to investigate the oviposition altering behavior of plants secondary metabolites against mosquitoes (Prajapati *et al*., 2005, Waliwitya *et al*., 2009).

Though many plants have been shown to possess insecticidal/larvicidal and growth inhibition activity against mosquitoes, most of these reports are based on laboratory observations only. One of the most commonly studied plant for control of mosquitoes is *Azadirachta indica* (Meliaceae), commonly known as neem in India.

Neem contains at least 35 biologically active principles (Mulla and Su, 1999), of which azadirachtin, a triterpenoid is the predominant insecticidal active ingredient in the seeds, leaves, and other parts of the tree. Neem products containing azadirachtin and other ingredients, have antifeedant, ovipositional deterrence, repellency, growth disruption, sterility and larvicidal action against insects (Schmutterer, 1990). Neem ased pesticides are now extensively used in agricultural practices all over the world. Neem oil and other commercial preparations of neem have been found as potential mosquito larvicide (Mittal *et al*., 1995). Dhar *et al*. (1996) demonstrated the effect of neem oil volatiles on gonotropic cycle and inhibition of oviposition in *An. stephensi* and *An. culicifacies*. Control of mosquito breeding has also been demonstrated in the field in some confined habitats using indigenous methods of application of neem oil in water and neem oil coated on wooden scraps (Nagpal *et al*., 1995; Batra *et al*., 1998).

Some review articles have been written on active medicinal plants that have been tested for activities against various stages of mosquitoes (ICMR, 2003; Maia and Moore, 2011; Ghosh *et al*., 2012). The activities reported included larvicidal,

adulticidal, oviposition inhibition and repellency. Some of these activities are discussed below.

Tagetes sp., commonly known as marigold has shown both larvicidal as well as adulticidal activity against mosquitoes (Perich *et al*., 1994; Macedo *et al*., 1997; Green *et al*., 1991; Pathak *et al*., 2000). Active components have been isolated from different parts of this plant. Green *et al*. (1991) reported mosquito larvicidal activity in the extract of *T. minuta* flowers. Perich *et al*., (1994) compared biocidal activities of the whole-plant extracts of three Tagetes species and showed that *T. minuta* had the greatest biocidal effect on the larvae and adults of *Ae. aegypti* (L.) and *An. stephensi* (L). Bioassays of simultaneous steam distillated extracts of *T. minuta* flowers showed larval mortality at LC_{90}, of 4 and 8ppm and against the adult at 0.4 and 0.45% against *Ae. aegypti* and *An. stephensi*, respectively (Perich *et al*., 1994). The extract from *T. minuta* was found to be most active among 83 plant species belonging to the C ompositae family, with a LC_{50} of 1mg/l against *Ae. fiuviatilis*. Active components of *T. minuta* have also been identified as thiophene derivatives, a class of compounds present in many plants of family asteraceae (Macedo *et al*., 1997). Pathak *et al*. (2000) reported 100% mortality with steam distillated oil extract from the whole plant of Terrecta, against larvae of *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti* at doses lower than 100ppm. Ethanol extract of another plant *Eclipta paniculata* belonging to family compositae, has also shown significant insecticidal activity, with LC_{90} of 17.2 mg/1 and LC50 of 3.3 mg/l (Macedo *et al*., 1997). Leaf extract of *Polyalthia longfolia* exhibits larvicidal and growth inhibition effect against larvae of *Cx. quinquefasciatus* (Murty *et al*., 1997). Application of the extract at the dose of 250 to 350ppm produced 64- 96% inhibition of adult emergence of *Cx. quinquefasciatus* in tanks and U-drains. Another plant, *Murraya koenigii* has also

showed mosquito larvicidal activity, due to the presence of carbazole alkaloids, mahanimbine, murrayanol, and mahanine. Volatile oil from the peel of citrus fruits has also shown toxic effects on mosquito larvae as well as adults (al Dakhil and Morsy, 1999; Ezeonu *et al*., 2001).

Susceptibility tests carried out against *Cx. quinquefasciatus* larvae and adults using peel oil extracts of bitter orange (*Citrus aurantium*), orange (*C. sinensis*) and lemon (*C. limon*) indicated that the extracts may contain potentially useful insecticides. Volatile extracts of *C. sinensis* showed greater insecticidal potency (Ezeonu *et al*., 2001). The larvicidal action of three ethanol extracts of peels of lemon, grapefruit and navel orange, against *Cx. pipiens* produced LC_{50} values as 18.5, 20.3 and 26.5ppm, respectively (al Dakhil and Morsy, 1999). The peel oil fulfilled other required specifications like suitable specific gravity, spreading pressure and viscosity. It is toxic at a wide pH range, stable to heat and light in terms of chemical change, which could alter larvicidal action. However, it is volatile and did not form a permanent film on water surfaces for long periods. This affected its larvicidal action (Mwaiko, 1992).

The effects (i.e. repellent, irritant and toxic) of 20 plant extracts, mainly essential oils, were assessed by Deletre *et al*. (2013) on adults of *Anopheles gambiae*, a primary vector of malaria. Their effects were compared to those of DEET and permethrin, used as positive controls. Most of the plant extracts had irritant, repellent and/or toxic effects on *An. gambiae* adults. The most promising extracts, i.e. those combining the three types of effects, were from *Cymbopogon winterianus* Jowitt, *Cinnarnomum zeylanicum* Blume and *Thymus vulgaris* L. The irritant, repellent and toxic effects occurred apparently independently of each other, and the behavioural

response of adult *An. gambiae* was significantly influenced by the concentration of the plant extracts.

Ghosh *et al*. (2012) summarized the mosquitocidal activities of various herbal products from edible crops, ornamental plants, trees, shrubs, herbs, grasses and marine plants according to the exaction procedure developed in eleven different solvent systems and the nature of mosquitocidal activities against different life stages of different vector species as a ready reference for further studies. From this review, the plant with the lowest LC_{50} value was reported in Solenostemma argel against *Cx. pipiens* (Al-Doghairi, *et al*., 2004). Several other plants such as *Nyctanthes arbotristis* Linn (Khatune, *et al*., 2001), *Atlantia monophylla* DC (Chowdhury *et al*., 2008), *Centella asiatica* L. (Matasyoh, *et al*., 2008), *Cryptotaenia paniculata* Hassk. (Rawani, *et al*., 2010) were also reported with promising LC_{50} values. These extracts may be fractioned in order to locate the particular bioactive toxic agent responsible for larval toxicity.

A lot of phytochemicals extracted from various plant species have been tested for their larvicidal and repellent actions against mosquitoes (Ansari *et al.*, 2000). Plant derived secondary materials include, alkanes, alkenes, alkynes and simple aromatics, lactones, essential oils and fatty acids, terpenes, alkaloids, steroids, isoflavonoids, pterocarpans and lignans. Saponins (Euphorbin - A) isolated from the crude extract of *Euphorbia hirta* were found to be effective against the II and IV instar larvae of *Culex quinquefasciatus* at LC_{50} of 596.765 and 446.4621ppm respectively (Arya *et al.*, 2011). Following the bioactivity-guided fractionation procedure, the active constituent isolated from *Cryptomeria japonica* sapwood was characterized as tectoquinone by spectroscopic analyses. The LC_{50} values of tectoquinone against *A. aegypti* and *A. albopictus* in 24 h were 3.3 and 5.4 mg/ml, respectively. In addition, comparisons of

mosquito larvicidal activity of anthraquinone congeners demonstrated that anthraquinone skeleton with a methyl group at C-2 position, such as tectoquinone, exhibited the strongest mosquito larvicidal activity (Cheng *et al*., 2008).

Perumalsamy *et al*. (2009) identified 26 compounds from *Asarum heterotropoides* of which safrole was the most toxic constituent to *Cx. p. pallens* and *Ae. aegypti* larvae. Thomas *et al*. (2014) reported bioassay- guided fractionation of *G. sepium* leaf extract led to the separation and identification of 8,11,14- eicosatrienoic acid as a potential new mosquito larvicidal compound with LC_{50} value 0.011 mg/ml and LC_{90} as 0.060 mg/ml against 4th instar larvae of *Culex quinquefasciatus*. While investigating mosquito larvicidal constituents from *Lantana viburnoides* sp *viburno ides* var *kisi*. Innocent *et al*. (2008) obtained furanonaphthaquinones region-isomers (LC_{50} = 5.48-5.70ppm in 72h) and the lantadene triterpenoid camaric acid (LC_{50} = 6.19ppm in 72h) as active principles while lupane triterpenoid betulinic acid (LC_{50} < 10ppm in 72h) was obtained from the least active fraction.

Bioassay-guided fractionation of *Abutilon indicum* led to the separation and identification of a betasitosterol as a potential new mosquito larvicidal compound with LC_{50} value of 11.49, 3.58 and 26.67ppm against *Aedes aegypti* L, *Anopheles stephensi* Liston and *C. quinquefasciatus* Say (Diptera: Culicidae), respectively (Abdul Rahuman *et al*., 2008). Based on Proton NMR spectrum of the eluted compounds and their comparison with published results, three different compounds were identified from *Spilanthes acmella*, N-isobutyl-2,6,8-decatrienamide (compound 1), undeca2E,7Z,9E-trienoic acid isobutylamide (compound 2) and (2E)-N-(2-methylbutyl)-2- undecene-8,10-diynamide (compound 3) (Pandev *et al*., 2011).

Generally, the active toxic ingredients of plant extracts are secondary metabolites that are evolved to protect them from herbivores. The insects feed on

these secondary metabolites potentially encountering toxic substances with relatively non-specific effects on a wide range of molecular targets. These targets range from proteins (enzymes, receptors, signaling molecules, ion-channels and structural proteins), nucleic acids, biomembranes, and other cellular components (Rattan, 2010). This in turn, affects insect physiology in many different ways and at various receptor sites, the principal of which is abnormality in the nervous system (such as, in neurotransmitter synthesis, storage, release, binding, and re-uptake, receptor activation and function, enzymes involved in signal transduction pathway) (Rattan, 2010).

Rey *et al*. (1999a) and David *et al*. (2000) found that botanical derivatives primarily affect the midgut epithelium and secondarily affect the gastric caeca and the malpighian tubules in mosquito larvae. Histopathological effects were observed to differ qualitatively according to their location along the midgut and quantitatively according to the concentration assayed, duration of the treatment, and mosquito species used. Rey *et al*. (1999b) observed that the overall destructive effects of tannins on the midgut epithelium of larvae were more efficient than those produced by *Bacillus thuringiensis var. israelensis.*

Rattan (2010) reviewed the mechanism of action of plant secondary metabolites on insect body and documented several physiological disruptions, such as inhibition of acetylecholinesterase (by essential oils), GABA-gated chloride channel (by thymol), sodium and potassium ion exchange disruption (by pyrethrin) and inhibition of cellular respiration (by rotenone). Such disruption also includes the blockage of calcium channels (by ryanodine), of nerve cell membrane action (by sabadilla), of octopamine receptors (by thymol), hormonal balance disruption, mitotic poisioning (by azadirachtin), disruption of the molecular events of morphogenesis and

alteration in the behaviour and memory of cholinergic system (by essential oil), etc. Of these, the most important activity is the inhibition of acetylcholinesterase activity (AChE) as it is a key enzyme responsible for terminating the nerve impulse transmission through synaptic pathway; AChE has been observed to be organophosphorus and carbamate resistant, and it is well-known that the alteration in AChE is one of the main resistance mechanisms in insect pests (Senthilnathan *et al*., 2008).

The effect of a phytochemical on the inhibition of mosquito growth and reproductive capacity is governed by mosquito species, plant species, plant parts, solvents used in extraction and fractions of the same solvent. Botanical extracts, termed Insect Growth Regulators (IGRs), can have a range of preemergent effects such as prolongation of instar and pupae durations, inhibition of larval and pupal molting, morphological abnormalities and mortality especially during molting and melanization processes. Over one thousand plant species contain bioactive substance with many of these containing phytoecdysones, phytojuvenoids and anti-juvenile hormones, which act as IGRs (Varma and Dubey, 1999). The use of ajugarins, isolated from Ajuga remota by Marcard *et al.* (1986) against mosquitoes is an example. Another example is provided by Neraliya and Srivastava (1996), who tested the insect growth regulatory activity of crude petroleum ether-acetone extracts of 25 angiosperm plants on *Cx. quinquefasciatus* larvae. In this case, 40% of the extracts possessed promising bioactivity. Mehdi *et al.* (2012) observed a wide range of morphogenetic deformities in different categories including larval pupal mosaic, abnormal pupae and pupal adult mosaic according to stage of metamorphosis when death occurred due to abnormal growth and moulting.

Ghosh *et al.* (2012) reviewed various compounds isolated from medicinal plants and tested on various stages and types of mosquitoes. These compounds include geranial, germacrene d, azardirachtin, dioncophylline-a, plumbagin, pachyrrhizine, marmesin, hugorosenone,α-terpinene, (E)-6-hydroxy-4,6- dimethyl-3-heptene-2-one, N-methyl-6β-(decal', 3',5'-trienyl)-3-β-methoxy- 2-β-methylpiperidine, neoduline, 4-methoxyneoduline, methyl-p-hydroxybenzoate, β-sitosterol and pipernonaline.

The degradation of natural products under field conditions is expected to take place faster than in the laboratory because of environmental factors, such as ultraviolet light, temperature, pH and microbial activity. Several botanical derivatives have shown efficacy in mosquito control field trials, but most trials have tested neem and few have included investigation into residual and non-target effects. Both degradation and residual effects of phytochemicals are important factors since they affect toxicity and suitability for field applications (Shaalan *et al*., 2005).

