LARVICIDAL EFFICACY OF AQUEOUS EXTRACT OF *Moringa oleifera* SEEDS ON MALARIA VECTOR, (*Anopeheles gambiae)* AND ITS TOXICITY EFFECTS ON MOSQUITO FISH, (*Poecilia reticulata*).

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Chinenyenwa Maria Dorathy OHIA
MATRIC NO: 97890

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August, 2014

CERTIFICATION

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DEDICATION

This work is dedicated to the **"I AM that I AM"**, the giver of all good things, the **Almighty God** and to the memory of two people dear to me; the best daddy in the world, Mr. James Okechukwu Kanu who believed in me from my beginning and set very high standards for me in all areas, and my beloved brother Emmanuel Okechukwu Kanu, both of whom death snatched away from me at the very beginning of this programme. The road to this achievement was turbulent but through it all dad, I made it. Till we meet across the river, your memory lives on dad.

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CMD

ABSTRACT

Malaria is one of the major public health problems in developing countries and its prevalence in Nigeria is dependent on the vector, *Anopheles gambiae.* The control of *Anopheles* is a major component of vector management but effectiveness has been limited by factors like insecticide resistance, cost, toxicity on non-target organisms and environmental pollution concerns. There is an increasing interest in developing plant-based insecticides as sustainable alternatives to chemical insecticides in mosquito control. This study was designed to determine the larvicidal efficacy of aqueous extract of *Moringa oleifera* seeds on the larvae of *Anopheles gambiae* and its acute toxicity effects on mosquito fish (*Poecilia reticulata*).

The study involved extraction of toxic components in *Moringa oleifera* seeds using aqueous extraction technique. Five aliquots of concentrations (1160, 1450, 2900, 5800 and 8700 µg/mL) were prepared by serial dilutions from the extract. Three independent experiments were run in quadruplicates on 1440 laboratory reared third instar larvae of *Anopheles gambiae;* twenty larvae per treatment were used and control group was exposed to distilled water. Larvicidal parameters, mortality and pupation were recorded 24-hourly for 5 days; larvae were considered dead if they were immobile and unable to reach water surface. Two independent toxicity experiments were run in triplicates on 480 male mosquito fishes exposed to three graded treatments (10, 20 and 30 mg/mL) of the extract. Behavioural responses, increased respiration, loss of orientation, discoloration, motility and mortality were observed hourly for 24 hours. Data were analysed using descriptive statistics, regression and probit analyses at $p \leq 0.05$. Probit was used to calculate the LC_{50} and LC_{90} values of the extract on *Anopheles* larvae and mosquito fish.

Larvicidal effects across the concentrations ranged from 59.0%-99.3%; at 2900 µg/ml, 59.0% mortality was observed within 24 hours and this increased with exposure duration across the different concentrations. There was a high linear relationship, (r=0.87) between larval mortality and concentration of the extract. The larval bioassay showed that the extract presented a 24hour-LC_{50} and LC_{90} values of 2505.8 and 6293.4µg/ml respectively. At lower concentrations (1160 and 1450 µg/ml), the larvae lived as long as 7 days before pupating while in the control pupation was not delayed. Acute toxicity evaluation on the mosquito fishes gave a 96 hour- LC_{50} and LC_{90} of 24.0 and 82.0 mg/ml respectively and also showed that the fishes exhibited varying degrees of changes such as concentration and time-dependent progressive declines in fish

motility, discoloration, with a corresponding increase in respiration and in the proportion of dead fishes as concentration increased unlike in the control.

Moringa extract was highly toxic to *Anopheles* larvae, inhibited pupae development and had low toxicity on *Poecilia reticulata*. This extract should be used to improve vector control with minimal toxicity effects on non-target organisms.

Keywords: Larvicidal efficacy, *Moringa oleifera*, *Anopheles gambiae*, Acute toxicity effects, *Poecilia reticulata*

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GLOSSARY OF ABBREVIATIONS AND ACRONYMS

AEMOS	Aqueous extract of *Moringa oleifera* seed
CDC	Centre for Disease Control
DALYs	Disability Adjusted Life Years
DDT	Dichloro-Diphenyl-Trichloroethane
DF	Degree of Freedom
FMH	Federal Ministry of Health
HCH	Hexachloro-Cyclohexane
IRS	Indoor residual spraying
MDGs	Millenium Development Goals
NRC	National Research Council
RBM	Roll Back Malaria
$U\text{-}_5$	Under Five Children
UNEP	United Nations Environment Programme
USA	United State of America
USEPA	United State of America Environmental Protection Agency
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Mosquito-borne diseases are among the leading causes of illnesses and deaths all over the world. The World Health Organization estimates that more than 300 million clinical cases each year are attributable to mosquito-borne illnesses (WHO, 1998). Mosquitoes are found throughout the world and are nuisances, hosts and or vectors of many disease-causing parasitic organisms of public health importance. These diseases include malaria, filariasis, dengue fever, yellow fever, mosquito-borne viral encephalitis among others.

Malaria is one of the most severe public health problems worldwide. It is a leading cause of death and disease in many developing countries including Nigeria, where young children and pregnant women are the groups most affected. According to Gilles and Warrell, 1993, WHO estimates that there are 300-500million cases of clinical malaria per year with 1.4-1.6 million deaths, mainly among African children and it is by far the most important insect transmitted disease. In Nigeria, malaria accounts for 25% of $U\text{-}_5$ mortality and 30% of child mortality and 11% of maternal mortality. At least 50% of the population will have at least one episode of malaria annually, while children that are aged below 5 years (about 24 million) will have 2 to 4 attacks of malaria annually. It is also the reason for hospital attendance in 7 out of every 10 patients seen in Nigerian Hospitals (Roll Back Malaria/Federal Ministry of Health, 2005).

The vectors of human malaria are mosquitoes and they belong to the genus *Anopheles*. There are about 430 species of *Anopheles* mosquito and of these only about 40 transmit malaria in nature (CDC, 2004) and from these only about 15 are vectors of major importance. Vector control is recognized as the major means of combating mosquito-borne diseases of public health importance. Insecticide based control measures such as indoor residual spraying (IRS) are the principal methods used to kill mosquitoes that bite indoors. However, prolonged exposure to synthetic insecticides increases development of resistance mechanisms in the mosquitoes, hence leading to the resurgence of malaria and mosquito-borne diseases in different communities.

Botanical insecticides have been long touted as alternatives to synthetic chemical insecticides for vector and pest management because they pose little threat to human and environmental health (Murray, 2006) and several plants such as *Azadirachta indica,* have been identified with insecticidal and pesticidal properties either in the seeds, fruits, barks, roots or in their root exudes. The plant *Moringa oleifera* (Lam) belongs to the family Moringacae and is commonly called drum stick tree, horse radish, West India Bern tree or "Miracle tree". It is a multipurpose crop indigenous to North West India (Cidamis et al., 2003). It is commonly planted in Africa as a living fence tree (Von Maydell, 1986). *M. oleifera* is a deciduous perennial tree with height between 10 and 15m, rather slender with drooping branches (F/FRED, 1992). It is a fast growing plant which produces leaves and pods that are edible by humans and livestock. All parts of the tree are used for their pharmacological, nutritional, and cosmetic properties. An alkaloid and triterpenoids have been reported in Moringa (Don Pedro, 1990; Isman, 1993).

1.2 Problem Statement

Mosquito-borne diseases represent a significant threat to human health despite considerable national and international control efforts. Mosquito breeding is a problem in Nigeria both in the rural and urban areas due to various reasons ranging from stagnant water bodies to blocked drainages and almost nothing is being done to destroy the mosquito habitats, most times only indoor prevention measures such as indoor spraying using aerosols, insecticide Treated Nets (ITNs) are used where available and affordable, also some medicinal plants are used traditionally to repel adult mosquitoes.

Control of mosquitoes has been a major component of vector and disease management but the effectiveness of the available vector control methods has been limited by various factors including insecticide resistance and environmental pollution concerns. Over the years, excessive exploitation of synthetic insecticides has resulted in serious problems like:

- Development of resistance mechanism.
- Insecticide induced resurgence of insect pests.
- Adverse effects on non-target organisms.
- Deleterious effects on the environment.

- Pesticide mishandling, abuses and consequences.
- Lack of local awareness on the use of natural products that are readily available.

1.3 Rationale for Study

The selective pressure of conventional synthetic insecticides has enhanced the resistance of mosquito populations at an alarming rate, resulting in wide spread resurgence, undesirable effects on non-target organisms and environmental and human health concerns. Hence, increasing the demand for new products that are environmentally safe, target specific and easily degradable.

Natural pesticides including plant derived products have received much attention due to their natural chemical defences against insect pests hence they now serve as effective alternative method to broad spectrum synthetic insecticides because they :

- Effectively reduce dependence on synthetic pesticides
- Reduce environmental pollution and injustice
- Enhance maximum protection and safety among producers and users.
- Are less prone to pest resistance and resurgence due to their subtle and target specific mechanisms of attacking pests
- Are biodegradable thus reducing the ability to bio-accumulate in the environment
- Are readily accessible, hence are cheap affordable and available.