From the conclusions of neem and tannin researchers, it can be surmised that botanical derivatives are not free of risk and further field investigations are necessary. Variable results have been found for neem formulations and extracts ranging from no effect to significant effects on non-target organisms. In India, Rao *et al*. (1995) applied *A. indica* to rice fields and found a marked reduction in the abundance of late instar culicine and anopheifle larvae and pupae. No significant reduction of most non-target organisms, including different aquatic insects, frog tadpoles and plant spiders was observed. Minimal non-target toxicity from neem formulations was recorded by Kreutzweiser (1987) for microinvertebrates.

Table 2.1 below contains the summary of some medicinal plants investigated against various species of mosquitoes and the level of their activities.

Table 2.1. Summary of some antimosquito plants and their activities

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Selection and Processing of Plants

After searching the internet for medicinal plants used traditionally in the treatment of various parasitic infection, the list compilled by Agarvee which contains medicinal plants used in East and West Africa was selected (APPENDIX I). A herbalist was also consulted who comfirmed some of the plants. The selected plants are those that are indigenous to this part of West Africa. They include *Combretum paniculata* Vent., *Euphorbia hirta* L., *Paulinia pinnata* L., *Aspilia Africana* C. D. Adams, *Cissampelos owariensis* P. Beauv, *Clerodendrum polycephalum* Baker, *Peltophorum pterocarpum* Backer ex Heyne, *Canivalia ensiformis* Linn. D. C., *Lycopersicon esculentum* Mill and *Phyllanthus mueleriana* (Kuntze) Exell. Plants were collected with the assistance of a taxonomist, Mr. A. T. Oladele (now Dr. A. T. Oladele) of the Department of Pharmacognosy, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife.

The leaves of each of the plants were collected from their natural habitats located in different parts of the city of Ile-Ife, between February, 2007, to July, 2007. They were oven-dried at 40^{o}C and powdered using a grinding machine, Daiki, 2002 model (APPENDIX XIX). Each of the powdered leaves were then soaked with 100% methanol and subjected to a shaker for 48 hrs. Each was filtered through a Buchner funnel with Whatman filter paper number 1 (24 cm in diameter). Crude extracts were evaporated to drynes using a rotary evaporator and stored in glass vials. Stock solutions were prepared using dimethylsulfoxide (DMSO).

3.2 Mosquito Source and Maintenance

The initial eggs used for the rearing of *A. aegypti* were obtained from Nigerian Institute for Malaria Research (NIMR), Lagos, in 2002. The eggs were immersed in water for 24 h. The newly emerged larvae, the first instar, were fed with powdered chicken feed called "grower". The feeding continued daily until the larvae passed through four instar developmental stages and then pupated. Adult mosquitoes emerged in a 30 x 30 x 30 cm cage. Emerged male adults were provided with sugar solution by soaking a cotton ball in 10 % glucose and wrapped in a muslin cloth. The sugar solutions were changed twice a week. Females were fed with blood three to four times a week, through a local fowl whose feather have been removed from one side. The exposed side was placed on the roof of the cage to provide access for female mosquitoes. Eggs were collected on moist filter paper. The filter papers were placed in plastic cups containing 50 mL of dechlorinated water. The eggs could be seen as black spots on the filter paper. The cage was checked daily for the presence of spider/cobweb which feed on adult mosquitoes. Any spider found was killed and removed immediately. The feet of the cage were surrounded with powdered insecticide (Rambo®) containing permethrin 0.6%, to prevent ants, which feed on the eggs, from entering. Colonies were maintained in the laboratory under standard conditions at (27 ± 1) °C; (75 ± 10) % relative humidity, and with a 12:12 h photoperiod.

Larvae of *Anopheles gambiae* and *Culex quinquefasciatus* mosquitoes used for the experiments were collected from their natural habitats in the field and brought to the laboratory, rinsed three times with water and allowed to aclimatise for 24 hrs before being used.

3.3 Screening of Selected Plants Using Larvicidal Test

Crude methanol extracts of selected plant leaves were tested against the larvae of *Aedes aegypti* mosquitoes obtained from the colony maintained in Drug Research and Production Unit, Obafemi Awolowo University, Ile-Ife. Test solutions of five concentrations 250, 500, 1000, 1500, 2000 ppm were prepared by diluting the stock with dechlorinated water. Tap water collected and stored for seven days was regarded as dechlorinated water. Any extract that did not show activity at these concentration range were considered to be inactive.

The larvicidal test was done according to WHO (2005b) with slight modifications. Plastic cups (Plate 3.1) with capacity of 120 ml each were arranged and 99 ml of dechlorinated water added to each. Ten mature larvae (late 3^{rd} and 4^{th} instars) were transferred into each of the cups. Larvae were first poured into a petri dish from which matured ones were picked using a cotton bud. Different quantities from the stock, equivalent to 250-2000 ppm, were transferred into cups and shaken gently to allow a good even dispersal of the extract. Mettler weighing balance was used to weigh 5 g of extract and 5 ml of DMSO added in a vial to dissolve it. From the stock, 0. 025, 0.05, 0.1, 0.2 ml were tranfered into experimental cups to give 250 – 2000 ppm. Each concentration was replicated thrice.

The leaf of a known potent natural insecticide, *Nicotiana tabacum* was collected, dried, powdered and extracted with methanol. This was concentrated to dryness before being used as positive control. Larvicidal activity was also compared with a synthetic insecticide, lambdacyhalothrin. Concentrations used for *N. tabacum*

varied from 62.50 to 1000 ppm, while that of lambdacyhalothrin (Lara Force®, manufactured by Agrotec) varied from 0.0625 to 4.00 ppm. Negative control received

Plate 3.1. Bio-assay cups containing larvae exposed to plant extract and lambdacyhalothrin

Source: Laboratory of Drug Research and Production Unit, O.A.U. (16/04/2014)

1 ml of DMSO. Mortalities were recorded after 24 and 48 h. Dead larvae were identified when they lied lifeless at the bottom of the cup or when they failed to move after disturbing the solution or after probing with a sharp object.

Regression formula was used to calculate the LC_{50} while Microsoft Excel statistical package was used to determine the significance difference.

3.4 Identification of *Clerodendrum polycephalum* Baker

The plant *Clerodendrum polycephalum* was selected for further activities being the one with the highest activity among the ten plants. Specimen sample of the plant was deposited at Herbarium Unit of the Department of Botany, O. A. U., Ile-Ife. A voucher number IFE 16962 was given to it.

3. 5 Larvicidal Assay Using Methanol Extract of *Clerodendrum polycephalum*

Larvicidal activity against *Anopheles gambiae* and *Culex quinquefasciatus* was done using procedure described above (section 3.3). Stock solution was prepared by weighing 10 g of *C. polycephalum* methanol extract into 10 ml of DMSO. Apropriate volume was transferred into test solution to attain different concentration. Thus, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8 ml of stock in 100 ml of test solution produced 250, 500, 1000, 2000, 4000, 8000 ppm respectively. Similarly, three replicates were used and negative controls received 1 ml of DMSO. Mortality was recorded after 24 and 48 hrs.

3.6 Phytochemical Analysis

Plant extraction was carried out by collecting 1.5kg of fresh leaf of *C. polycephalum* which was dried in the oven at 40°C, powdered and soaked with 100%

methanol for 72 hr under electric shaker. It was concentrated to dryness using Rotary evaporator, stored in a glass vial and kept in the refrigerator until when needed.

Phytochemical analysis of the crude methanolic extract was done to determine the presence of group of compounds such as terpene, alkaloids, saponin, anthraquinones and tannins. The tests were performed according to the methods of Edeoga *et al.* (2005) and methods of Odebiyi and Sofowora, (1978 and 1979).

3.6.1 Tests for alkaloids

Alkaloid test was done by weighing 0.5 g of the extract in 5 ml of 1% aqueous hydrochloric acid on a steam bath and stirred. lml of the filtrate was treated with three drops each of Mayer's reagent (potassium mercuric iodide solution, 1.36 g mercuric chloride and 5 g of potassium iodide, dissolved in 100 ml distilled water), Wagner's reagent (solution of iodine in potassium iodide, 2 g of iodine and 6 g of potassium iodide were dissolved in 100 ml distilled water), and Dragendorff's reagents (Dragendorff's reagent was made of two solutions. Solution A contained 1.7 g basic bismuth nitrate in 100 ml water/ glacial acetic acid (80 ml water and 20 ml glacial acetic acid in a 4:1 ratio), and solution B contained 40.0 g potassium iodide in 100 ml of water. Both solutions were mixed in the following manner to produce 100 ml Dragendorff's reagent (5 ml solution A + 5 ml solution B + 20 ml glacial acetic acid + 70 ml water). Turbidity or precipitation with the reagents was taken as evidence for the presence of alkaloids in the extract.

3.6.2 Tests for flavonoids

Ferric chloride tests: This was done by adding 1.0 g of the extract with 200 m1 of water, 12 drops of ferric chloride solution were then added. A dark colouration indicated the presence of phenolic compound.

Free flavonoid test: A mixture of 4 m1 of aqeous solution of the extract and 2 m1 of Ethyl acetate was shaken together and allowed to stand for 3 minutes. The presence of free flavonoids was indicated by production of yellow colour in ethyl acetate layer.

Lead acetate test: Aqueous solution of the extract (5 mL) was added to 1 ml of 10 % lead acetate solution. Yellow precipitate indicated presence of flavonoids.

3.6.3 Test for saponins (Frothing test)

The crude extract (1g) was dissolved in l00 ml of distilled water in a test tube. The tube was stopped and shaken vigorously for at least 30 seconds. The test tube was allowed to stand in a vertical position and observed over a 30 minutes' period. Persistence of the honeycomb froth above the surface of the liquid after 30 minutes is indicated of the presence of saponins.

3.6.4 Test for tannins

Ferric chloride test: Ferric chloride test was done by adding 5 g of extract to 10 ml of distilled water, stirred thoroughly and then filtered through a filter paper inserted into a glass funnel. Ferric chloride reagent (4 m1) was then added to the filtrate. A blue-black precipate was taken as evidence for the presence of tannins.

Bromine water test: Five drops of bromine water were added to 1 ml of the water extract. A precipitate indicated the presence of condensed tannins.

Formaldehyde test: To a small portion (0.2 g) of each of the water extract was added a drop of formaldehyde solution and 3 drops of 10% hydrochloric acid. A precipitate was taken as evidence of the presence of hydrolysable tannins.

3.6.5 Test for anthraquinones derivatives

Borntrager's test: From the crude extract stored in a bottle, 0.5 g of the extract was weighed in a separate dry test tube and 10 ml of chloroform was added and shaken for 5 minutes. The extract was filtered and equal volume of ammonia was added to the

filtrate and shaken. A bright pink colour in the upper aqueous layer is an indication for the presence of free anthraquinones.

Modified Borntrager's test: From the crude extract, 0.5 g was weighed and boiled with 10 ml of 10 % hydrochloric acid for 2 minutes. The extract was filtered. To the filtrate was added equal volume of chloroform. The solution was transferred into a separating funnel and the two layers were allowed to separate. The lower chloroform layer was poured into a clean test tube and 10 % ammonia solution was added and shaken. The two layers were again allowed to separate. A bright pink colour in the upper aqueous layer is an indication for the presence of free and/or combined anthraquinones.

3.7 Chromatographic Techniques

3.7.1 Vacuum Liquid Chromatography (VLC) separation of methanolic extract of *Clerodendrum polycephalum* leaf

After determining the phytochemical content of the crude methanol extract, it was subjected to VLC separating techniques. From the crude extract, 50 g was chromatographed on a column of silical gel (60 - 120 mesh). The gel containing the extract was packed into a separating funnel and connected to a vacuum pump (APPENDIX XVI). A filter paper of the same width with the funnel was placed on top of the mixture. The extract was eluted using N-hexane, Dichloromethane, ethyl acetate and ethanol successively (Table 3.1). The eluents were monitored by Thin Layer Chromatography (TLC). The TLC was developed in a TLC tank (APPENDIX XVII) containing appropriate solvent that separated the mixture. Those that have similar TLC components were bulked together. Eleven fractions were obtained and after pooling them together, they were reduced to four: CPH1- CPH3; CPH4 – CPD3;

CPA1-CPA2; CPE1 – 2. Each was evaporated to dryness and tested for larvicidal activities.

Table 3.1. Solvent system for separation of crude extract of *Clerodendrum polycephalum* by Vacuum Liquid Chromatography (VLC)

Solvent	Volume used	Code
a. N-Hexane	0.35L	CPH1
b. N-Hexane	0.65L	CPH2
c. N-Hexane	1.0L	CPH3
d. N-Hexane	0.5L	CPH4
e. Dichloromethane (DCM)	1.0L	CPD1
f. DCM	2.0L	CPD2
g. DCM	1.0L	CPD3
h. Ethyl acetate	0.6L	CPA1
i. Ethyl acetate	0.3L	CPA2
j. Ethanol	1.75L	CPE1
k. Ethanol	1.9L	CPE2

3.7.2 Spray Reagent (Vanillin sulphuric acid)

This is a universal detector that detects a wide range of compounds by producing different colours after spraying. The reagent was made by dissolving 1 g of Vanillin in 100 ml of concentrated sulphuric acid to make Vanillin sulphuric acid spray. After spoting of the fractions on a TLC plate, it was immersed in a glass iodine tank containing appropriate solvent (DCM: Ethanol 3:2/7:3) to separate the components. The TLC plate was brought out and sprayed with Vanillin sulphuric acid, which was heated on a hot plate for 1-2 minutes. Flavonoids appear as yellow/orange spots, terpenoids generally produce pink/orange/purple reactions and phenolics appear as yellow/grey colour.