Larviciding is a key strategy in vector control and cost can be reduced if larvicides are manufactured locally. Little is known about the bio-insecticidal effects of plant extracts on mosquitoes of public health importance in Nigeria, though several studies have been carried out in other countries like India and Iran where plant extracts have been utilized by communities as appropriate technology for curbing disease at the grassroots.

The study seeks to integrate the use of *Moringa* into public health; employing its extract as an effective bio-insecticide on Anopheles mosquito and recommending the Moringa extract in mosquito control as an alternative and accessible bio-insecticide to the proven toxic and

expensive synthetic insecticides. Anopheles mosquito was selected for this study because it has been implicated as a notorious vector of malaria and filariasis, two of the diseases of critical public health importance in Nigeria.

1.4 Broad Objective of the study

To test the relative bio-insecticidal potency of aqueous extracts of *Moring oleifera* on larval development of Anopheles mosquito and assess its toxicological effects on *Poecilia reticulata.*

1.5 Specific Objectives of the study

The specific objectives were to:

1. Extract the active aqueous ingredients of the seeds.
2. Test the efficacy of the aqueous extract on the Anopheles species.
3. Determine the level of susceptibility of the mosquito species population to the aqueous extract.
4. Establish the bio-efficacy of larvicide components of the test seed on the mosquito population.
5. Determine the impact of the extract on non-target organisms using *Poecilia reticulata* (Mosquito/Guppy fish) as a model.

CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria Epidemiology

Malaria has been with man since prehistoric times. It has killed and still kills billions of people, the urban preterm and term babies, infants and adults including pregnant women (Adetokunbo and Herbert, 2003, Hanson *et.al*, 2003). There are 400 million cases of malaria illness each year and at least one million people die annually from malaria. Malaria is a public health problem in more than 90 countries inhabited by about 2,400 million people i.e. about 40% of the world's population. It is endemic in a total 101 countries and territories with the largest percentage in Africa (WHO, 1998). Worldwide prevalence of the disease is estimated at 300-500 million clinical cases each year (WHO, 1998; WHO, 2004, Malaviya *et al*, 2006). Mortality estimates are between 1.5-2.7 million people every year (WHO, 2004).

In Africa, malaria is a leading health problem in Africa with almost the entire population being at risk (Vantadoost and Vaziri, 2004). More than 90% of all malaria disease is in sub-Saharan Africa; Very few countries are free from the malaria and more than 80% of the population is at risk of it. Mortality due to malaria is estimated at over 1 million deaths each year, the vast majority occurring among young children especially in remote rural areas with poor access to health services (WHO, 1998). Africa has the most effective vector of the disease, i.e. the Anopheles mosquito, the most deadly species of the plasmodium as well as the most conducive climatic condition for the multiplication of both.

Also up to 30% of malaria deaths in Africa are in countries undergoing complex emergencies i.e. situation in which war, civil strife, food shortage and displacement affect large civilian populations (Roll Back Malaria & Complex Emergencies, 2005). During these events, deaths due to malaria far exceeds those caused by the issues or conflicts at the root of the emergency; poor living conditions in temporary camps and affected towns can both increase vectors and water borne disease transmission thus eroding an individual's immunity to malaria.

Malaria in Nigeria accounts for 30-50 % of morbidity, 25% mortality in Under- Five (U_{-5}) children, 30% of childhood mortality and 11% maternal mortality. Approximately 50% of Nigeria population have at least one episode of malaria per year, while U_{-5} children (making up about 24 million) will have 2-4 episodes of malaria annually. It is also the reason for hospital attendance in 7 out of every 10 patients seen in Nigerian hospitals (Roll Back Malaria, 2005).Malaria incidence is stable and endemic all year round (WHO, 2007).

Epidemiological factors are hosts (man or animal), vector (mosquito), parasites (*Plasmodia*), favourable environment for vector and parasite multiplication, social and economic factors. Malaria is non-immune to both sexes and all ages are susceptible to malaria infection. Infants may have relative protection due to maternal antibodies acquired transplacentally and also due to lower rates of biting by vectors due to conscious protection or shielding from direct mosquito bites. Females have been found to have lower parasite rate and mount stronger humoural immune responses than males.

2.2 Malaria and vulnerable groups

Malaria affects vulnerable groups including U_{-5} children and pregnant women due to the lessened immunity in both groups. It thereby contributes to poverty and underdevelopment both for the individual, family, community and nation as a whole because a large bulk of yearly income is spent on malaria and related symptoms, prevention and treatment. It also reduces economic productivity due to absenteeism from school and work during malaria bouts.

2.2.1 Malaria and Children

Malaria kills one child every 30 seconds, in absolute numbers, malaria kills 3000 U_{-5} children per day (WHO, 1998). This far exceeds the mortality rate of HIV/AIDS. U_{-5} African children are chronic victims of malaria, suffering an average of 6 bouts a year. In fatally afflicted children mortality is usually in less than 72hours after symptoms development. In children of school age malaria is usually one of the principal reasons for poor school attendance.

2.2.2 Malaria and Pregnant women

Malaria is particularly dangerous in pregnancy. It causes severe anaemia is a major contributor to maternal deaths in endemic areas, hence pregnant women are at high risk of malaria. Non-immune pregnant women risk both acute and severe clinical disease resulting in up to 60% foetal loss and over 10% maternal deaths, including 50% mortality for severe disease. Semi-immune pregnant women with malaria infection risk severe anaemia and impaired foetal growth even when they do not show signs of acute clinical disease. An estimated 10,000 of these women and 200,000 of their infants die annually as a result of malaria infection in pregnancy. HIV infected women are at increased risk (WHO, 1998).

2.2.3 Malaria and Other high risk groups

Other high risk groups are non-immune travellers, refugees, displaced persons, labourers entering endemic areas. Malaria epidemics related to political upheaval, economic difficulties and environmental problems also contribute dramatically to death tolls and human suffering (WHO, 1998).

2.3 Impact of malaria on public health

Mosquito borne diseases such as malaria, filariasis, dengue fever and dengue haemorrhagic fever are serious public health problems in tropical regions especially Africa and Asia and these are transmitted to humans through mosquito bites only (Apiwat *et.al*, 2006). Of all these diseases, malaria is the most important especially in developing countries (Vatadoost and Vaziri, 2004); it continues to be a major public health problem causing enormous morbidity and mortality in many tropical and sub-tropical countries. Malaria affects the poor of the society thus exacerbating inequity in health and impeding development of any nation. The economic consequence of malaria related diseases are high (WHO Factsheet, 2007).

Malaria is a disease caused by members of the protozoan genus Plasmodium, a wide spread group of sporozoans that parasites cells. Four species can infect humans: *P. ovale* that causes malariae or quartian malaria, *P.vivax,* similar form causing ovale malaria, *P.malariae* which causes benign tertian malaria and *P.falciparum,* which causes malignant tertian malaria.

A person gets malaria when bitten by a female mosquito that is looking for blood meal and is infected with malaria. The parasite enters into the blood stream and travel to the liver, where they multiply. When they re-emerge into the blood, symptoms of it are characterized by chills, fever and enlarged spleen, aches and headaches (CDC, 2004). Diarrhoea, coughing and skin discoloration (yellowing of skin) sometimes occur. These are all indicators that the parasites have reproduced very rapidly, thereby clogging blood vessels and rupturing blood cells. Person with severe falciparum malaria may develop haemorrhaging (bleeding problems), shock, kidney or liver failure, central nervous system problems and eventual death (CDC, 2004, Adetokunbo and Herbert, 2003)

The evolution of resistance to cheap and easily available drugs and insecticides , changes in environmental conditions especially due to climate changes leading to increasing epidemics, malnutrition, economic factors, population movements among many others has worsened the situation and made malaria not just a disease of public health importance but also of great socio-economic relevance and importance.

2.4 Economic implication of malaria.

The economic cost of malaria due to cost of treatment, loss of productivity and reduced earnings due to lost days from work may be as high as 1.3% of economic growth per annum in countries with intense transmission (including Nigeria), compounding these over the years this loss has led to substantial difference in GDP between countries with or without malaria.

Malaria traps families and communities in a downward spiral of poverty and disproportionately affecting marginalized population and poor people who cannot afford treatment or who have limited access to health care (WHO, 2007). Direct costs include a combination of personal and public expenditures on both prevention and treatment of disease. In countries with very high malaria burden (e.g. Nigeria), the disease may account for as much as 40% of public health expenditure, 30-50% of in-patient admissions and up to 60% of outpatient visits. Malaria has lifelong effects through increased poverty, impaired learning and decreases attendance in work place and schools (WHO, 2007), it is also a major cause of poor child development (Roll Back Malaria/FMH, 2005).

The entire Nigeria population estimated at about 168 million are considered to be at risk of malaria and this is compounded by increasing resistance to the seemingly cost effective anti-malarial drugs and insecticides. Poverty, ignorance of the public, weak health infrastructure at all levels (LGAs, state and national levels) are constraints to effectively combating the disease and this is manifested in weak surveillance systems in place, shortage of drugs and lab supplies, lack of environmental management and poor sanitary conditions among many others, above all , operational finances are inadequate. Traditionally, malaria is seen as a challenge for the health sector alone with little or no involvement by other sectors or the general community (WHO, 2007, Roll Back Malaria, 2005).