3.7.3 Thin layer Chromatographic (TLC) plates

Pre-coated Merk silical gel 60 F_{254} aluminium plates (20 x 20cm) were used to monitor the various fractions. Small sizes were cut each time to carry out the monitoring.

3.7.4 Larvicidal testing of Vacuum Liquid Chromatography (VLC) fractions

Four fractions obtained from the VLC fractions were tested against the larvae of *Aedes aegypti* in order to determine which solvent extracted the active ingredients. The four fractions were N-hexane, Dichloromethane, Ethyl-acetate and ethanol. Larvicidal tests were carried as described in section 3.3 (page 75 above). Experiments were set up in triplicate using 250 – 2000 ppm. Mortality was read after 24 h.

3.7.5 Column Chromatography

The ethanolic fraction obtained from the VLC was subjected to column chromatographic separation and purification. 11 g of the fraction was weighed and chromatographed on a column of silica gel (60 - 120 mesh). The components were eluted using DCM and Methanol in varying combinations (from non-polar to polar

solvents) as contained in Table 2. The first solvent used was 100 % DCM, followed by DCM: Methanol (9:1); then DCM: Methanol (8:2) until it reached DCM: Methanol (1:9); and finally, 100 % Methanol (Table 3.2). The eluents were monitored by TLC and those that had similar components according to UV spectroscopy, were pooled together. Two hundred and thirteen (213) sub-fractions (20 m1 each) were obtained and after pooling them together, they were reduced to 8 (CP1-8). Each was evaporated to dryness, spotted on a TLC plate and examined under the UV. The solvent system that gave the best separation on the TLC plate was DCM 7:3 Ethanol. Other DCM and Ethanol solvent system ratios tried included 9:1, 4:1, 5:5 and 4:2.

The subfraction that was UV active, by virtue of its characteristic light blue flourecence at 366 nm, was identified. The subfraction, CP 4 was found to be UV active and was processed further using Gas Chromatography - Mass Spectroscopy (GC-MS) in order to identify specific compounds in it.

3.7.6 Detection Methods

Ultra-violet (U. V.) light was used for locating fluorescent compounds. The chromatograms on the TLC plates were examined under short (254 nm) or long (366 nm) wavelengths.

3.7.7 TLC - Retention factor (R_f)

The retention factor, or R_f is defined as the distance traveled by the compound divided by the distance traveled by the solvent (Sofowora, 1993) on the plate.

$$R_f = \frac{\text{distance traveled by the compound}}{\text{distance traveled by the solvent front}}$$

Table 3.2. Solvent system for column chromatographic purification of ethanolic fraction of *Clerodendrum polycephalum*

Solvent system		Tube number	Volume used (ml)
a. DCM	(100%)	1 – 27	540
b. DCM:Methanol	(9:1)	28 – 68	800
c. DCM : Methanol	(8:2)	68 - 86	360
d. DCM:Methanol	(7:3)	87 - 117	600
e. DCM : Methanol	(6:4)	118 - 145	540
f. DCM: Methanol	(5:5)	146 - 159	260
g. DCM : Methanol	(4:6)	160 - 172	240
h. DCM : Methanol	(3:7)	173 - 184	220
i. DCM:Methanol	(2:8)	185 - 191	120
j. DCM : Methanol	(1:9)	192 - 195	60
k. Methanol	(100%)	196 – 213	340

3.7.8 Analysis of subfraction CP4 by Gass Chromatography-Mass Spectroscopy

The analysis of the sub fraction was carried out on an Agilent Technologies (GC 7890; MSD 5975) (APPENDIX XVI). An HP-5 MS fused silica capillary column (30 m, 0.25 mm, 0.2511 m film thickness) was directly coupled to the mass spectrometer. It was composed of 5% phenyl methyl siloxane. The carrier gas was Helium at a constant flow of 1.4123 mi/mm and split ratio of 50:1 was employed. The temperature programme used was 9 minutes isothermal at 50°C, then 50 - 280 °C at a rate of 5°C/minute then held isothermal for 5 minutes. The pressure was 1.5 psi and average velocity of 43.311 cm/sec. The total run time was 60 minutes. The individual constituents were identified by the ir identical retention indices, by comparing their mass spectra with the Mass Spectroscopy data bank. The entire fractionation and isolation procedures, from the beginning to the end is summarized in a flow chat (Figure 3.1).

3.7.9 Flow chart of bioactivity-guided fractionation and isolation of active compound in *Clerodendrum polycephalum*.

Bioactivity-guided fractionation and steps leading to the isolation of active compound are summarized in the flow chart (Fig. 3.1).

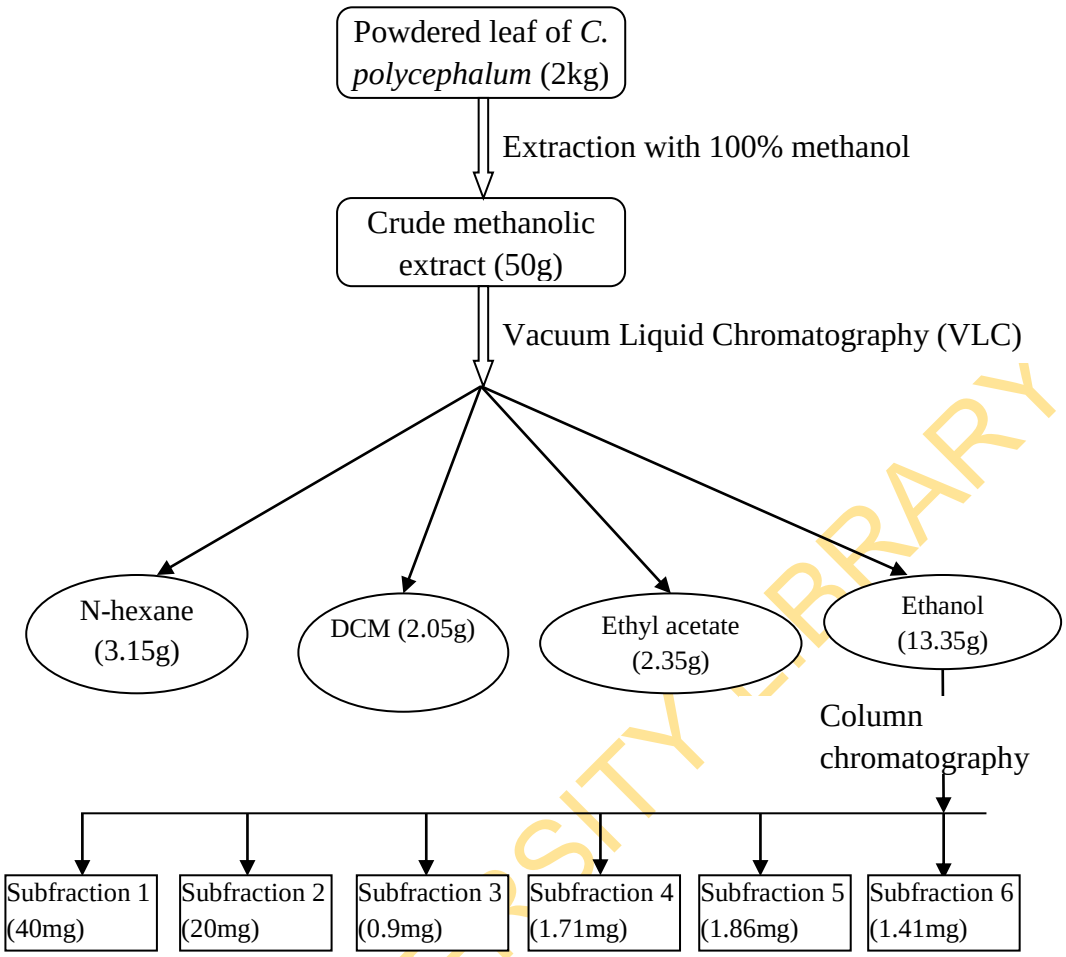


Figure 3.1. Flow chart summarizing bioactivity-guided fraction and identification of active compound

3.8 Other Biological Activities Carried Out With Crude Extract of *Clerodendrum polycephalum*

3.8.1 Effects on growth and development of mosquito larvae

Insect growth regulator assay of WHO (1996) was followed. Third instar larvae of each of *Aedes aegypti*, *Culex quinquefasciatus* and *Anopheles gambiae* mosquitoes were exposed to three sub-lethal concentrations (LC_{25}, LC_{50} and LC_{75}) obtained from their larvicidal activities results. Against *A. aegypti* mosquito, sub-lethal concentrations used were 560, 1120 and 1675 ppm; *C. quinquefasciatus* sub-lethal concentrations were 240, 4800 and 7200 ppm while *A. gambiae* sub-lethal concentrations included 1875, 3750 and 5625 ppm. Concentrations were determined by weighing respectively, 0.56, 1.12, 1.675, 0.24, 4.80, 7.2, 1.875, 3.75 and 5.625 g separately and dissolving it in 1 ml of DMSO before introducing it into 99 ml of experimental water. Four replicates of 10 larvae each were used for each concentration. Larva and pupa mortality as well as adult emergence were recorded daily up to emergence of all the adults or death of the last larva or pupa. From the test results, percentages of both emerged and dead pupae and percentage of adult emergence, larval duration, and adult emergence inhibition (% IE) were determined.

Percent adult emergence inhibition is calculated thus:

$$\%IE = 100 - \frac{(\text{T x } 100)}{C}$$

where T adult survival in treated medium and

C = adult survival in control.

3.8.2 Oviposition preference

The method employed was that of Mohsen *et al*. (1990). Four 200ml cups containing 100 ml of the crude extract in solution with sub lethal concentrations and controls were placed inside a one - cubed cage. Twenty (20) blood-fed female *Aedes aegyti*

mosquitoes were exposed to the extract in solution. The mosquitoes were fed for at least three days before the commencement of the test. The cups were observed daily for eggs for 7 days.

Percent inhibition of oviposition is expressed as anti-oviposition index 1 and calculated as follows:

100 x [(Ec - Ee)/ Ec] where

Ec is the number of eggs laid in the control, Ee is the number of eggs laid in the treated dishes.

3.8.3 Effects on mosquito pupae

Ten (10) pupae of each species of mosquito were transferred into plastic cups containing different concentrations of the extract and left for 48h. For each concentration, there were four replicates. Each replicate was set up in a separate cage. Observations were made on number of adults that emerged and alive, dead or moribund pupae. Moribund pupae were those that showed discoloration or occasional jerking movement when the water was disturbed.

3.8.4 Effects on non-target organisms

Two representative organisms were selected for testing. They included the tadpoles of *Bufo regularis* and fresh water snails, *Biophalaria glabrata. B. regularis* tadpoles spend a short time in water, while *B. glabrata* spends its entire life in water. Tadpoles were collected from a nearby stream behind our laboratory at Drug Research and Production U nit, O. A. U., Ile-Ife. *B. glabrata* were picked from a laboratory colony.

3.8.5 Rearing and maintenance of *B. glabrata*

Rearing was done using dechlorinated water in plastic bowls. Decchlorination was done by collecting tap water in big plastic drums and allowing it to stand for a week. To feed the snails, waterleaf (*Talinum triangulare*) plants were collected, dried

in the oven and powdered. Small quantities, two pinches to start with, were introduced into the water. Lettuce were also used occasionally to feed the snails. Egg masses of the snails were laid on the bottom, walls of containing bowls as well as pieces of polythene bags cuts from used sachet water bags and inserted into the water.

Once weekly, the water was changed to remove waste and ensure provision of well-aerated water for the snails. Once monthly, the containers were washed thoroughly to ensure a clean environment for maximal growth and fecundity. Mature snails were removed from the bowls and transfered into separate bowls to allow juvenile snails feed and grow separately.

3.8.6 Molluscidal activity of the methanol extract of *Clerodendrum polycephalum*

WHO (1996) procedure for testing was also used. The extract was diluted in DMSO (dimethyl sulphoxide) and varying quantities delivered into experimental glass beakers containing snail water (250 ml) to attain 250, 500, 1000, and 2000 ppm. Ten snails (3 weeks old) were transferred into the beakers in duplicates. The mouths of the beakers were covered with net to prevent snails from crawling out of the test solutions. Mortality was checked after 24 h. Dead snails were kept in clean water for another 24 h to confirm their mortality. Those than did not show any sign of life were then regarded as dead. Positive control received niclosamide, while negative control received 2 mL of DMSO. The LC_{50} was calculated.

3.8.7 Testing of the methanol extract of *Clerodenrum polycephalum* on tadpoles

In testing for the activity of *C. polycephalum* against tadpoles, concentrations from 250, 500, 1000 and 2000 ppm were also used in four replicates. Stock solution was also prepared as in section 3.5 above. Tadpoles were also washed by transferring them into fresh dechlorinated water thrice before being used. Each experimental

plastic cup received 100 ml of water into which right volume of extract, already dissolved in DMSO, was introduced. Each cup was shaken gently to spread the extract. Ten tadpoles were transferred into each cup. Mortality was checked after 24 h.

CHAPTER FOUR

RESULTS

4.1 Screening of Selected Plant

The result of the ten selected plants that were tested for larvicidal activity is presented in Table 4.1. Five plants were active within the the concentration range 250 – 6,000 ppm, and five were inactive. The order of increasing activity (LC_{50} values) were *Cissampelos owariensis* (5060 ppm) < *Lycopersicum esculentum* (2702 ppm) < *Euphorbia hirta* (2400 ppm) < *Phyllanthus mueleriana* (1458 ppm) < *Clerodendrum polycephalum* (1122 ppm). The inactive plants were *Combretum paniculata, Paulinia pinnata, Aspilia africana, Peltophorum pterocarpum* and *Canavalia ensiformis*.

4.2 Larvicidal Activities

Table 4.2 shows the larvicidal activities of the methanolic crude extract of *C. polycephalum* leaf on *Aedes aegypti* mosquito. The extract showed a concentration dependent activity against the larvae. As the concentration increased, mortality also increased. The minimum effective concentration, after 24 h, was recorded at 250 ppm which resulted in 46.67 % mortality. At 500 ppm, the mortality increased to 50 %, while at l000ppm, it went further up to 76.6 %. The l500 ppm and the 2000 ppm resulted in 83.33 and 96.67 % mortality respectively. Negative control did not produce any mortality.

The observed effect of the extract of *C. polycephalum* on *C. quiquefasciatus* was also dependent on the concentration used (Table 4.2). However, the concentration required for effective mortality doubled the concentration required for *Aedes aegypti* larvae (250 – 2000 ppm). At 24h, the minimum concentration producing 13.33% mortality was 2000 ppm. The mortality went up to 30% at 2,000ppm. While 4,000 and 8,000 ppm produced 40 and 90 mortalities repectively.

Table 4.1. Screening of leaves of selected medicinal plants for larvicidal activities

Plant species (Family)	Common name	LC_{50}(ppm)
Clerodendrum polycephalum (Lamiaceae)	Bagflower	1122
Phyllanthus mueleriana (Euphorbiaceae)	Gooseberry	1458
Euphorbia hirta (Euphorbiaceae)	Asthma plant	2400
Lycopersicum esculentum (Solanaceae)	Tomato	2702
Cissampelos owariensis (Menispermaceae)	Velvet leaf	5060
Combretum paniculata (Combretaceae)	Forest flame-creeper	NA
Paulinia pinnata (Sapindaceae)		NA
Aspilia africana (Asteraceae)	Sweet gum	NA
Peltophorum pterocarpum (Fabaceae)	Haemorrhage plant	NA
Canavalia ensiformis (Fabaceae)	Yellow Flame tree Jack Bean	NA

NA= Not active

Table 4.2. Larvicidal activity of the crude methanol extract of *Clerodendrum polycephalum* leaf against *Aedes aegypti.*

Concentration (ppm)	24h %mortality±SD	48h %mortality±SD
250	46.67+0.50	50.00±0.82*
500	50.00±0.82	53.33+0.47*
1000	76.67±1.24	80.00±0.0*
1,500	83.33±0.47	86.67±0.47*
2,000	96.67±0.47	100*
Control	0.00	0.00
LC_{50}	1122ppm	1231ppm
95% confidence interval (lower and upper limits) =	50.2969 – 116.3731	53.3744 – 124.402

*significant at $p<0.05$.

The effects of the extract on *A. gambiae* was similar to that of *Culex quinquefasciatus* (Table 4.3). The larvae of *C. quinquefasciatus* and *A. gambiae* were more resistant to the toxic effect of the extract than *A. aegypti* larvae. At 24h, the mortalities ranged from 23.33 to 100% mortality when the concentration used varied from 1,000 to 8,000 ppm. Complete mortality was recorded at 8,000 pppm while control mortality was zero.

Mortalities at 48 h were significantly different from 24 h mortalities for the three mosquito species.

The extract of *Nicotiana tabacum* was used as a positive control of a natural agent. It was also compared to a synthetic compound lambdacyhalothrin. The effects of the two materials are shown in Tables 4.5 and 4.6. It showed concentration-depended mortality. Activities of a natural agent ranged from 62.5 - 1000 ppm resulting in 6.67 – 100 % mortality for *A. aegypti*, 0.0 – 87.5 % mortality for *C. quinquefasciatus* and 3.3 – 90.0 % mortality for *A. gambiae* mosquitoes within 24 hr. That of the synthethic agent ranged from 0.0625 - 4.0 ppm. *Aedes aegypti* mosquitoes were more susceptible to the lethal effects of *N. tabacum* than *Anopheles gambiae* and *Culex quinquefasciatus*.

Figure 4.1 summarises the larvicidal activities of the methanol extract against the three species of mosquitoes with that of *Nicotiana tabacum* as positive control.

Table 4.3. Larvicidal activity of crude methanol extract of *Clerodendrum polycephalum* leaf against *Culex quinquefasciatus*

Concentration (ppm)	24h %mortality±SD	48h %mortality±SD
250	0	0
500	0	0
1000	0	0
2,000	13.33±0.47	26.67±0.47*
3,000	30.00±0.00	36.67±0.47*
4,000	40.00±0.87	46.67±0.96*
8,000	90.00±0.00	100±0.00*
LC_{50}	4794.4ppm	4152.25ppm
95% confidence interval (lower and upper limits) =	0.73613 -21.760	10.217 -15.759

* Significant at $p<0.05$.

Table 4.4. Larvicidal activity of crude extract of methanol extract of *Clerodendrum polycephalum* leaf against *Anopheles gambiae*

Concentration (ppm)	**24h %mortality±SD**	**48h %mortality±SD**
250	0.00	0.00
500	0.00	0.00
1,000	23.33±0.47	36.67±0.47*
2,000	30.00±0.0	50.00±0.00*
4,000	53.33±0.47	73.33±0.47*
8,000	100	100
Negative Control	0.00	0.00
Positive control (0.4ppm)	100	100
LC_{50}	3750 ppm	3760 ppm
95% confidence interval		
(lower and upper limits) =	0.2069,19.0640	12.0 140, 51.32329

*significant at $p<0.05$.

Table 4.5. Larvicidal activities of *Nicotiana tabacum* against the larvae of *Aedes aegypti, Anopheles gambiae* and *Culex quinquefasciatus.*

Conc. (ppm)	*Aedes aegypti*		*Culex quinquefasciatus*		*Anopheles gambiae*	
	24h (% mortality)	48h (% mortality)	24h (% mortality)	48h (% mortality)	24h (% mortality)	48h (% mortality)
62.5	6.67±0.47	10.00±0.81	0.0	3.33±0.47	3.33±0.47	6.67±0.47
125	20.0±0.00	26.67±0.47	13.33±0.	20.00±0.8	16.67±0.4	20.00±0.8
250	36.67±0.4	43.33±0.47	58	1	7	1
500	7	70.00±0.0	30.00±0.	36.67±0.4	33.33±0.4	40.00±0.4
1000	63.33±0.4	100	81	7	7	7
	7		50.00±0.	66.67±0.4	53.33±0.4	76.67±0.4
	100		0	7	7	7
			83.33±0.	100	90.0±0.81	96.67±0.4
			47			7

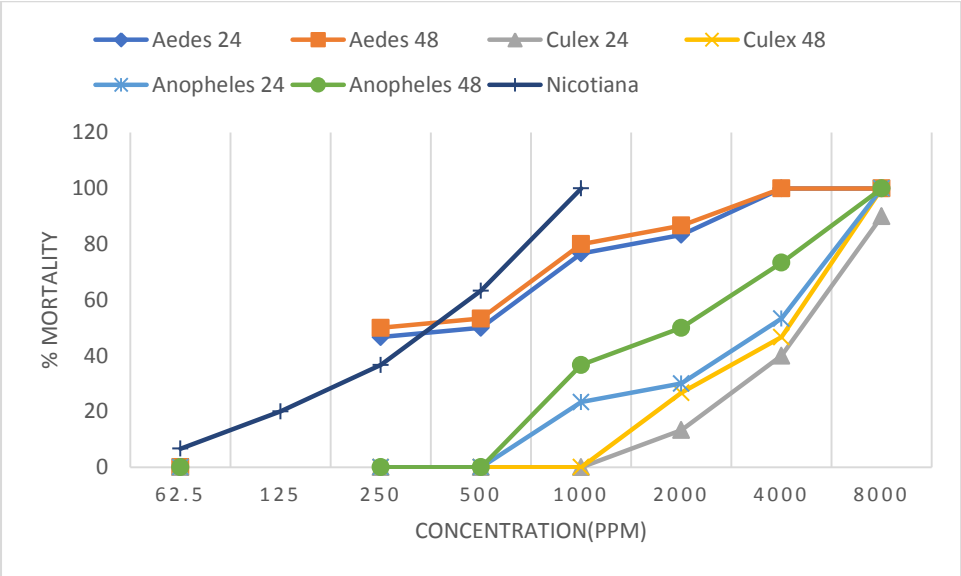


Figure 4.1. Larvicidal activities of methanol extract of *C. polycephalum* against *Aedes aegypti, Anopheles gambiae,* and *Culex quinquefasciatus*

Table 4.6. Larvicidal activity (±SD) of *Aedes aegypti* larvae exposed to Lambdacyhalothrin

Concentration (ppm)	% mortality
0.0625	26.26±0.47
0.125	43.33±0.47
0.25	53.33±0.47
0.50	70.00±0.00
1.00	80.00±0.81
2.00	93.33±0.47
4.00	100.00

LC_{50} = 0.44 ppm

4.3 Phytochemical analysis

Screening of the crude extract for the presence or absence some group of compounds revealed that the leaf extract is rich in flavonoids, alkaloids, tannins and saponins (Table 4.7). Three out of four tests carried out to detect flavonoids were positive, while two out of four tests carried out to detect alkaloids were positive. The two anthraquinone tests done were negative. Tests carried out to detect saponins and tannin were, however, positive.

4.4 Extract yield

When 700 g of the powdered leaf were soaked in methanol for three days, after filteration and concentration, it yielded 68g of crude extract i. e. 9.7 % yield (Table 4.8). The different solvents used to elute from Vucuum Liquid Chromatography (VLC) gave rise to the following: N-hexane, 3.15 g (4.6%) DCM, 0.65 g (0.96%), ethyl acetate, 2.35 g (3.46%) and ethanol, 13.75 g (20.22%).

4.5 Larvicidal activity of VLC fractions

In order to determine the most active fraction, the fractions were tested for their larvicidal activities against the larvae of *Aedes aegypti* mosquito (Table 4.9). Dichloromethane (DCM) fraction and ethyl acetate fractions were not active at l000 ppm while N-hcxane and ethanolic fractions showed some levels of activities. The activity of N-hexane fraction was very low with 10 % mortality at 2000 ppm. Ethanol fraction however was the most active with 100 % mortality at 2000 ppm.

Table 4.7. Phytochemical constituents of the crude methanol extract of *Clerodendrum polycephalum*

Compound	Test	Positive/negative
Flavonoids	Ferric chloride/Aluminium chloride	+
	NaOH	+
	Free flavonids	-
	Lead acetate	+
Alkaloids	Draggendroff	+
	Mayer	-
	Wagner	+
Anthraquinone	Borntrager's	-
	Modified Borntrager's	-
Saponin	Froth test	+
Tannins	Ferric chloride	+

Table 4.8. Percentage yield of crude extract and Vacuum Liquid Chromatoraphy fractions

Fractions	Weight (g)	Percent (%) Yield
Crude methanolic extract	68	9.7
N-hexane extract	3.15	4.60
Dichloromethane (DCM)	0.65	0.96
Ethyl-acetate	2.35	3.46
Ethanol	13.75	20.22

Table 4.9 Percent mortalities (±SD) of Vacuum Liquid Chromatography (VLC) fractions of *Clerodendrum polycephalum* against the larvae of *Aedes aegypti*

Conc. (ppm)	DCM	N-hexane	Ethyl-acetate	Ethanolic
250	0.0	0.0	0.0	0.0
500	0.0	3.33±0.47	0.0	33.33±0.47
1000	0.0	6.67±0.47	0.0	73.33±0.47
2000	0.0	10.00±0.81	0.0	100.0

4.6 Purification and Identification of compounds

The chromatographic properties of the bulked fractions eluted from the VLC are presented in Table 4.10 and as seen on Plate 4.1. The solvent system that gave the best separation was DCM 7: 3 Ethanol. Other solvent systems that did not separate distinctly were DCM: Ethanol in ratios 4:1, 4:2, 5:5 and 9:1. The R_f values of the fractions ranged from 0.0 to 0.29. After spraying with vanillin-sulphuric acid, the different compounds contained were separated with different colours. Noticeable colours were purple blue and brown. The first three colour showed purple, followed by blue and the last three showed brown.

The active ethanolic fraction was subjected to column chromatography in order to purify and isolate active compounds. A total of two hundred and thirteen fractions were obtained. After monitoring them using TLC plates (APPENDIXES II – VI), those with similar profiles were bulked together, and were reduced to eight. The chromatographic properties of the eight bulked fractions are presented in Table 4.11 and as seen on Plate 4.2. The ethanol fraction was separately spotted on another TLC plate in order to properly separate the content using another solvent system (Plate 4.3). The R_f values of the eight compounds ranged from 0.0 to 0.92.The TLC of the most active fraction is shown on Plate 4.4.