2.5 Malaria Control

Malaria control in Africa decreased tremendously with the end of the eradication era of the 60s when effective control methods were developed and implemented. The little gains in vector control at the time declined quickly to almost nothing by the early 1970s. Renewed interest in tackling malaria problem started again towards the end of the 1980s with the call to African countries to evaluate or re-evaluate their malaria control situation and in addition re-allocate adequate resources towards malaria control efforts.

In 1992, the global ministerial conference in Amsterdam adopted a global strategy for malaria control which was also later adopted in 1993 by the World assembly, with four basic technical elements:

1. To provide early diagnosis and prompt treatment of malaria through provision of drugs and treatment of those infected.
2. To plan and implement selective effective and sustainable preventive measures including vector control
3. To detect early, contain or prevent malaria epidemics in high risk areas
4. To strengthen local capacity in basic, applied research and development. In particular the ecological, social and economic determinants of the disease.

The objective of vector control in malaria control is to reduce levels of transmission through reduction or targeting of the mosquito vectors thus reducing malaria morbidity and mortality to the barest minimum.

2.5.1 Vector control

Effective vector control is defined as the application of targeted site-specific activities that are cost effective. Malaria control with respect to vector control is a big challenge due to many factors which include:

- Complexity of disease control process
- Complexity of the vectors.
- Expensiveness of control programs.
- Variation of disease patterns and transmission dynamics from place to place, by and according to climate and environmental circumstances.

Concerns about the environment give rise to the need to develop and maintain environmentally sustainable methods of vector control that is aimed at reducing reliance on chemical insecticides and involving inter-sectorial collaboration.

2.5.2 Larviciding

Larviciding involves the killing of larval stages of mosquitoes by the application of various forms of chemicals to the breeding sites. The most common chemicals are insecticides of various groups, insect growth regulators (IGRs) or bacterial larvicides. Larviciding is effective in localized well accessible breeding sites and may be applied in conjunction with other methods.

2.6 MOSQUITOES

2.6.1 Scientific classification

Kingdom:	Animalia
Phylum:	Arthropoda
Class:	Insecta
Order:	Diptera
Family:	Culicidae
Sub Families:	Toxohynchitinae
	Culicinae
	Anophelinae
Genera:	*Anopheles*
	Culex
	Aedes
	Mansonia
	Haemagogus
	Sabethes
	Psorophora (Knight & Stone, 1977)

The genera listed above are the ones that are most important as man- biting mosquitoes. In all there are 34 genera of mosquitoes with about 1300 species.

Arthropods have the following characteristics

- Composed of several parts or segments some of which may be jointed or fused and consequently segmentation may not be clearly visible.
- Body is covered with exoskeleton (tough skin), a chemically hardened cuticle that forms a protective shield
- The body normally has paired jointed legs and antennae
- The heart is simple and dorsal while the ganglionated nerve cord is ventral but connects to a dorsal large ganglion in the head region called the 'brain'.

- The coelom (body cavity) is the space between the alimentary canal and body wall and is often called a haemocoele because it contains the insect's blood.

Within the Phylum Arthropoda there are several classes including the Class Insecta which is the largest and mosquitoes belong to this class (WHO, 2003). There are over 2500 species of mosquitoes throughout the world. Mosquitoes are responsible for more human deaths than any other living creature making them the most dangerous creatures on earth.

2.6.2 Distribution of mosquitoes

Mosquitoes have world-wide distribution they occur throughout the temperate and tropical regions and extend their range northward into the Arctic Circle; the only area from which they are absent is the Antarctica. They are found at elevations as high as 5500m and in mines at depths of 1250m below sea level. Some genera have a restricted distribution and may be confined to certain regions and areas of the globe for example, the genus *Haemagogus* is found only in South and Central America. Some mosquitoes may be found in only a few countries or regions while others are widespread in wider regions an example is *Aedes aegypti* and *Anopheles* species that is widespread in the tropical regions of the world (Service, 1980).

2.6.3 Public Health/Medical Importance of Mosquitoes

Anopheles species are primarily of medical importance as vectors of human malaria (*Plasmodia* spp.) but they are also vectors of filariasis (*Wuchereria bancrofti*) and some arboviruses. The genus *Aedes* contains important vectors of yellow fever, encephalitis viruses, dengue and several other arboviruses (Service, 1980). Some *Culex* species transmit *W. bancrofti* and a variety of arboviruses and *Mansonia* species transmit *Brugiamalayi*. *Psorophora* species are important mainly as nuisance mosquitoes but also occasionally transmit yellow fever and arboviruses. *Haemagogus* and S*abethes* species are also vectors of yellow fever and a few arborviruses. Several other mosquitoes in other genera are also minor vectors but of importance as they are troublesome because of the serious biting nuisance they cause to man and animals (Service, 1980).

2.6.4 General Biology and life cycle of mosquitoes

All mosquitoes must have water to complete their life cycle; these can range in quality from melted snow to sewage effluent and can be in any container. Mosquitoes of different species lay their eggs in a variety of water sources that range from small containers to large expanse of marshland. In mosquito species identification, the type of water in which the larvae is found can be an aid to its identification. Adult female mosquitoes show distinct preference for the type of water sources in which to lay their eggs hence each species has its unique environmental requirement for maintenance of its life cycle (CDC, 2004).

Mosquitoes have unique feeding habits in that it is only the adult females that bite man and other animals, the males only feed on plant juices. In some species females prefer to feed on only one type of animal while in some they feed on a variety of animals. The female mosquitoes have to feed on sufficient blood meal to be able to develop egg. If not they die without laying viable eggs. However, some species of mosquitoes have developed a means to lay viable eggs without getting blood meal (McCafferty, 1983).

The flight habits of mosquitoes depend on species type, most domestic species remain fairly close to their point of origin while some species known for their migration habits are often a nuisance far from their breeding sites (McCafferty, 1983). The flight range for females is usually longer than that of males. Wind is also a major factor in the migration of mosquitoes though most mosquitoes stay within a mile or two of their source, some have been recorded as far as 75 miles from their breeding source (Service, 1980, CDC, 2004, McCafferty, 1983).

The life span of an adult mosquito is about a week for the males and about a month for females depending on factors such as temperature, humidity, time of the year and sex of the mosquito. Under the best condition in the tropics, the average life span of a female *Anopheles* mosquito is about 3weeks and a female continues to lay eggs throughout her lifetime and most will lay between 1-3 batches of eggs during their life, though some may lay as many as seven batches. Species of mosquitoes which overwinter as fertilized and hibernating adults usually live for many months (McCafferty, 1983).

There are four common groups of mosquitoes viz: *Aedes*, *Anopheles*, *Culex* and *Culiseta*. And these all go through four separate and distinct stages of their life cycle (Plate 2.1) (Complete Metamorphosis) i.e. Egg →Larva→ Pupa → Adult. Each of these stages are recognizable by their special appearance, the larval and pupal stages are usually spent in water (McCafferty, 1983; CDC, 2004).

Egg

Eggs are usually laid one at a time and they float on the surface of the water or damp soil. *Anopheles* and *Aedes* species do not make egg rafts but lay the eggs separately while *Culex* and *Culiseta spp* lay eggs in rafts of hundred or more eggs. *Culex, Culiseta* and *Anopheles* lay their eggs on damp soil or flood plains .mosquito larvae usually emerge within 24-48 hours (CDC, 2004).

Larva

Mosquito larvae are commonly called wrigglers and must usually live in water for 7-14 days depending on water temperature. Larvae have a well-developed head with mouth brushes used for feeding, a large thorax and segmented abdomen with no legs. They come to the surface at frequent intervals to obtain oxygen through a breathing apparatus (tube) called siphon. Most larvae can be found hanging from the water surface. In contrast to other mosquito larvae, *Anopheles* larvae lack this respiratory siphon and for this they position themselves parallel to the water surface. Larvae eat algae and micro-organisms as well as organic matter in the water. Larvae molt four times and with each molt their size increases, the stages between molts are called instars. At the 4^{th} instar the larva reaches a length of almost ½ inch and when it molts it becomes a pupa (Service, 1980, CDC, 2004).

Pupa

The pupal stage is an aquatic resting non-feeding stage that ranges from 1-4 days depending on species and temperatures. The mosquito pupae are commonly called tumblers because when disturbed they dive in a jerking tumbling motion and then floats back to the surface. This is the time when the mosquito turns into an adult. It takes about 2 days before the adult s fully developed. When development is complete the pupal skin splits and the adult emerges.