Table 4.10. Chromatographic properties of Vacuum Liquid Chromatography fractions

Spots	Retention factor (R_f values)	Colour after spraying
1	0.0	Purple
2	0.0	Purple
3	0.0	Purple
4	0.29	Blue
5	0.29	Blue
6	0.29	Brown
7	0.0	Brown
8	0.0	Brown

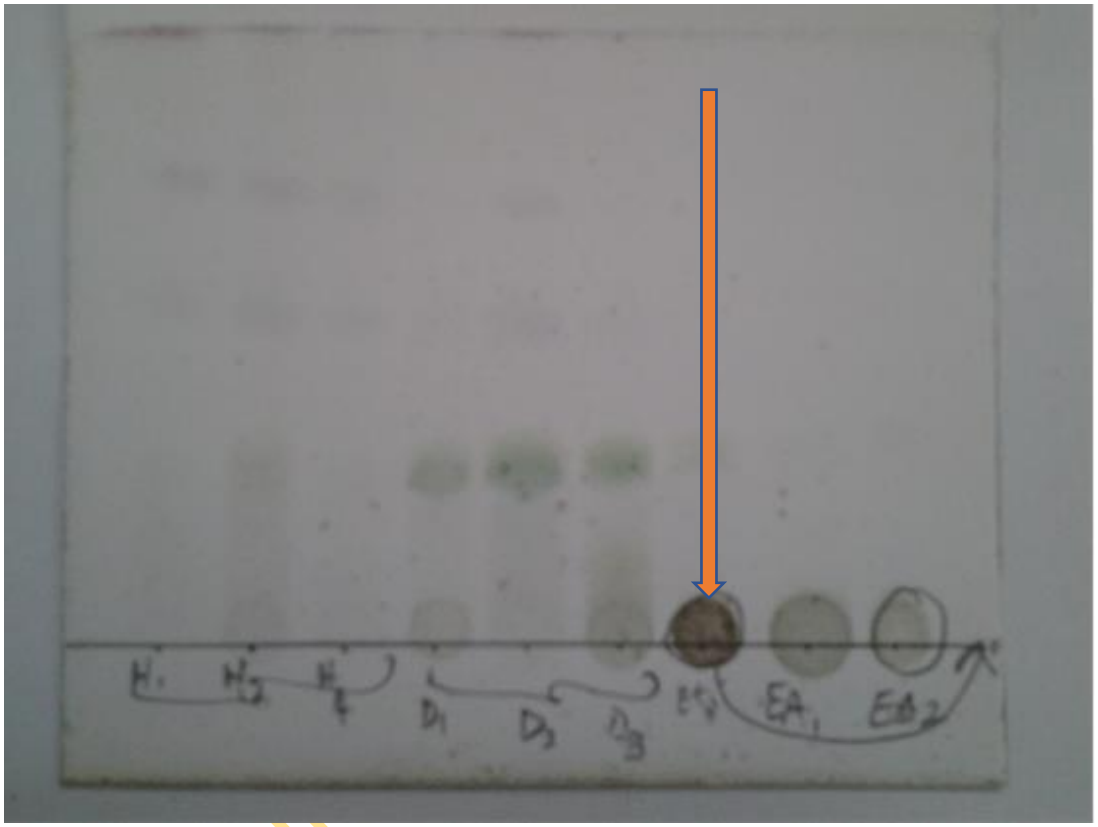


Plate 4.1. Thin Layer Chromatography (TLC) plate of fractions obtained from Vacuum Liquid Chromatography (VLC) of *Clerodendrum polycephalum* after spraying with Vanillin-sulphuric acid (ethanol spot arrowed)

Key: H_1 – EA_2 represent nine fractions eluted from VLC fractions. H_1 – H_4 are N-Hexane fractions; D_1 – D_3 are DCM fractions; EA_1 and EA_2 are Ethyl acetate fractions; E_t is ethanol fraction obtained last. (Viewed at long wave length, 366nm)

Plate 4.2. Thin Layer Chromatography **(**TLC) Plate showing separation of ethanol fraction arrowed in Plate 4.1.

Key: EA= ethyl acetate fraction; ET= ethanol fraction

Table 4.11 Chromatographic properties of bulked fractions obtained from column

Spots	Retention factor (R_f values)	Colour before spraying	Colour after spraying
1	0.92	Dark grey	Purple
2	0.0	Grey	Blue
3	0.0	Grey	Blue black
4	0.83	Light brown	Chocolate
5	0.48	Brown	Light brown
6	0.55	Grey	Brown
7	0.0	Grey	Grey
8	0.0	Grey	Grey

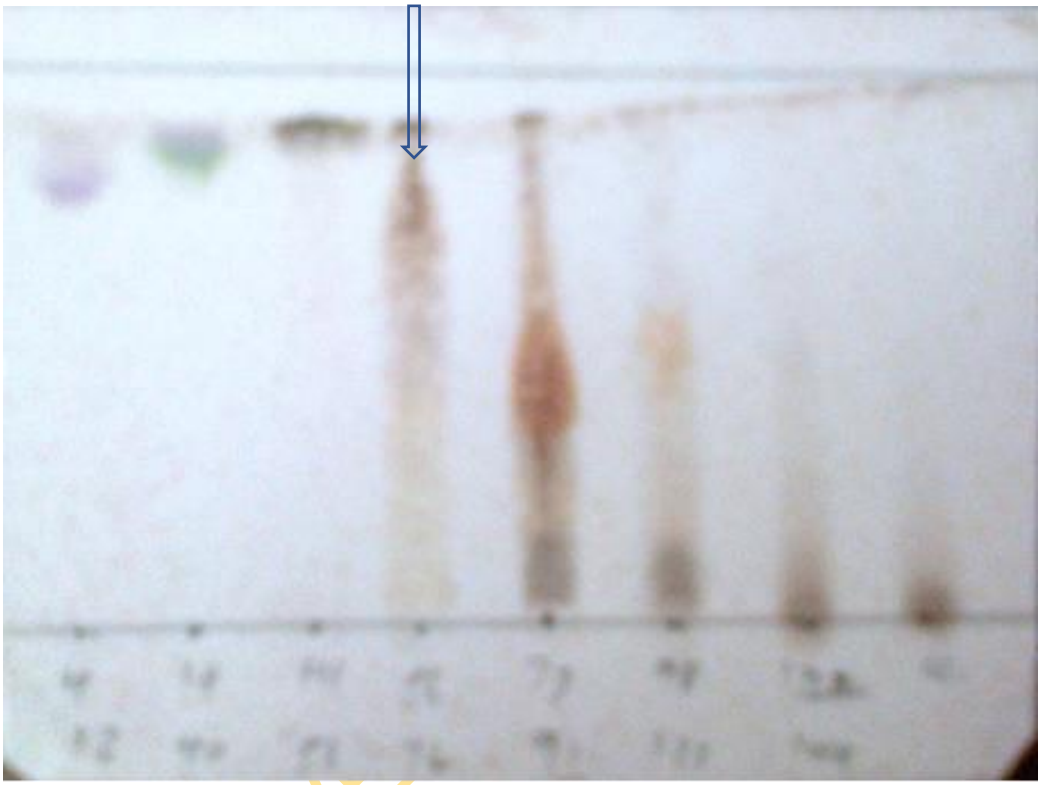


Plate 4.3. Thin Layer Chromatography (TLC) plate of the most active Fraction

Key: Eight spots were seen in this plate. The 4th spot (arrowed) showed characteristic blue flourescence under UV and was scrapped, filtered and sent for GC-MS analysis

4.7 Profiles of Compounds Identified By GC-MS

Seventeen compounds were identified from the subfraction 4. The GC-MS profile of the compounds is highlighted in Table 4.12 while the chromatogram is shown in Figure 4.2. Some of these compounds' characteristics did not fully match those in the GC-MS database, while some matched with appreciable percentages and their identities are also known. Five compounds had ≥90 % matching, five had between 50 and 90 % matching; the remaining seven had below 50 % matching. The first compound has a Retention Time (RT) of 23.926 min while the last compound has a RT of 58.813 min since the experiment was done within an hour. The one with the highest quantity (23.69 %) was the eighth compound with a RT of 48.984 min while the compound with the smallest amount is number fourteen on the list with a RT of 51.103 min, % area of 0.05 and 42 % in quality. Those compounds that matched the content in the database of the GC-MS machine and of highest qualities were identified. They include: 2- Hydroxy-5-methylbenzaldehyde, Ethyl iso-allocholate and 4-((1E)-3-Hydroxy-1- propenyl)-2-methoxyphenol with molecular weight of 136.14792, 436.62456 and 180.20048 g/mol respectively (Table 4.13). Their structures are also presented in Figures 4.3 – 4.5.

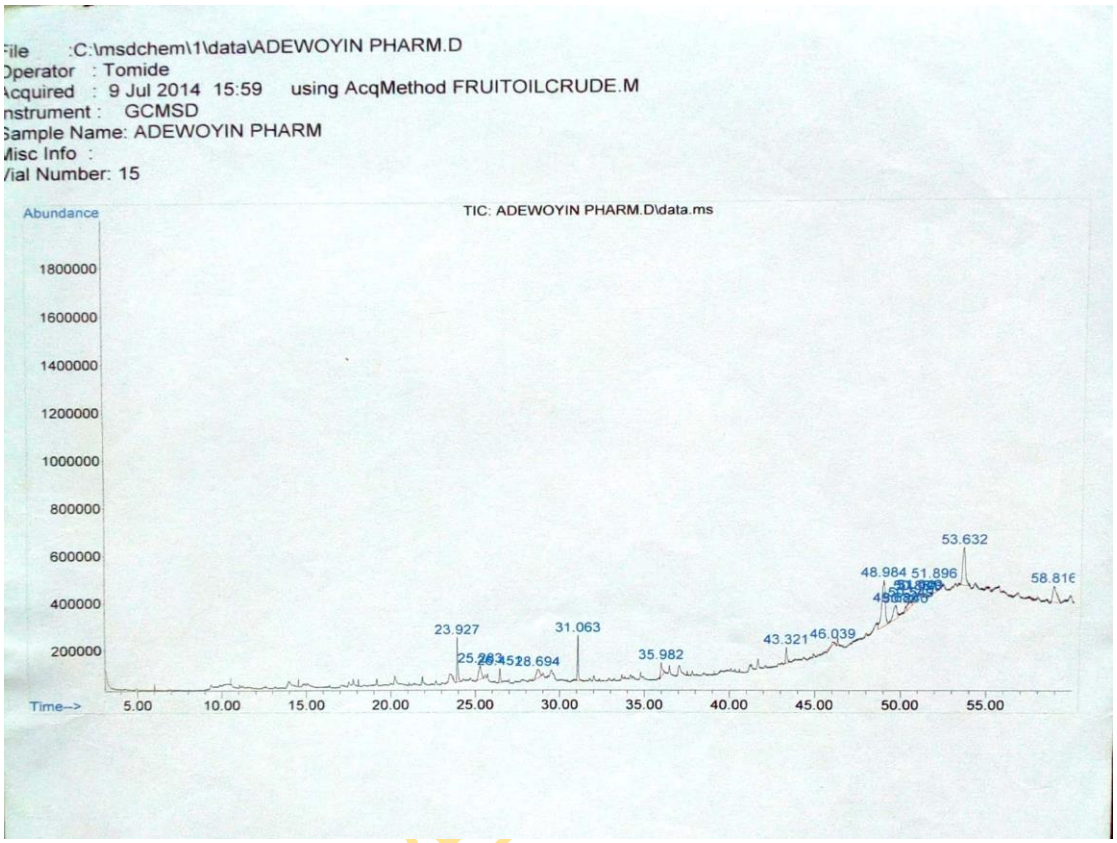


Figure 4.2. Chromatogram of ethanol subfraction of *Clerodendrum polycephalum*

Table 4.12. Profiles of compounds identified by GC-MS machine

Compd	Retention Time (min.)	Area (concentration)	% Matching Quality
1	23.926	7.57	50
2	25.285	5.64	53
3	26.448	2.32	94
4	28.692	4.30	38
5	31.066	6.07	92
6	3.980	3.37	95
7	43.322	3.16	35
8	48.984	23.69	95
9	49.691	7.50	47
10	50.243	1.14	46
11	50.551	3.19	55
12	50.860	3.62	64
13	51.050	1.00	49
14	51.103	0.05	42
15	51.899	3.31	43
16	53.632	15.12	93
17	58.813	8.58	70

Table 4.13. Profiles of three notable compounds identified by GC-MS

Compound	RT (min)	Molecular formular	Molecular weight	Peak (%)
2-Hydroxy-5-methylbenzaldehyde	23.926	$C_8H_8O_2$	136.14792 g/mol	7.5
Ethyl iso-allocholate	48.990	$C_{26}H_{44}O_5$	436.62456 g/mol	23.69
4-((l E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	3 1.060	$C_{10}H_{12}O_3$	180.20048 g/mol	6.07

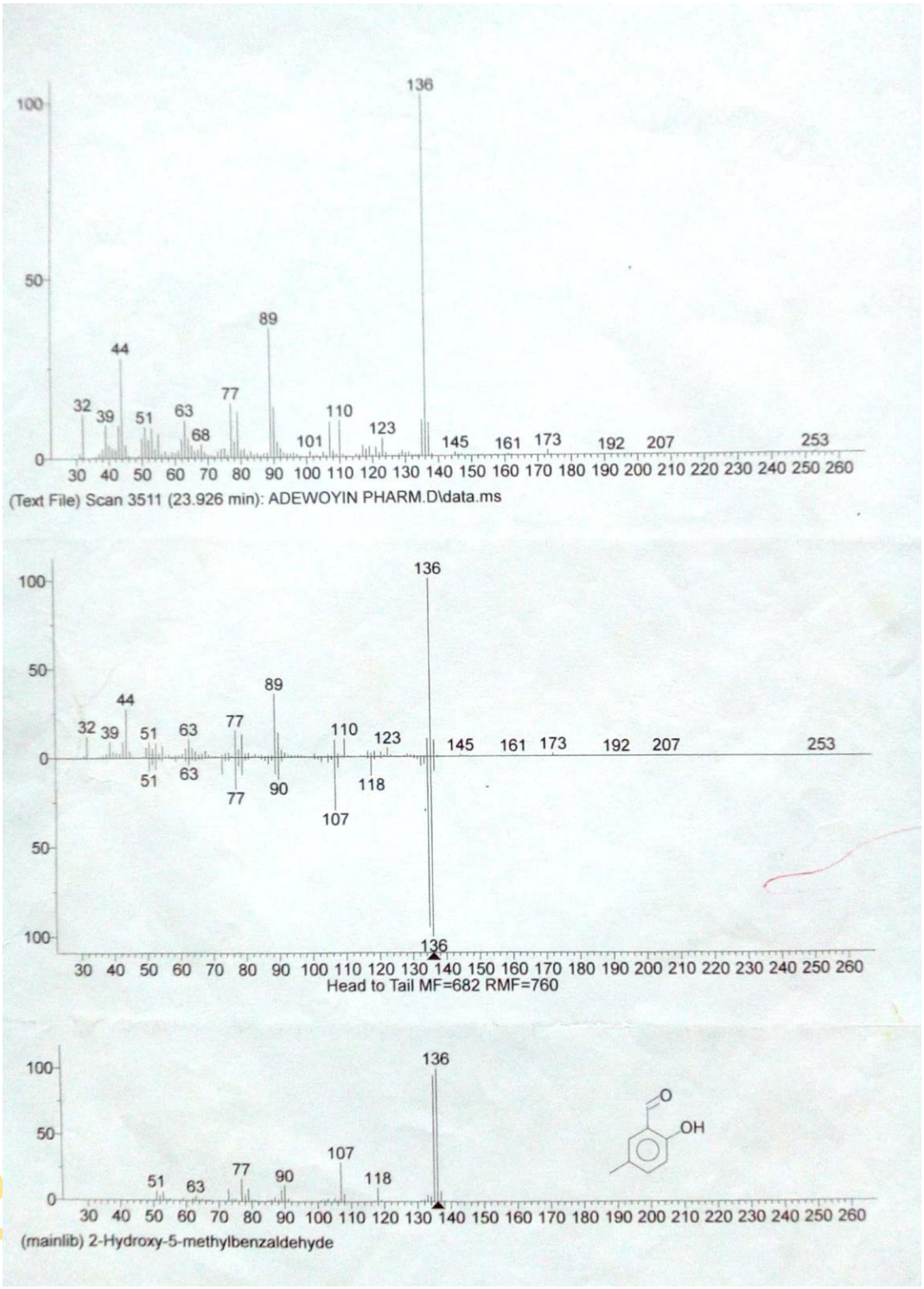


Figure 4.3. Structure of 2-Hydroxy-5-methylbenzaldehyde

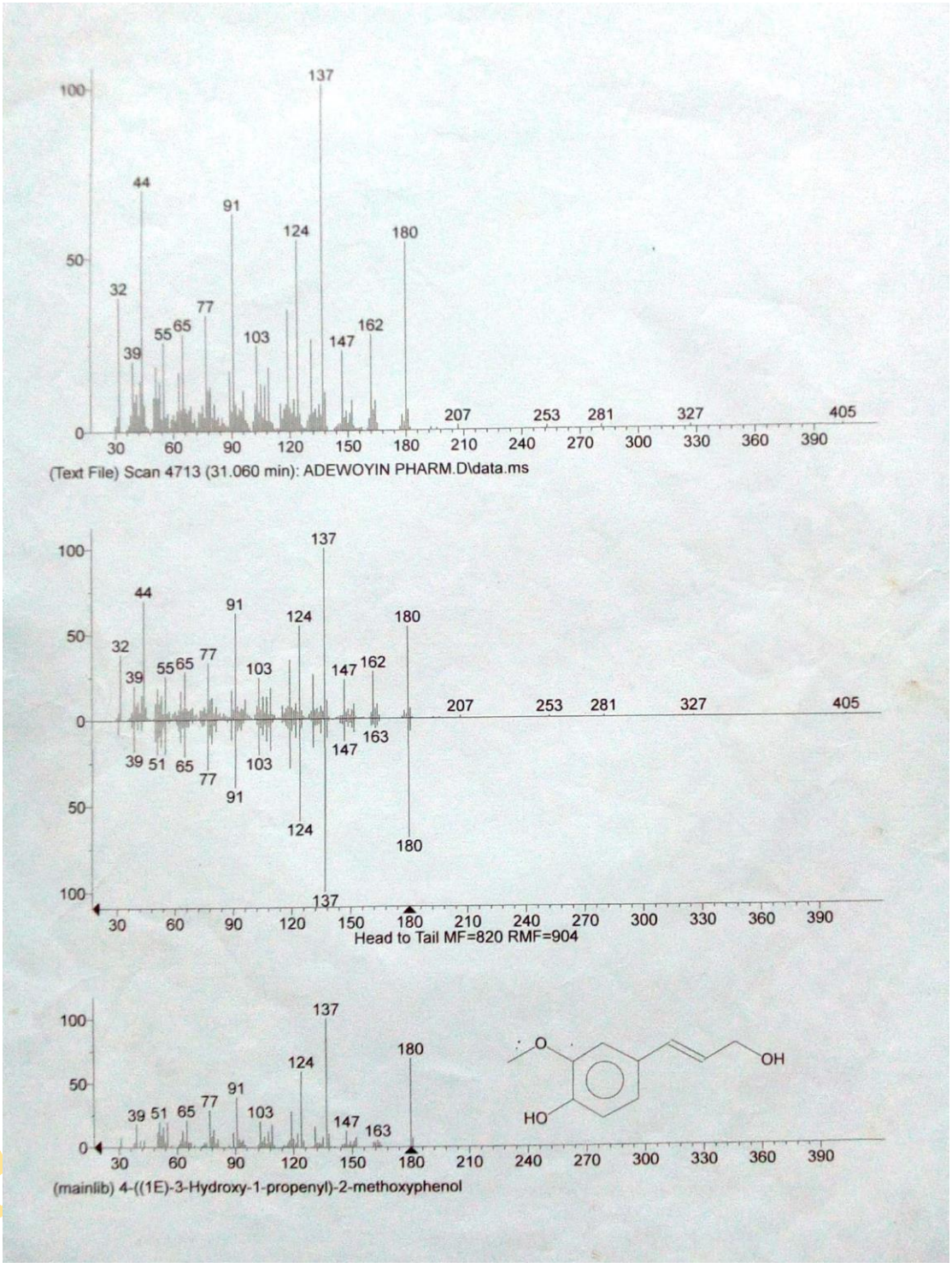


Figure 4.4. Structure of Ethyl iso-allocholate

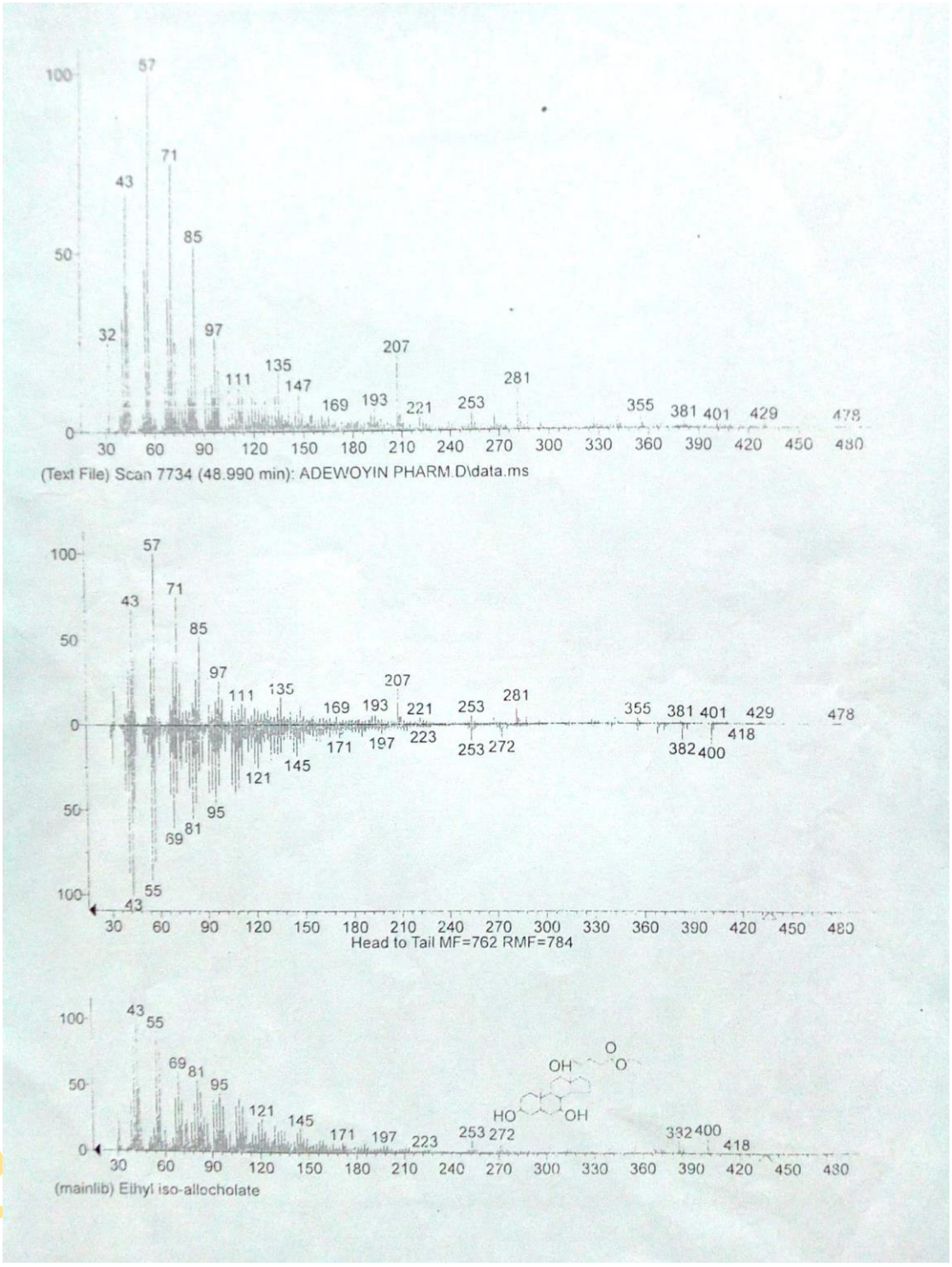


Figure 4.5. Structure of 4-((1E)-3-Hydroxyl- 1 -propenyl)-2-methoxyphenol

4.8 Effects of Methanol Extract of *Clerodendrum polycephalum* on Growth and Development of Mosquito Larvae

The effects of the crude methanol extract of *C. polycephalum* on growth and development of mosquito larvae are shown in Tables 4.14, 4.15 and 4.16. At the time of larva death, discolouration of larvae occurred. Some turned black from the living light pink colour. Larva duration (in days) was the same with control at lowest concentrations for all the three mosquitoes. However, at sublethal concentrations, larval durations were slightly reduced which was significantly different from the control ($p<0.05$). At the same concentration levels, there were effective inhibitions of adult emergence in the three mosquitoes used. Emergence inhibition was low (22.8 – 26.3 %) at lowest concentrations for all the mosquitoes. The concentration that killed 50% larvae was sufficient enough to inhibit greater number from emerging. In all, *Anopheles gambiae* was the most affected, recording complete (100 %) adult emergence inhibition at 1675 ppm (LC_{75}).

Table 4.14 Effect of methanol extract of *Clerodendrum polycephalum* on growth and development of *Aedes aegypti* larvae

Conc. (ppm)	Larva duration (Days)	Dead Larvae	Dead Pupae	Dead Adult	Total Emerged Adults
560	2.75±0.5	12	0	0	28(24.33)*
1120	2.5±0.5	23	7	0	10(72.98)
1675	2.25±0.5	27	7	0	6(83.8)
Control	2.75±0.5	2	0	0	37

*Figure in parenthesis = % IE (Emergence inhibition)

Table 4.15. Effect of methanol extract of *Clerodendrum polycephalum* on growth and development of *Culex quinquefasciatus* larvae

Conc. (ppm)	Larva duration (Days)	Dead Larvae	Dead Pupae	Dead Adult	Total Emerged Adults
2400	3.25±0.5	12	1	0	27(22.8)*
4800	2.75±0.5	23	6	2	8(77.2)
7200	2.5±0.57	28	8	2	2(94.6)
Control	3.25±0.5	1	0	0	39

*Figure in parenthesis = % IE (Emergence inhibition)

Table 4.16. Effect of methanol extract of *Clerodendrum polycephalum* on growth and development of *Anopheles gambiae* larvae

Conc. (ppm)	Larva duration (Days)	Dead Larvae	Dead Pupae	Dead Adult	Total Emerged Adults
1875	3.25±0.5	8	4	0	28(26.3)*
3750	3.00±0.0	18	10	4	1(97.4)
5625	2.75±0.5	30	10	0	0(100)
Control	3.25±0.5	2	0	0	38

*Figure in parenthesis = % IE (Emergence Inhibition)