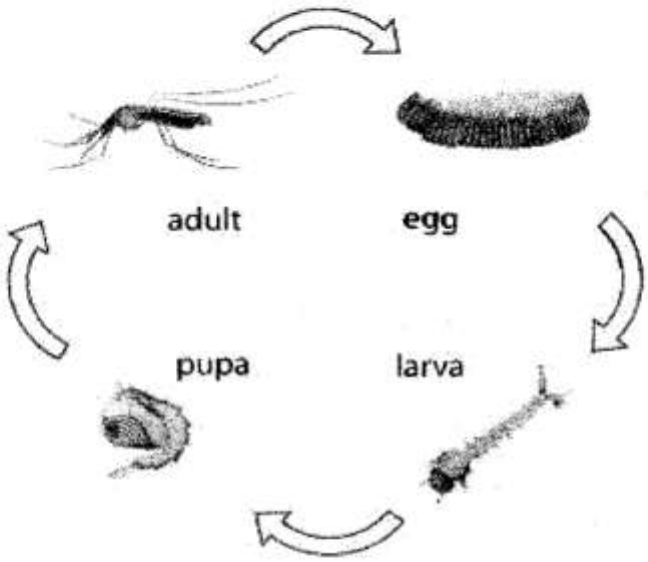


Plate 2.1: The lifecycle of Mosquito

Source: McCafferty, 1983

The pupa is lighter than water and therefore floats at the surface, oxygen supply is through two breathing tubes called ‘’trumpets’’ (Service, 1980, CDC, 2004, McCafferty, 1983).

Adult

The newly emerged adults rest on the surface of the water for a short time to dry, for all its parts to harden; also to spread the wings out to dry properly before it can fly. When adults emerge from the aquatic stages they mate and the female go in search of blood meal to obtain the protein necessary for the development of the eggs (Service, 1980, CDC, 2004, McCafferty, 1983).

Culex are generally weak fliers and generally do not move far from their habitats, they are painful and persistent biters but prefer to attack at dusk and after dark, they readily enter dwellings blood meals. *Culiseta* mosquitoes are moderately aggressive biters, hiding in shade during the day (CDC, 2004, McCafferty, 1983).

Aedes are painful and persistent biters, attacking during daylight hours, they do not enter dwellings and they prefer to bite mammals and humans. They are strong fliers and are known to fly many miles from their breeding sources. *Anopheles* are also persistent biters and are the only mosquito species which are successful vectors of the malaria parasite to man (Service, 1980, CDC, 2004, McCafferty, 1983).

2.7 *Anopheles* mosquito

There are about 430 species of *Anopheles* mosquitoes of these approximately 40 species can transmit malaria in nature (CDC, 2004) and from these only about 15 are vectors of major importance. Some *Anophelines* prefer to bite animals and rarely transmit malaria parasite to humans, others do not live long enough to allow the development of the parasite or the parasite does not seem able to develop in them.

The vectors of human malaria all belong to the genus *Anopheles*. *Anopheles* mosquitoes are found worldwide except Antarctica, different species transmit the parasite depending on the region and environment. E.g. *A. gambiae* is the principal vector of malaria a disease which afflicts over 500 million people and causes more than 1 million deaths each year (Pubmed, 2008). *Anopheles* mosquitoes are important vectors of the malaria parasites, *Plasmodium spp*

and lymphatic filariasis parasite *W. bancrofti* in sub Saharan Africa. In Nigeria *W. bancrofti* is the etiological agent of lymphatic filariasis and *Anopheles* mosquitoes act as the effective vector of this parasite.

2.7.1 Life stages of *Anopheles* mosquito.

The *Anopheles* mosquito undergoes complete metamorphosis i.e. Egg →Larva→ Pupa → Adult.

Anopheles egg

As in other mosquitoes only female anopheles bite and they use the proteins from their blood meal to produce a batch of eggs that are laid in relatively clean water such as irrigation water, puddles, and marshes.

A female *Anopheline* normally mates only once in a lifetime and usually requires a blood meal before the eggs can develop. Blood meals are usually taken 2-3 days before the next batch of eggs are laid. About 100-150 eggs are laid on the water surface during oviposition (Plate 2.2). Oviposition sites vary from small hoof prints and rain pools to streams, swamps, canals, rivers, ponds, lakes and rice fields. *Anopheles* eggs are not resistant to drying and hatch within 2-3 days although it may take up to 2-3 weeks in colder climates (CDC, 2004).

Anopheles larva

Larva hatches from the egg after about 1 or 2 days and generally floats parallel to the water surface unlike most other mosquito larvae (Plate 2.2). *Anopheles* larvae develop through four larval instars. The small larva emerging from the egg is called the 1st instar. After 1 or 2 days it molts into the second instar, followed by the 3rd instar and 4th instar at further intervals of about 2 days each (CDC, 2004).

The larva remains in the 4th instar stage for 3 or 4 days before molting onto the pupal stage. The total time spent in the larval phase is generally 8-10 days at normal tropical water temperature. At lower temperatures, the aquatic (larval) stage takes longer to develop. Larvae spend most of the time feeding on algae, bacteria and other microorganism on the surface

micro layer of the water and they breathe through spiracles located on the 8th abdominal segment and hence must come to the surface frequently (CDC, 2004).

When disturbed, larva quickly swims towards the bottom but soon returns to the surface to trap oxygen. They swim either by jerking movements of the entire body or through propulsion with the mouth brushes (CDC, 2004).

***Anopheles* pupa**

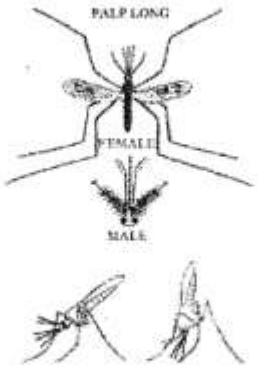
This is the stage in which a major transformation takes place i.e. from living in water to becoming a flying adult mosquito. It is a transitional, motile stage that is uniquely short lived. The pupa is shaped like a comma when viewed from the side; the head and thorax are merged into a cephalothorax with the abdomen curving around underneath (CDC, 2004, Service, 1980).

As with the larvae, pupae must come to the surface frequently to breathe through a pair of respiratory trumpets on the cephalothorax. It stays just under the surface and swims down when disturbed but does not feed. The pupal stage lasts for 2-3 days after which the dorsal surface of the cephalothorax splits to allow emergence of the adult mosquito (Service, 1980).

Anopheles adult

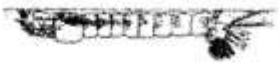
The adult emerges from the pupal stage and temporarily rests on the water surface until it is dried and able to fly. Soon after emergence, the adults mate and the female goes in search of its first blood meal. If these contain gametocytes of the malaria parasite i.e. *Plasmoodium spp*, male and female gametes of the parasite undergo fertilization in the mosquito stomach, the zygotes develop into ooccysts on the outer surface of the stomach wall and sporozoites develop in the oocysts over a period of 12 days before populating the mosquito's salivary glands. During subsequent feeds the injection of saliva into the host carries spoorozoites, which may establish a new malaria infection in the host. Approximately 12 days is required for sporozoites development (CDC, 2004).

The female usually mates only once because she receives sufficient sperm from a single mating for all subsequent egg batches. Normally the female takes her first blood meal only



(a): Anopheles Adults: Diagram showing the top the different male and female and the bottom row shows typical resting position.

(b): Eggs: Diagram showing the lateral floats and bottom row eggs being laid singly



(c): Larva: Diagram showing larva in position parallel to the surface

Plate 2.2: The distinguishing features of *Anopheles* Mosquito

(Source: CDC, 2004)

after mating but sometimes the first blood meal can be taken by young virgin females. The first batch of eggs develops after one or two blood meals, while successive batches usually require only one blood meal. Males live for about a week, feeding on nectar and other sources of sugar. Females also feed on sugar sources for energy (CDC, 2004).

After obtaining a full blood meal females will rest for a few days while the blood is digested and eggs are developed. The process depends on temperature but usually takes 2-3 days in tropical conditions. Once eggs are fully developed they lay them and resume host seeking. This cycle is repeated until the female dies. Life span of female *Anopheles* is about a month or longer in captivity but most probably do not live longer than 1-2 weeks in nature. Their chances of survival depend on temperature, humidity, environmental factors but also their ability to successfully obtain blood meal while avoiding host defences (CDC, 2004, McCafferty, 1983).

2.7.2 Identification of *Anopheles* mosquito.

The identification of pupae of *Anopheles* species is very difficult, so it is essential that one is able to identify the adults correctly. As a result when the pupae are obtained from the field they should be kept alive and allowed to emerge into adults to allow for proper and correct identification.

Some *Anopheline* species are similar in morphology, while they are actually different species. These species are genetically related and known as sibling species and are morphologically grouped under the same complex: An example is the *Anopheles gambiae* complex also called *A. gambiae* sensu lato or s.l., which has seven different species viz*: A. gambiae* sensu stricto(s.s.), *A. arabiensis, A. quadriannulatus species* A , *A. quadriannulatus species* B, *A. bwambae, A. merus and A.melas.* However, it is not possible to differentiate between these species by using an identification key that is based on external morphology (WHO, 2003). External characteristics of adult and larval anophelines that are useful in species identification are reviewed below:

Head: The head has a pair of large compound eyes with a pair of antennae joined to the head between the eyes. Below the antennae is a pair of palps and it is composed of five parts in *Anopheline* mosquitoes. The palps of *Anopheles* mosquito are a distinguishing feature from other mosquitoes as they are as long as the proboscis, another distinct feature is the spotted wings due to the presence of discrete blocks of black and white scales on the wings, the number, length and arrangement of these dark and pale areas differs considerably in different species and provide useful characters for species identification. The palps are covered with scales which may be of different colours and used in species identification. A proboscis protrudes from the ventral part of the head and extends forward. In both sexes the palps are about as long as the proboscis and in males not in females they are enlarged (i.e. clubbed) at their ends (Plate 2.2) (Service, 1980, CDC, 2004).