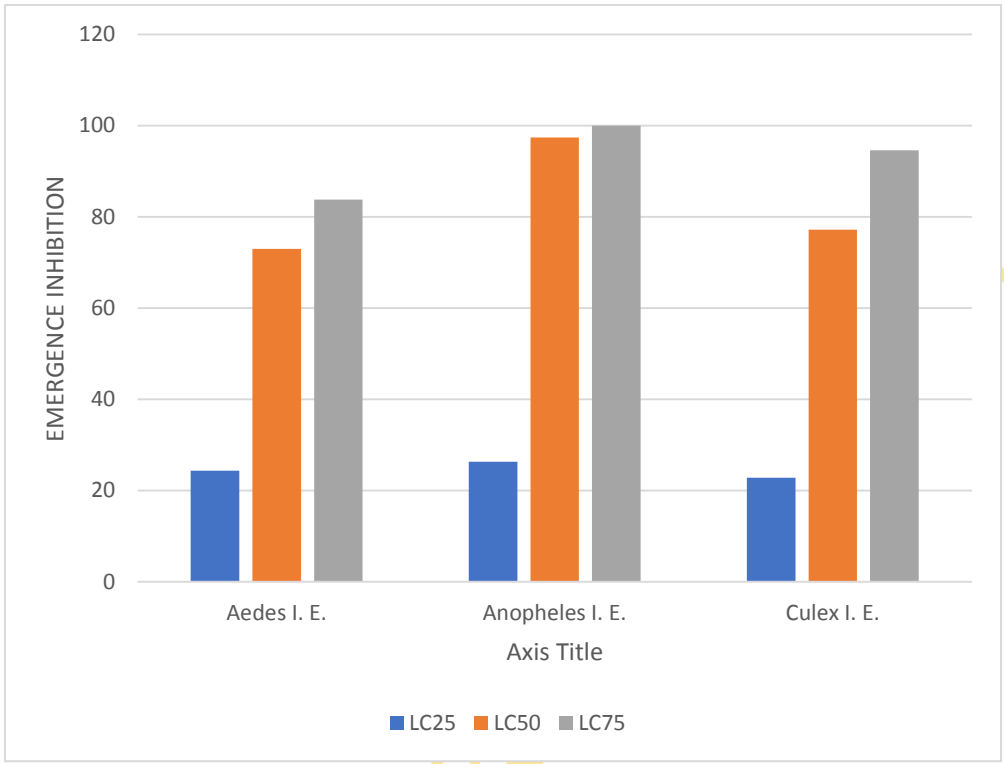


Figure 4.6. Effect of methanol extract of *Clerodendrum polycephalum* on Emergence Inhibition (IE) of *Aedes aegypti, Anopheles gambiae* and *Culex quinquefasciatus*

4.9 Effects of Methanol Extract of *Clerodendrum polycephalum* on Oviposition

The result of antioviposition is as shown in Table 4.17. The extract also showed concentration-dependent antioviposition i.e. antioviposition index (I) increased (46.4 - 89.9) with concentration (250 – 2000 ppm). There was an effective prevention of egg- laying in higher concentrations (500 – 2000 ppm). A greater percentage (67.5 - 87.25 %) of eggs laid eventually hatched.

4.10 Effects on Pupae

The effects of the methanolic extract of *C. polycephalum* on pupae are presented in Tables 4.18, 4.19 and 4.20. The extract showed concentration dependent activity against the pupae. The higher the concentration, the higher the number of pupae that did not survived after emergence. Many emerged adults were found dead on the water surface, while some others successfully emerged. At 250 ppm, the percentage of *Aedes aegypti* that emerged and died was 2.5 % which increased to 7.5 % at 500 ppm. At 1000 and 2000 ppm, the percentages of inhibition were 45.0 and 77.5 respectively. The percentages of emerged but dead *A. gambiae* and *C. quinquefasciatus* mosquitoes at 100 – 8000 ppm varied from 0 – 5 % and 0 – 77% respectively.

Generally, the leaf extract inhibited the moulting process of the pupae with different degrees of morphological changes. Death of adult occurred either right after partial emergence, where some parts of the pupal remain attached to thorax and head region or after incomplete emergence where the main trunk exuviated but the rest of the adult body is retained within the pupal exuvia. These obeservations of morphological changes were observed at the higher doses of 2000 ppm for *Ae. aegypti*, 8,000 ppm for *C. quiquefasciatus* and 8,000 ppm for *An. gambiae*. The pupae of *A. aegypti* were the most susceptible of the three mosquitoes.

Table 4.17. Effects of methanol extract of *Clerodendrum polycephalum* leaf on oviposition of *Aedes aegypti*.

Conc. (ppm)	Total no of eggs laid	% hatching	Antioviposition index (I)
250	881	67.5	46.4
500	319	68.75	80.5
1000	190	78.75	88.4
2000	165	84.25	89.9
Control	1644	86.25	

Anti-oviposition Index (I) = 100 X [(Ec – Ee)/Ec]

Where Ec is eggs laid in the control

Ee is eggs laid in each of the concentration

Table 4.18. Effect of crude methanol leaf extract of *Clerodendrum polycephalum* on pupae of *Aedes aegypti*.

Conc. (ppm)	% emerged and dead	Sex ratio of emerged adults	
		Total Male (%)	Total Female (%)
250	2.5±0.43	17(42.5)	22(55)
500	7.5±0.43	16(40)	21(52.5)
1000	45.0±1.5	11(27.5)	11(27.5)
2,000	77.5±0.43	3(7.5)	6(15)
Control	0.25±0.43	20(50)	19(47.5)

Table 4.19. Effect of crude ethanol leaf extract of *Clerodendrum polycephalum* on pupae of *Culex quinquefasciatus*.

Conc. (ppm)	% emerged and dead	Sex ratio of emerged adults	
		Total Male (%)	Total Female (%)
1,000	0.00±0.0	18(45)	22(55)
2,000	37.5±0.43	11(27.5)	16(40)
4,000	57.5±1.08	4(10)	7(17.5)
8,000	77.5±0.43	3(7.5)	6(15)
Control	0.0±0	17(42.5)	23(57.5)

Table 4.20. Effect of crude extract of methanol extract of *Clerodendrum polycephalum* leaf on the pupae of *Anopheles gambiae*

Conc. (ppm)	% emerged and dead	Sex ratio of emerged adults	
		Total Male (%)	Total Female (%)
1,000	0.0±0.0	17(42.5)	23(57.5)
2,000	2.5±0.43	16(40)	23(57.5)
4,000	2.5±0.43	17(42.5)	22(55)
8,000	5.0±0.50	14(35)	24(60)
Control	0.0±0	18(45)	22(55)

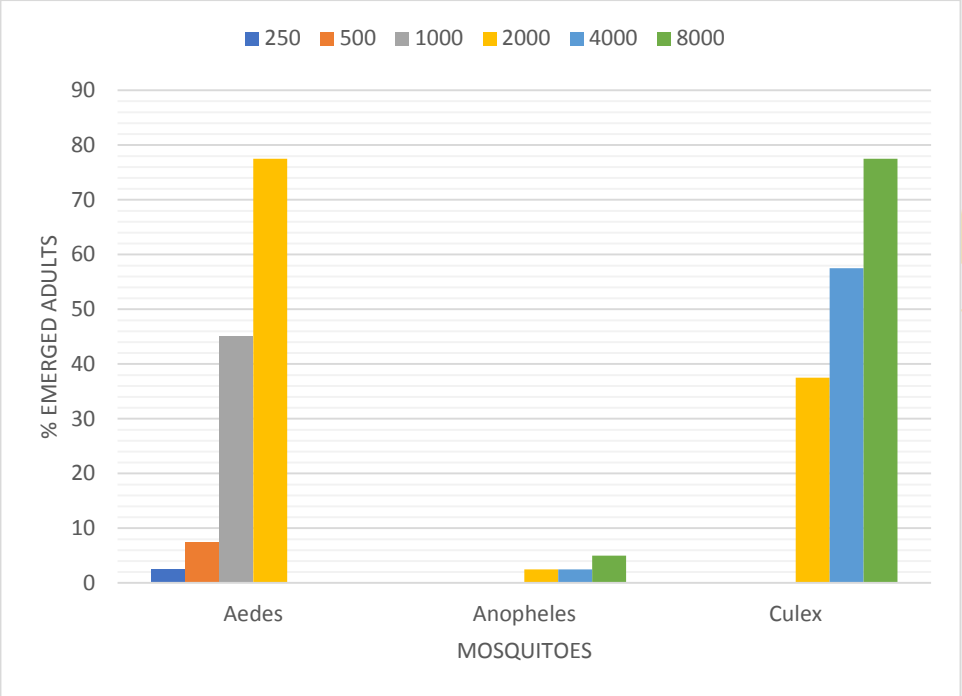
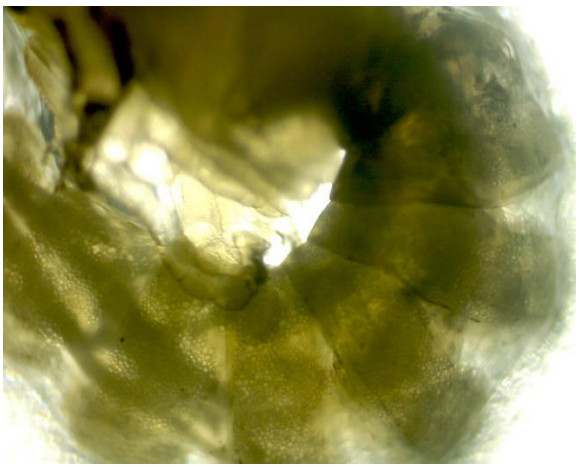
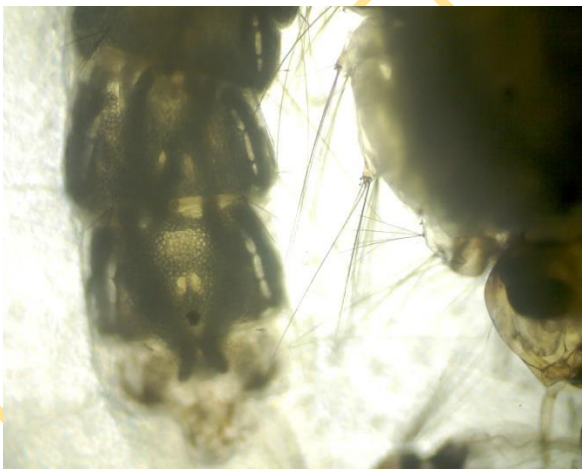


Figure 4. 7. Effects of methanol extract on adult emergence of *Aedes aegypti*, *Anopheles gambiae* and *Culex quinquefasciatus*



A

Damaged head region in mosquito larva



B

Discoloration of tail region of mosquito larva

Plate 4.4. Morphological changes in mosquito larvae exposed to methanol extract of *Clerodendrum polycephalum*

4.11 Effects of Methanolic Extract of *C. polycephalum* on Non-Target Organisms

The methanol extract was tested on Toad tadpoles and fresh water snail, *Biophalaria glabrata*. The result is presented in Table 4.21. The extract did not have any effect on tadpole of *Bufe regularis* until the concentration was increased to 2000ppm. The minimum concentration that produced snail mortality was 250ppm which resulted into 10% mortality. The mortality increased with increasing concentrations. However, the same set of concentrations produced appreciable snail mortalities. Mortality at 250 ppm was 10 %, at 500 ppm, it was 35 % while at 1000 and 2000 ppm, it resulted in 90 and 95 % mortalities respectively. The LC_{50} for snail mortality was 815.4 ppm.

Table 4.21. Effects of methanol extract of *Clerodendrum polycephalum* on non-target organisms

Conc. (ppm)	***Bufo regularis*** **mortality** **% mortality ±S D**	***Biomphalaria*** **glabrata** **% mortality** ±SD
250	0.0	10.0±1.14
500	0.0	35.0±0.7
1000	0.0	90.0±1.14
2000	10.0±0.7	95.0±0.7
Negative Control	0.0	0.0
Niclosamide(0.2mg/L)		100
		LC_{50} 815.4 ppm

CHAPTER FIVE

DISCUSSION

Clerodendrum polycephalum was investigated in this study being the best out of ten medicinal plants initially screened for larvicidal activites. The ten plants had been selected from the list of antiparasitic plants. There are several approaches to selection of medicinal plants for the purpose of carrying out antimosquitoes or insecticidal activities. One popular approach is to select those plants which are used locally in order to authenticate such claims. Another is to select a plant family which have been noted to possess insecticidal activities, an example is the Meliacea family, of which *Azadirachta indica* (Neem) is a member. Sometimes, some plants are selected because they are rarely attacked by herbivorous insects as a result of poisonous and repellent compounds they possess. It is a common practice to investigate different plant parts. Thus, leaf, root, stem bark, root bark and flower of medicinal plants can be investigated.

Several studies have been conducted on plant derived chemicals which are non-toxic to man and domestic animals and serve as useful basis for the development of safer and more selective mosquito insecticides (Sukumar *et al*., 1991, Alouani *et al* 2017, Amira, *et al.*, 2018).

The screening of ten plants in this research, showed that *Clerodendrum polycephalum* was the most potent with LC_{50} of 1120ppm. The order of increasing susceptibility of mosquito larvae (in terms of LC_{50} at 24 h) to the extract is *Aedes aegypti*>*Anopheles gambiae*>*Culex quinquefasciatus*. This order of susceptibility has also been reported severally (Ali *et al*., 2013; Chelela *et al*., 2014). However, the order of susceptibility did not follow the same pattern as observed by Govindarajan (2010). Differences in mortality between the three species of mosquitoes caused by

the extract could be due to ecological factors and inequalities in larva and pupa sizes as they were not raised from the same environment.

The 24/48 h bioassay was recommended by WHO (1996) as a tool for evaluating toxicity of phytotoxins however, a number of researchers have used either the 24 h bioassay (Remia and Logaswamy, 2010) or 48 h (Candido *et al*., 2013). The 48 h bioassay is actually necessary when the toxicity at 24h is low (WHO, 1999). Some have extended the period to even 72h (Haldar *et al*., 2011; Granados-Echegoyen *et al*., 2014).

Longer duration of exposure (48h) allowed greater contact with the active ingredients and perhaps the case slow release of the compound to the experimental media at 48h also contributed to the increase in mortality at 48h. In addition to the duration, the concentrations used for the larvicidal was on the high side (1000 – 4000 ppm). The use of crude extract, in some cases, requires large amount to produce mortality (Nour *et al*., 2012) except in highly toxic plant (Perich *et al*., 1994). Little amounts are required by the time the various components are separated and tested. Very little amount is also needed if the plant material is volatile oil (al Dakhil and Morsy 1999, Ezeonu *et al*., 2001, Deletre *et al*., 2013) which is more or less an isolate.