The head is specialized for acquiring sensory information and for feeding. The antennae are important for detecting host odour as well as odours of breeding sites where the females lay their eggs. The antennae are also useful to distinguish between the female and male mosquito. On the female, the hairs of the antennae are few in number and short while the male has very long hairs on the antennae, which consequently gives them a bushy moustache-like appearance on examination (WHO, 2004).

Thorax: the thorax has a pair of wings and a pair of halters on the upper surface and three pairs of legs on the lower or ventral surface. The wings have several veins on them. Each vein is given a number name. The vein along the front edge of the wing is called the costa and the short vein behind it is called the subcosta. There are six other veins numbered 1-6 of which veins 2, 4 and 5 are forked (WHO, 2004, Service, 1980).

These veins are covered with scales which are usually brown, black, white or cream in color. The back edge of the wing has fine scales. Many *Anophelines* have wings spotted with dark and pale patches which together with these other characteristics are used in species determination. The thorax is specialized for locomotion and in all *Anopheleses*, the scutellum is rounded in outline (WHO, 2004, Service, 1980).

Abdomen: the abdomen has eight similar segments and two modified segments, the 9th and 10th. The 9th segment has a pair of spiracles while the 10th is the anal part, on segments IV-VI are found well developed fan-shaped hairs, called palmate hairs, these are also sometimes present on segments I-III. Each segment has up to four tergal plates on its dorsal side. There is usually a pair at the anterior and a second pair at the posterior of each segment and two accessory plates (Service 1980).

The 9th abdominal segment is fused with the 8th segment and carries the spiracles through which the larva breathes. On each side of the 9th segment is a pecten, which is a triangular plate with comb-like teeth. Most of the upper surface of the anal segment is occupied by a large tergal plate called the saddle. Hairs may arise from the saddle or from the anal segment. On the lower surface of the anal segment is a series of hairs called the ventral brush, four anal gills extend from the anal segment (WHO, 2004, Service, 1980).

The abdomen is specialized for food digestion and egg development. This segmented body part expands considerably when a female takes a blood meal. The blood is digested over time serving as a source of protein for the production of eggs, which gradually fill the abdomen (Service, 1980).

2.7.3 Life span of mosquito

Once ingested by a mosquito, malaria parasites must undergo development within the mosquito before they are infectious to humans. The extrinsic incubation period, i.e. the time required for development in the mosquito ranges from 10-21 days, depending on the parasite species and the temperature. If a mosquito does not survive longer than the extrinsic incubation period, then she will not be able to transmit any malaria parasites. However, it is not possible to measure the life span of mosquitoes directly in nature, but indirect estimates of daily survivorship have been made for several *Anopheles* species.

The mean probability of daily survivorship of *A. gambiae* in Nigeria is 0.80 with annual mean life expectancy of 12.24 days (Olayemi and Ande, 2008) while estimates of daily survivorship of *A. gambiae* from Tanzania ranged from 0.77 to 0.84, this means that at the end of a day between 77% and 84 % will have survived. Assuming this is constant through

the adult life of a mosquito, less than10% of the female population of *A. gambiae* would survive longer than a 14- day extrinsic incubation period. If daily survivorship increased to 0.9, over 20% of mosquitoes would survive longer than a 14-day extrinsic incubation period. Control measures that rely on insecticides (e.g. indoor residual spraying and larviciding) may actually impact malaria transmission more through their effect on adult longevity than through their effect on the adult mosquito population (CDC,2004).

2.8 CONTROL OF MOSQUITOES

More effort has been made to control mosquitoes than any other biting insect, such control measures have been directed to specific vectors such the malaria vectors, yellow fever vectors and a host of other insect vectors of public health importance. These control measures can be directed at either the mature (adult) or immature aquatic stages or at both stages simultaneously.

These control measures can be broadly grouped into two, viz:

1. Control targeted at immature stages

These include all control measures targeted at the aquatic stage of development of the mosquito vector, thus it targets the egg, larval and possibly the pupal stage of the mosquito and these include:

a) Biological control: the introduction of predators, parasites or pathogen to mosquito habitat to control mosquito population, though often considered a naturalist control method, it is a process that involves the manipulation of the environment

   Even though this control method does not cause any chemical pollution, they are usually difficult to implement and maintain. More over if predators are used it is unlikely that they will prey exclusively on the mosquito larvae and pupae; they may also feed on beneficial and or harmless insects.

b) Genetic method: involves the use of genetic manipulation and selective rearing to produce mosquitoes that are refractory to infection with human diseases, such as malaria or filarial and then released into the environment to compete with the natural population of susceptible ones and eventually replace them. Other methods include species replacement, sterile-male release techniques. None of these methods are simple and they

may prove difficult to implement than more conventional insecticidal methods (Service, 1980).

2.8.1 Pesticides

Pesticides can be broadly defined as substances or a mixture of substances used in preventing, repelling, mitigating or destroying pests; substances derived from plants, microorganisms, organic and inorganic molecules are also included. Pesticides are used in virtually everywhere; it is therefore not restricted to use on agricultural fields. They are used because the pests they are designed to control, compete with man for food, spread disease, destroy properties or just pose a nuisance. The modes of action of pesticides vary depending on activity of pest, life cycle spectrum of attack. Some work by interfering with the life cycle of pests, they may not necessarily kill the pest but they stop them from reproducing effectively.

2.8.2 Insecticides

These are pesticides used specifically against insect pests. By definition, they are agents of chemical, biological origin that control insects (Ware & Whitacre, 2004). Control may result in killing the insect or otherwise preventing it from engaging in behaviours deemed harmful. Insecticides may be natural i.e. bio-insecticides or manmade i.e. synthetic and are applied to target insects in a myriad of formulations and delivery systems in form of sprays, baits, slow release diffusion. Toxicity levels of pesticides vary depending on active ingredient, risk to human and environmental health, ability to break down rapidly and accumulation in the ecosystem.

2.8.3 Synthetic insecticides

The discovery of synthetic insecticides several decades ago led to a revolution in pest and vector management. The first synthetic broad spectrum pesticides were the organo-chlorides which offered a revolution in the efficacy of pest control and were hailed as “wonder sprays’’ in the 1950’s to combat disease carrying insects like fleas and mosquitoes (Annol, 2003). These insecticides held their sway, helping to rid many countries of the scourge of diseases like malaria, bubonic fever amongst many others. Other synthetic insecticides were the

organophosphates, carbamates and more recently pyrethroids. These chemical pesticides were very effective and became the main tool in vector and pest management in crop protection, livestock and public health.

However the wide scale use of the early synthetic broad-spectrum pesticides particularly organo-chlorines with their prized characteristic of long persistence produced obviously damaging side effects and while the pests also developed resistance to its continued use. Hence their effectiveness is over shadowed by the serious deleterious effects they pose on non-target organisms, human, animals and the environment in general.

These pesticides were discovered to be lethal to non-target organisms such as beneficial insect vertebrates both in aquatic and terrestrial ecosystem. Some were extremely persistent organic pollutants (POPs) and undergo bioaccumulation in the food chain. Their active ingredients and metabolites were also found to impair mammalian endocrine system, nervous system and some were carcinogenic (Birech, *et al*.2006).

These pesticides are ubiquitous and spread to every part of the environment and many of these, especially organochlorines such as DDT leave residues in terrestrial and aquatic biota, accumulate in the food chain and lead to biological magnification (Lincer *et al*, 1981), they are found in soils, sediments, rivers, food, plant and air, all of which constitute the effective biotic and abiotic components of the environment.

The indiscriminate use of synthetic insecticide has caused untold deleterious damage to our environmental integrity." In any war, one must have a sword (offensive weapon) and a shield (defensive protection). When we choose to only use volatile, synthetic pesticide poisons to "control" pests, we have no shield, no protection and our only weapon is attacking us not our enemy. Since the advent of volatile synthetic pesticide poisons in the 1940s a protracted war against pest population have been waged and now our air, water, food ,mother's milk, blood and adipose tissue all "normally" contain significant residues of these poisons, their metabolites, their "inerts" and contaminants! We have suffered an ever increasing array of health effects, damages and deaths- yet our pest "enemy" continues not only to flourish but to

increase. We have continually killed our own allies (the beneficial), poisoned our own water, air, and food and thereby sickened, wounded or killed ourselves and our own forces and continually ignored our enemy's natural weaknesses and engaged in warfare using only one (useless) weapon! We have totally forgotten how to protect ourselves and how to successfully wage war on our pest enemy. Indeed, we are losing the war against these thousand pests mainly because we insist on using only synthetic pesticides" (Tvendten, 2007).