The efficacy of a synthetic insecticide, lambdacyhalothrin, was found to be several folds higher than the extract. Pure compounds are usually very potent materials. That of known natural insecticide, the extract of *Nicotiana tabacum*, was three/four times better than the extract used for this study. The plant is known to be highly toxic to insects because of the high content of nicotine. It is even planted around the house to ward off dangerous pests and reptiles in some places in Western Nigeria.

The effects on the morphological changes on pupae and shortening of larvae duration in this study suggested that the mode of action of the extract could be by contact and that the plant extract contains Insect Growth Regulator (IGR) components which could be exploited in mosquito control.

In a similar study, Mehdi *et al*. (2012) observed a wide range of morphogenetic deformities in different categories including larval pupal mosaic, abnormal pupae and pupal adult mosaic according to stage of metamorphosis when death occurred due to abnormal growth and moulting. In a recent study by Aouinty *et al.* (2018) microscopic observations revealed significant lesions on larval cuts treated with aqueous extract of *Ricinus communis*, it also induced histopathological changes at different levels of the body leading to disorganization of movements followed by immobilization and subsequent death.

The acetone extract of *N. indicum* and *T. orientalis* have been studied with LC 50 values of 200.87, 127.23, 209.00 and 155.97 ppm against third instar larvae of *A. stephensi* and *C. quinquefasciatus*. The acetaone extract has proved to be much more effective over other plant systems reported earlier (Siddiqui, *et al.*, 2003) where significantly higher doses of plant extracts were required for achieving 100% mortality against *C. quinquefasciatus* and *A. stephensi* larvae from *Vitex*, *Azadirachta* and *Feronia* plant extracts respectively. The acetone leaf extract of *C. fistula* and *S. indica* also exhibited significant mortality and also showed morhogenetic effects in 4^{th} instar larvae of *Anopheles* and *Culex*.

The extract used in this study prevented successful egg-laying of some mosquitoes in higher concentrations of the extract used. This suggests that the extract produced some volatile compounds, which may act as repellents. Certain strong odour often repell mosquito from feeding and from breeding successfully. *Aedes* is a

container breeder mosquito, follows visual and olfactory cues to find appropriate oviposition sites and then both physical and chemical factors of water, to assess the suitability of potential larval habitats in order to maximize the fitness of offspring (Sumba *et al.*, 2004). The extract did not affect the hatching of the majority of the eggs laid. Impurities provided by the extract might have prevented those that did not hatch.

Although the extract studied had a deleterious effect on fresh water snail, it did not affect *Bufo regularis* tadpoles. A natural product to be introduced into an aquatic environment must not be harmful to humans while handling and must not be toxic to other organisms in the environment (Boudjelidah *et al*., 2005). Mortality on fresh water snails may be an added advantage to the ability of the extract in that while the extract is being used as larvicides, it has the ability to eradicate the vector of schistosomes, causative organism of schistosomosis in an environment where the disease is problem.

Vacuum Liquid Chromatography was used to separate the components using non-polar to more polar solvents. This system of extraction has the advantage of bringing out the components faster than the regular column chromatography because it uses electric pumping machine. The differences in the extract yields from the crude could be linked with the different availability of extratable components resulting from the varied chemical composition of plants (Hsu *et al*., 2006). The use of vigor during extraction also contributed to the yields (Siddhuraju *et al*., 2003) as it was subjected to a shaker for 72hrs. The fraction obtained by the most polar of the solvents used, ethanol, happened to have the highest yield (20.22 %) and containing the active components. It has been discovered that phenolics (one of which was isolated from

the extract of C. polycephalum) are often extracted in higher amounts in more polar solvents such as ethanol (Siddhuraju *et al*., 2003).

Phytochemical analysis conducted on many extracts revealed the presence of constituents which are known to exhibit medicinal as well as physiological activities (Sofowora, 1993). Analysis of *C. polycephalum* extract shows that the leaves are rich in, tannins, flavonoids, saponins, and alkaloids. However, Anthraquinones were completely absent form the extract, even though they are mainly found in dicot plants like *C. polycephalum* (Dominyn *et al*., 2008). Tannins are naturally occurring polyphenols and are the most abundant secondary metabolites made by plants. They are responsible for the astringent and bitter taste of some leaves thereby protecting leaves from attack by insects. The fact that tannins give some leaves astringent and bitter taste is one of the criteria used for selecting a candidate plant for insecticidal study. Their main characteristic is that they bind and precipitate proteins which contribute to death of insects (Barbehenn and Peter 2011). They are also used to treat tonsillitis and skin eruption. Tannin has been administered internally to check diarrhoea and intestinal bleeding (Thorington Jr. and Ferrell, 2006).

Flavonoids are hydroxylated phenolic substances known to be synthesized by plants in response to microbial infection and they have been found to be antimicrobial substances against a wide array of microorganisms *in vitro*. The phenolic compounds are one of the largest and most ubiquitous groups of plant metabolites (Singh *et al*., 2007). They possess biological properties such as antiapoptosis, antiaging, anticarcinogen, antiinflammation, antiatherosclerosis, cardiovascular protection and improvement of endothelial function, as well as inhibition of angiogenesis and cell proliferation activities (Singh *et al*., 2007). Their activity is probably due to their

ability to complex with extracellular and soluble proteins and to complex with bacterial cell wall (Marjorie, 1999). They also are effective antioxidant and show strong anticancer activities (Salah *et al*., 1995; Del-Rio *et al*., 1997). Natural antioxidant mainly come from plants in the form of phenolic compounds such as flavonoid, phenolic acids, tocopherols etc. (Ali *et al*., 2008).

Saponins are glucosides with foaming characteristics. They are also present in many plants' leaves. Toxicity to fish, tadpoles and snails is a characteristic property of saponins. Traditionally, saponins have been extensively used as detergents, piscicides and molluscicides, in addition to their industrial applications as foaming and surface active agents and also have beneficial health effects (Shi *et al*., 2004). Saponins are used to treat yeast and fungal infections (Roa *et al*., 1995). Khanna and Kannabiran (2007) carried out qualitative analysis of the phytochemicals in three plants and found out that saponin was the major component that was responsible for the observed larvicidal activity. Wiesman and Chapagain (2006) reported that saponin extracted from the fruit of *Balanites aegyptiaca* Del. showed 100 % larvicidal activity against *A. aegypti* mosquito larvae. Morrissey and Osbourn (1999) have suggested that the saponin molecules interact with the cuticle membrane of the larvae, ultimately disarranging the membrane could be the most probable reason for the larval death.

Lastly, alkaloids are chemical substance of plant origin composed of carbon, nitrogen and oxygen some of which include caffein, cocaine, morphine. All these substances have strong effcts on the nervous system of both man and animals including insects such as mosquitoes. Alkaloids affect membrane structure and cytoskeletal structure - cellular weaking or collapse as well as leaky membrane (Qui *et al*., 2014). This particular insight gives an idea about the possible mode of action of the plant extract. The activity of crude extract is often attributed to the complex

mixture of these active compounds. These activities were seen on the different stages of mosquitoes used in my experiments.

The biochemical as well as the chemical compositions of plants parts or tissues is often influenced by different origins, environmental, and seasonal factors. Randrianalijaona *et al.* (2005) has reported seasonal changes in the chemical composition of essential oils in more than seventy *Lantana camara* from different parts of the world.

Prominent among the compounds identified from *C. polycephalum* in this study, by GC-MS analysis, are two phenolics and a steroid. Ethyl iso-allocholate is a steroid. It has antimicrobial, diuretic and anti-inflammatory activity, antibacterial, antioxidant, anti-turner, Cancer preventive, Chemo preventive and Pesticide (Singariya *et al*., 2012, Saravanan *et al*., 2014). Ethyl iso-allocholates has been identified as constituents of some medicinal plants in varying quantities by some workers who also used GC-MS analysis as a means. The compound has been found to be present in leaf and bark (Sarada *et al*., 2011; Muthulakshmi, *et al*., 2012), grass (Singariya *et al*., 2012), flowers (Hema *et al*., 2010) and volatile oil (Ogunlesi *et al*., 2010). Jasim *et al*. (2015) also reported the antimicrobial activity of alkaloidal constituents from *Solanum nigrum* of which ethyl iso-allocholate was one of the alkaloids.

The retention time of ethyl iso-allocholates obtained in this present work was longer (48.990) compared with what was recorded by the aforementioned researchers. For instance, in Muthulaskshmi *et al*. (2012), the retention time was 33.22, in Sarada *et al*. (2011), it was 25.14, while Hema *et al*. (2010) and Singariya *et al*. (2012) recorded 21.67 and 29.08 minutes respectively. This could be attributed to different running time of individual GC-MS machines used. Longer running time will result in

longer Retention Time (RT) and vice versa. The amount of ethyl iso-allocholate in the present study was much, compared to previous works. While it is the main (23 .69 %) component in *Clerodendrum* polycephalum leaves, the quantities were very minimal (0.01 - 0.49 %) in others (Sarada *et al*., 2011, Singariya *et al*., 2012).

The second compound, 4-((IE)-3-Hydroxy-1-propenyl)-2-methoxyphenol is a phenolic compound which has antimicrobial, anti-oxidant, anti-inflammatory and anagelsic property (Deryabin and Tolmacheva, 2015). The same has been identified in *Polygonatum odoratum* (Chunsriimyatav *et al*., 2013) and *Mussaenda frondosa* (Gopalakrishnan and Vadivel, 2011) as one of the major constituents of the aerial parts and leaves respectively. The RT for 4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol in this work was 31.060 minutes, whereas Gopalakrishnan and Vadivel, 2011 reported 10.020 mins while Chunsriimyatav *et al*., (2013) reported 11.050 mins. Furthermore, the concentration of this compound in the present study (6.07%) was similar (8.3 0%) to the finding of Gopalakrishnan and Vadivel (2011) but different (0.30% - 0.70%) from that of Ravikumar *et al.* (2012).

The third important component, 2-Hydroxy-5-methylbenzaldehyde is also a phenolic compound that has antieczematic, immunosuppressant, hepatoprotectant, antihypotensive, antiviral (picornavirus), antiparasitic, antiprotozoal, antiperistaltic, antitreponemal, antiulcerative, and insecticidal properties (Janakiram and Johnson, 2016). The NCBI pubchem biassays also confirmed the anticancer properties of 2-hydroxy-5-methylbenzaldehyde. (Matsunaga *et al.*, 2015). However, information on isolation and identification of this compound from a medicinal plant is very scarce.

In the present study, 17 compounds were recognized by the GC-MS analysis. Previous analysis of fractions from medicinal plants have identified different quantity of compounds. Sarada *et al*. (2011) identified 17 compounds from the leaves and 23

from bark of *Naringi crenulata*. Gopalakrishnan and Vadivel (2011) also reported 20 constituents from the leaves of *Mussaenda frondosa* while Arivoli *et al.* (2018) identified 6 compounds from the leaves and 12 compounds from the flower of *Jasminnum fluminense.* The type of solvent and level of purity of sample could determine the number of compounds identified by GC-MS spectrometry. Purer samples are expected to yield fewer number of compounds.

The medicinal plant studied extensively in this work, *C. polycephalum,* has demonstrated great potential for the control of mosquito species. The plant was obtained free of charge throughout the period of investigation, which makes it cheap. It does not pose any risk to human health as I handled it several times without suffering from any toxic effect from the plant. It usually leaves a bitter taste in one's mouth after handling the fresh leaves. This may be a comfirmattion of tannin content of the leaves. The plant is however gradually becoming scarce in urban communities. This is because of the difficulty involved in collection as they are available after searching deeper into the bush. The extract obtained from the plant is also biodegradable as it had no effect on any larvae after some days. Degradations and decomposition had taken place afterall.

There is no doubt that the plant *C. polycephalum* is a plant that could be exploited in mosquito control. Control programme could focus on elimination of larva stage. The advantage of targeting larvae is that they cannot escape from their breeding sites and grow to adult. This will also reduce overall pesticide use in control of adult mosquitoes.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

The use of medicinal plants as alternatives to orthodox medicines has attracted more attention in recent years. Many of these natural products have found their way into the Nigerian markets with ease to the detriment of our own traditional and efficacious medicinal plants. From the study conducted, *C. polycephalum* is one of our locally available medicinal plants that has shown biological activities. There abound several of such plant in our local forest waiting to be discovered.

The result of the present study reveals the presence of medicinally active constituents that are insecticidal in nature. The administration of the methanolic extract of *C. polycephalum* was able to cause mosquito larva mortality, caused morphorlogical changes on the pupae and inhibited effective laying of eggs by female mosquitoes. The plant *C. polycephalum* therefore contains insecticidal (including larvicidal and IGR ability) properties which could be used exploited in mosquito control programme.

The various components identified in the extract are indications that the plant is rich in bioactive compounds. The plant is especially rich in phenolic compounds such as Ethyl iso-allocholates which is most abundant in the most effctive fraction.

Ethanol, a polar solvent, effectively extracted the bioactive compounds. Since the GC-MS machine library information could only match few compounds at a very high level to confirm their identities, there is need to subject the extract to a more sophisticated machine that could reveal the identities of more compounds. It is possible for novel compounds to be isolated through such machine.

However, the fact that the plant is not easy to come by makes it an endangered species which may become extinct with time if care is not taken. Throughout the course of study, the plant was collected from abandoned plots of land and on subsequent visits to such places one found out that the place has been cleared for building activities. At a time, I had to travel to Osogbo, along Ilobu road for more collection when I couldn't find any in Ile-Ife. Efforts are being made to cultivate it in our medicinal plant garden.

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