Extensive use of synthetic chemical insecticides for the control of vector borne diseases has created problems related to but not limited to;

- Physiological resistance of vectors including Insect resistance.
- Adverse effect on non-target organisms and environment
- High Operational cost
- Pesticide mishandling, abuse and misuse
- Overdependence on synthetic insecticide and
- Low Community Acceptance

(Brown, 1986)

2.8.4 Impact of synthetic insecticides on the Environment

2.8.4.1 Development of insect resistance

Selection pressure on insect populations due to indiscriminate use of insecticide have caused many arthropod pest species, including human disease vectors like mosquitoes, to develop mechanisms of resistance to withstand insecticide treatments (Nauen, 2007). Prolonged exposure of insecticide over several generations of mosquito like most other insects leads to resistant development, a capacity to survive contact with an insecticide. Mosquitoes can have many generations per year hence high levels of resistance arise very quickly.

Resistance of mosquitoes to particular insecticides has been documented within few years of introduction of such insecticides. Use of insecticides in agriculture has often been implicated as contributing to resistance in mosquito populations and over 400 mosquito species have documented resistance to one or more insecticides and pesticides in agriculture.

In terms of production of synthetic insecticides, the cost of developing and registering new pesticides is awesome, almost 60 million US dollar, pesticide manufacturers are unwilling to risk investment on products whose market life could be shortened by development of pest resistance (Shand, 1989). Insecticide resistance is a very important problem because out of 50-60 million species of insect on earth only about 1 million has been classified and only about one thousand are pest of importance and already over 50% of these one thousand pests are resistant to these volatile (dangerous) synthetic pesticide (Tvendten, 2007).

2.8.4.2 Overdependence on synthetic insecticide

Synthetic insecticides are fast acting, usually broad spectrum with effectiveness against wide variety of pests and hence for this reason they are commonly used in and around homes. In terms of economics, about 87 million pounds of pesticides are used in and around homes annually. Of this about 43% and 32% are insecticides and herbicides respectively. The remaining 25% is made up of fungicides, rodenticides, disinfectants and other chemicals (US EPA, 1978). Also, it has been shown that 85% of homeowners store at least one or more household pesticide products (Subramanian, 2004).

2.8.4.3 Pesticide mishandling and Abuse

Although the use of pesticides especially insecticides in Africa represents a small fraction of the global total, its misuse and abuse is disproportionately high. Factors that lead to this high rate of misuse include the high illiteracy level, inaccessibility to suitably protective clothing (Birech *et al*., 2006) and the need to speed track effect on pests and vectors.

The resultant effects of this misuse include human poisoning, destruction of natural predators of pests, and non-target organisms, insecticide resistance, crop pollination problems due to honey bee losses, domestic animal poisonings, contaminated livestock products, aquatic and wildlife losses and contamination of underground water and rivers (Pimentel *et. al*., 1980). Annually about 1 million cases of pesticide poisoning are estimated with about 20,0000 deaths mostly in developing countries, in the USA about 70,000 children were involved in common house-hold pesticide-related (acute) poisoning or exposure in 2004 (Tvendten, 2007). Also in some regions use of particular pesticides have become popular methods of

self-harm, especially gaining notoriety among health care workers and the public (Eddleston, 2000).

Lack of adequate knowledge among pesticide handlers in developing countries have led to misuse in all steps of application as is obvious in all stages from point of purchase transporting, storage, application and subsequent disposal of empty pesticide containers (WHO, 1994).

Synthetic pesticides affect wildlife and humans even babies' health but a concern more specific to these pesticides is the danger attached to the chronic regular intake as residues in the environment, thus the environmental concerns about residual insecticides.

2.8.4.4 Effect on non-target organisms and environment.

Various adverse effects on non-target organisms have been linked to the use of synthetic insecticides in the environment, these includes:

1. **Distribution of insecticides and insecticide residues in water bodies due to their ubiquitous nature**

   A major source of water contamination is the use of large quantities of pesticides, insecticides included especially in developing countries to control insect vectors of public health importance. The persistent chemical residues in the waters to which they are applied are carried to adjacent areas by water movement and the contamination maybe of local nature or spread to considerable distances in time and space.

   Other sources of these residues in water include:

   1. Land runoff into rivers and
   2. Percolation into ground waters during the rainy season as well as
   3. Leachates from domestic refuse dumps into ground water.

Osibanjo (1990) reported that the maximum residue values of organochlorine pesticides in ground water were higher than in surface water and concluded that there was greater contamination of ground water than surface water, an example is DDT which was the least soluble of the pesticides surveyed hence it was not detected in surface or ground water samples

however the more soluble metabolite DDE was detected in ground water. There are direct and indirect effects arising from the intrusion of pesticides into water, for instance fish kill is documented and at least some aspects of its effect (e.g. economic, recreational aesthetic) are readily apparent. However, the major subtle but significant effects that are in the form of residues in aquatic biota which biomagnifies and bio accumulates along the food chain are not so apparent.

2. Distribution of insecticides in air

The finding of ubiquitous DDT in areas remote from point of application points to the fact that there are other methods of transport of these products. It was found that DDT was present in rain and snow collected in various parts of the world and in atmospheric dust over the land and ocean. Hence, atmospheric route is accepted as the major route of long distance movement of pesticides in the environment.

The level of pesticides in the air depends to a large extent on the intensity of pest control programmes using pesticides in that area. Koeman *et al*. (1972**)** in a survey of pollution of Lake Nakuru in Kenya suggested that certain tropical regions where pesticides were extensively used may be important sources for the observed atmospheric pollution. Thus a persistent insecticide like DDT is globally distributed considering its continued use and obviously in developing countries, continued use will extend its stay in the environment with all its attendant health hazards.

3. Effect on man

A substantial part of applied chemicals and their degraded products may persist for years in the bodies of animals including man. These small concentrations have substantial biological consequences. They may cause cancer (Carcinogenic), may be responsible for birth defects (Teratogenic) or cause genetic alterations (Mutagenic).

Literature reports on confirmed or suspected chronic effects of pesticide residue contamination on human health exists worldwide and the list gives serious cause for concern globally.

1. Embryo malformation possibly caused by contact during pregnancy with the herbicide, Dinoset.
2. Danger of cancer from fungicide, the American environmental protection agency (EPA), found five active substances that are either carcinogenic or potentially carcinogenic. 28 pesticides containing these five active substances were further analyzed. It was found that the carcinogenic potential was most frequent in fungicides with 60% followed by herbicides and insecticides.
3. A rare type of cancer (non-Hodkins' Lymphoma) was found six times as often in farmers who had been spraying herbicides especially 2 ,4-D for more than 20 years than in non- farmers
4. Bronchial cancer is diagonised twice as often in those exposed to pesticide in agriculture than those who are not.
5. Alterations in brain waves and neurological abnormalities were found in workers whose blood serum showed sub-acute toxic level in Lindane
6. It is interesting to note that children whose homes and gardens are treated with pesticides have 6.5 times risk of Leukaemia than children living in untreated environment (Rea, 1996).

Organochlorine pesticides grossly contaminates human tissues and relatively high levels of DDT, HCB, Lindane and Endosulfam in human breast milk has been reported(UNEP,2002) and this is of concern in view of WHO's rigorous campaign that mothers' breast milk is best for children. It has also been established from studies in South Africa that organochlorines can be transferred to infants via breast milk and adipose tissue showed high levels of DDT (6375 µg/kg) and HCB(4650 µg/kg).

In Nigeria, occupational exposure affected levels in blood as high as 11,565 µg/kg of DDC, 958 µg/kg for Aldrin and 92 µg/kg for Dieldrin(UNEP, 2002).In fact the problem is mainly their chronic effects, for example DDT in the blood indicated very recent exposure whereas DDE (a metabolite of DDT) reflects the chronic level of DDT exposure. One year was required for the metabolism of DDT into DDE when volunteers were fed with DDT dosages (5-35 mg/day) (El-Zorgani & Musa, 1976).

4. Effect on the Environment

One of the major ways by which danger arise due to insecticide usage is when the environment becomes contaminated with a persistent material so that food and water all carry a low concentration, much too low to be acutely poisonous but enough to maintain a constant level in the bodies of the population. This is the problem about which nations are mostly concerned because the whole human population and natural fauna can be affected.

5. Damage to non- target, non-adjacent animals and plants through the food chain

The effects of insecticide residues on animals and wildlife include death of avians after eating granular pesticides; animals may develop cancer, abnormal thyroid function, decreased fertility, decreased hatching success, demasculinization and feminization of males, alteration of immune function (Hammond, 1995). Dietary intakes of meat, milk and milk products and other animals form the greatest sources of human exposure to these persistent toxic substances in form of residues.

Sometimes these insecticides are applied directly to plants to control feeding pests, in such cases residues may be found much more in the aerial than in the plant root. However, with soils incorporated pesticides a vast majority of evidence indicates that more residues are found in the root than in the aerial portion.

Osibanjo and Adegeye(1989) in a survey of organochlorine pesticide levels in 9 fruit species and 16 vegetable species from12 major towns in Nigeria compared values with those of developed countries and WHO/UNEP GEMS results and found that the residue concentrations detected were much lower than the codex limits (Codex Alimentarius Maximum Residue Limits)(FAO/WHO, 1986).

6. Effect on the soil

The amount of pesticides, insecticides inclusive depends on the rate and frequency of application and also on the type of chemical used i.e. whether persistent or non-persistent.

A comparison of occurrence and concentration of organochlorine pesticides in Nigerian soil to agricultural soils in the United States of America showed that the residue levels in Nigeria was higher than those in the U.S. (Osibanjo, 1990). Understandably, the use of pesticides in the U.S as in most developed countries has been restricted hence the seemingly low residue levels whereas in Nigeria and in most developing countries such chemicals are still been used in high quantities. In Sudan, Dichloro-Diphenyl-Trichloroethane (DDT) and Hexachloro-Cyclohexane (HCH) at levels of 17,400*n*g/g and 880*n*g/g respectively have been reported (UNEP, 2002).

However, the fact that some quantities are still been detected after long periods of discontinued use even in the advanced countries is a reflection of the prolonged persistence of organochlorine pesticides. With other classes of insecticides like the organophosphates, carbamates and pyrethroids there are usually no residue problems though they are highly toxic to mammals.

Edwards (1973) listed four possible effects on living organisms in soils contaminated with these chemicals and these are:

1. Residues may be directly toxic to animal or plant life in the soil hence affecting soil fertility by eradication of fauna,
2. They may affect the organisms generally to produce populations resistant to the insecticides,
3. They may have sub-lethal effects that result in alterations in behaviour or changes in metabolic or reproductive activity and
4. They may be taken into the bodies of soil flora and or fauna and passed on to their organisms and hence may also affect neighbouring and successive crops.

7. Effect on food

Animals and plants constitute the major food for man and pesticides applied to the soil are absorbed by plants, which in turn are eaten by animals. During the residence of these chemicals in the body of the organisms they undergo several chemical and biological transformations to

form new products. If crops are sprayed immediately prior to harvest without an appropriate waiting period even organophosphate residues can persist until the food is in the hands of the consumer.

Aside from all the above, many researchers around the world have identified various adverse effect of the use of synthetic insecticides which include:

8. Damage to decomposers and pollinators
9. Secondary pests outbreaks due to competitors being more affected or vulnerable to pesticides than the target pests or causing damages to predators or other controls of competitors .
10. Damage to natural predators of target pests or other control mechanisms thus leading to resurgence of pests to higher pre-treatment densities.
11. Deposition of pesticides on buildings, walls, paint works, machineries leads to damages. (Ecological Agriculture Projects, 1978)

Due to these attendant problems of synthetic insecticides, there is a current inclination to greener technology than ever since the advent of modern science. The era of dependency on synthetic chemicals of the early and middle 20th century prompted the synthesis of newer chemicals as a panacea for all diseases and ailments. The conservative attitudes of some communities which depended on some natural products in preference to the synthetic were often credited to inertia or "backwardness". But today modern societies finding themselves entwined in this web of their own creation are willing to revert to nature for remedies

Thus in response to this all-pervasive use of synthetics in every walk of life be it agriculture, clothing, preservation or healthcare is now paving way for a search for eco-friendly products. The side effects of the synthetic pesticides are often not less serious than the problem themselves because they cause environmental contamination and they are a great risk to human health, as a consequence there has been an intense search for safer pesticides.

Synthetic chemical insecticides have been used for controlling insects with great success in the past. But the problems associated with their use and procurement has necessitated the exploration of a more sustainable alternative (Echezona, 1997).

In analyzing the problems and the health impacts associated with leaving the insect pest unchecked there is absolutely a need for the search of bioactive substances which have satisfactory properties concerning their effect on the target pest, not expensive to produce and also environmentally safe nowadays and not later on (Shand, 1989).

The problems associated with the use of synthetic insecticides have given rise to the need for effective, biodegradable pest control materials with minimal toxicity to the environment. One of the best ways to reduce synthetic chemical insecticides is to eliminate those products replacing them with alternative control method (Baker, 1984).

Unless we desire death of our race, we must stop releasing tonnes of virtually untested, unstable, synthetic pesticide poisons that are creating synergistic contamination that no one can honestly say they can truly assess all of the human health risks for and which still cannot even control our pest enemy (Stephen, 2005).

Recently there are reported environmental and economic pressures that are fostering heightening interest in the development of commercial bio-pesticides, because a growing number of once highly effective chemical pesticides have become useless due to alarming increase in the resistance of insects to synthetic insecticides (Shand, 1989)

2.8.5. Botanical insecticides

Botanical pesticides are natural pesticidal products derivable from plants and are available as alternative to synthetic pesticides.

The attendant problems associated with the continued use of synthetic insecticides and the increased concern for the protection of the environment in its entirety has necessitated a reduction in use of the synthetic and an increase in the search for better efficient, ecologically sound and environmentally safe insecticides. In recent times, more attention has been paid to studies of natural pesticides in pest and vector control because they are the most cost effective and environmentally safe inputs in pest and vector management.

Plants were first used against biting insects by the ancient Greeks and are still being used by a large number of people today (Moore and Lenglet, 2004) even in Africa. Advantages of botanical pesticides include that most are plant based hence making them readily accessible and they have no persistent detrimental effects on the environment as they rapidly degrade to harmless substances.

Botanical insecticides have long been touted as attractive alternatives to synthetic insecticides in pest and vector management because they pose little threat to the environment or human health (Murray, 2006). Numerous plant products have been reported either as insecticides for killing larvae or adult mosquitoes or as repellents for mosquito biting and are one of the best alternatives for mosquito control (Brown, 1986, Sukumar *et. al*., 1991).

There are about 3,000 plants and trees with insecticidal and repellent properties in the world (The Hindu, 2002). These fall into several categories including repellents, feed deterrents, toxins, growth regulators. So many plant trees have been investigated and proven to be active as insecticides. Some of the common botanicals include pyrethruim, neem, Ryania, Red squll, Derris Nicotine, Rotenone, Limonene, Ocimum, Citrus. So many plant trees and shrub parts have been investigated and proven to be active against insect pests. Natural pesticidal products, also called botanical pesticides are available as alternatives to synthetic chemical formulations. Because most botanical insecticides must be eaten by the insect pests, they are primarily harmful to these pests and do little harm to beneficial insects.

Most botanicals are rapid acting and are of low to moderate toxicity to mammals. The mode of action of botanical insecticides gives it a comparative advantage over synthetic insecticides that are usually broad spectrum in action. Botanical insecticides usually must be ingested by the insect pest hence they are specific in action and primarily harmful to these pests and do little harm to beneficial insects or animals.

One limitation of botanical insecticides is in their rapid breakdown ability while this makes it less risky to health and the environment; this often creates a need for precise timing or more frequent applications (Karen, 2005). Also although often thought as natural and hence

assumed harmless, safety clothing must be worn when spraying these, even though their toxicity is low to warm blooded animals some botanical pesticides have been found to be toxic to fish and other cold blooded creatures and should be handled with care (Relf & Luna, 1997), some have also been found to be lethal to a wide range of insects including natural predators and parasitoids.

Examples of available insecticides of plant origin include but are not limited to the following: Derris dust is a product derived from the root of several species of tropical legumes (Ordish, 1967), especially *Derris elliptica* (Angus, 1978) and *D. trifoliate* which grows in tropical Asia and parts of the Congo Basin. The active ingredient of derris dust is found to be lethal to a wide range of insect species as well as earthworms and fish; therefore it is advisable not to use the product near dams and waterways (US EPA, 1991, Endersby and Morgan, 1991). Quassia is made from the bark and root of *Picrasma quassioides,* a South American tree. The spray is effective against aphids and caterpillars which are small in size and also found to be safe on the larvae and adults of ladybirds. However as an insecticide, quassia is lethal to the larvae of hoverflies and may taint food crops if applied just before harvesting (Conacher, 1986).

An insecticide can be extracted from the seeds and leaves of *Azadirachta indica A.Juss*, popularly called Neem. The tree occur naturally in the hot, dry tropics of Southern and South Eastern Asia and their seed extract has been used as an insecticide throughout these regions for thousands of years (Rice, 1989). The seeds contain triterpenoids known as azadirachtin which has deterrent, anti-feedant, growth disrupting, anti-ovipositional and fecundity reducing properties on a range of insects (Schmutterer, 1990). Nymphs or larvae of phytophagus insects and Lepidopterous larvae are susceptible to neem derivatives when they are formulated as feeding poisons. This property makes neem suitable for use in pest management as parasitoids are spared, it does not have an immediate knockdown effect on pests, but reduces feeding and death occurs within several days while the residual effect may persist for two to seven days (Schmutterer, 1990).

Azadirachtin, the key insecticidal ingredient found in the neem tree is a naturally occurring substance that belongs to an organic molecule class called tetranortriterpenoids (Grace-Sierra, 1990) and the tree's major agent of battling insects is one of the most powerful and safest natural bio-molecule as pesticide. In agriculture it is so potent that a mere trace of it can prevent some insects from touching the plants. It is similar in structure to insect hormone called "Ecdysones" which controls the process of metamorphosis as the insects pass from larva to pupa to adult and azadiracthin seems to be an "ecdysone blocker". As it blocks the production and release of vital hormones, leading to inability of insect to molt, thus breaking their life cycle.

Nicotine extracted from the tobacco plant, *Nicotianaa tabacum* L, is highly toxic to mammals but breaks down within 24hours; it is also a powerful insecticide on the larvae of Lepidoptera and other pests while also been lethal to some beneficial insects and earthworms (Conacher, 1986; Bennett, 1988).

Rotenone acts as a stomach poison and is effective up to 48 hours after application; it has a synergistic effect when applied with pyrethrum which is extracted from the flowers of *Chrysanthemum cinerariaefolium* or *C.roseum* (Conacher, 1986, Bennett, 1988). It kills aphids and caterpillars but also affects other arthropod predators such as hover birds, lacewings and ladybird larvae.

2.9 Moringa oleifera Lam.

2.9.1 Scientific classification

Kingdom:	Plantae
(Unranked):	Angiosperms
(Unranked):	Eudicots
(Unranked):	Rosids
Order:	Brassicales
Family:	Moringaceae
Genus:	Moringa
Species	*Moringa arborea*
	Moringa borziana
	Moringa concanensis

Moringa drouhardii

Moringa hildebrandtii

Moringa longituba

Moringa oleifera

Moringa ovalifolia

Moringa peregrina

Moringa pygmaea

Moringa rivae

Moringa ruspoliana

Moringa stenopetala

2.9.2 Description

Moringa is the only genus in the family Moringaceae. And it comprises 13 species, which span a range of life forms, from tiny herbs to massive trees; all from tropical and sub-tropical climates. The taxon name M*oringa* comes from the Tamil/Malayalam word *murungakkAi* (Wikipedia, 2009).

The most widely known species is *Moringa oleifera,* a multi-purpose tree native to north western India (Gidamis *et al*., 2003, Olson, 2001), and which is commonly referred to when the name *Moringa* is used. The African species, *M. stenopetala*, is also widely grown, but to a much lesser extent than *M. oleifera. M. oleifera,* is commonly referred to as "*Moringa*", Drumstick tree, horse radish or West Indian Ben tree. Its other names such as Miracle tree, mother's best friend e.t.c. are in recognition of its versatility and use. In Nigeria it has so many names depending on ethnic or local languages including Ewé ilé (Yoruba), Okochi egbu (Ibo) and Zogale (Hausa). It is commonly planted in Africa as a living fence tree (Von-Maydell, 1986) and also used as forage for livestock.

2.9.3 Botany

A decidous, perennial tree with height between 10 and 15 m, rather slender with drooping branches (F/FRED, 1992). It is a fast growing plant which produces leaves and pods that are edible by humans and livestock. It has a soft white wood and corky, gummy bark. Root has

the taste of horseradish. Each compound leaf contains 3-9 very thin leaflets dispersed on a compound tripinnate (3 times pinnate) stalk. Flowers are white and fragrant, producing long pendulous, 9 ribbed pods, 3- angled winged seeds. Propagation is by seeds and stem cuttings. In cultivation, it is often cut back annually to 1m or less and allowed to re-grow so that pods and leaves remain within arm's reach.

2.9.4 Distribution

Moringa is grown mainly in semi-arid, tropical and sub-tropical areas. It grows best in dry sandy soil, tolerates poor soil including coastal areas. It is drought resistant and grows in practically all kinds of well drained soils and conserves water by shedding leaves during the dry season. It is widely cultivated and naturalized in tropical Africa, Central and South America, Sri Lanka, India, Mexico, Malaysia and the Philippines. It is grown in settled areas as a backyard vegetable and as a border plant.

The *Moringa* tree is considered one of the world's most useful trees; almost every part of it can be used for food or has some beneficial property. It is a traditional food plant which has potentials of improving nutrition, boosting food security, fostering rural development and support sustainable land care (National Research Council, 2006).

2.9.5 Ecology

Moringa ecology ranges from sub- tropical Dry to moist through tropical very dry to moist forest life zones, it prefers neutral to slightly acidic soils (pH of 4.5-8) and grows best in well drained loam clay loam and tolerates clay soils but does not grow well if water logged., it thrives in sub-tropical and tropical climates flowering fruiting freely continuously. It grows best on dry sandy soil (James, 1983).

It requires an annual rainfall of between 250-3000mm. it is drought resistant tree though in drought conditions it may lose its leaves but recovers when the rains arrive. It grows best at altitudes up to 600m but will grow at altitudes of 1000m. It will survive in a temperature range of 25-40˚C but is known to tolerate temperature of 48˚C and light frosts (HDRA, 2002).

It loses its leaves from December to January and new growth starts in February – March. It produces cream coloured flowers at 8 months and the flowering season begins in January and continues through to March. Fruits ripen from April to June. The pods are triangular in cross section, 30-50 cm long and contain oily, black, 3 winged seeds (HDRA, 2002).

2.9.6 Chemistry

The pod contains per 100g, 86.9g H2O, 2.5g protein, 0.1g fat, 8.5g total carbohydrate, 4.8g fibre, 2.0g ash, 30mg Ca, 110mg P, 5.3mg Fe, 184IU Vitamin A, 0.2mg niacin, 120mg ascorbic acid, 310µg Cu and 1.8µg I.

Leaves contain 7.5g H2O, 6.7g protein, 1.7g fat, 14.3g total carbohydrate, 0.9g fibre, 2.3g ash, 440mg Ca, 70mg P, 7mg Fe, 11,300 IU vitamin A, 120 µg vitamin B, 0.8mg nicotinic acid, 220mg ascorbic acid and 7.4mg tocopherol per 100g. Estrogenic substances, including β-sitosterol, an anti-tumour compound and a pectinestarase are reported. Pterrygospermin, a bactericidal and fungicidal compound, isolated from *Moringa* has an LD_{50} subcutaneously injected in mice and rats of 350 to 400 mg/kg body weight.

2.10.7 Utilization

All parts of the tree can be used in a variety of ways *viz*:

a. Human food

The leaves are full of vitamins, are good as food, low in fats and carbohydrates and rich in minerals, iron and vitamin B (HDRA, 2002). Of all the plant parts used, the leaves are the most utilized. The leaves can be used in the same way as spinach and are used to make tea for a variety of medicinal purposes and are also consumed as salad. The seed contains 35-40 % oil which can be used for cooking, gives good soap, does not turn rancid and also burns without smoke (HDRA, 2002; James, 1983; Burkhill, 1966).

In Asia, the flowers of *M. oleifera* are mixed together with other foods since they are rich in Ca^{++}, K^{+}, waxes, alkaloids, quercetin and Kaempferol (Rangaswani & Sankarasubramian, 1946; Ramachandran *et. al*., 1980). Roots have taste similar to horseradish and can be used to make sauce similar to horseradish sauce at the seedling stage of the plant. Gum from the plant bark can be used to season food.

Plate 2.3: **Fruiting branch of *Moringa oleifera* tree**

(Source: **Google images)**

b. Animal fodder

Ruminants and poultry browse the bark, leaves and young shoots of *Moringa*, livestock diets are improved by the addition of *Moringa* plants parts (HDRA, 2002). The dry pods have adequate characteristics to be used as substratum for lab animal bedding (Farias *et. al.*, 2004).

c. Water purification

Leaves and fruits of *Moringa* possess coagulatory properties hence are used in water purification. Water extracts obtained from dry seeds have been used due to their excellent turbid water coagulation properties attributable to the presence of cationic electrolytes (Jahn, 1988; Gassenschmidt *et. al*., 1995; Ndabigengesere *et. al*., 1995).

Treatment of impure water with *Moringa* seed powder removes 90-99 % of bacteria in contaminated water (HDRA, 2002; Von Maydell, 1986; Olayemi and Alabi, 1994) and offers the advantage of being a natural purification agent with minimal environmental hazard. Its use in water purification replaces chemicals such as aluminium sulphate which are dangerous to the environment and expensive. It is a cheaper alternative to mechanical filtration.

In Brazil, seed powder suspension have been introduced efficiently into the North East region due to the tree's good adaptation to arid areas as an attempt to improve hygiene habits and life quality, help to reduce child mortality and collaborate with the sustainable development of the region (Morton, 1991; Gerdes, 1997; Ferreira *et. al*. 2008)

Plate 2.4: Pods of *Moringa oleifera*

d. Natural medicines

Every part of *Moringa* has been effectively used against various ailments. Leaf extracts show antioxidant and hypocholesterolaemic activities (Iqbal & Bhanger, 2006; Chumark *et. al.*2008). Leaves can be rubbed against the temple to relieve headaches, around bleeding sites to stop bleeding; extracts can be used against bacterial or fungal skin infection (HDRA, 2002; James, 1983).

Moringa is also good in treating malnutrition due to its high fibre content (HDRA, 2002 and James, 1983), this is particularly of interest in region where malnutrition is a problem and in people living with HIV/AIDS infection and it is a current plant promoted by the WHO in combating malnutrition in children and other vulnerable groups to boost immunity to infections. *Moringa* popularly called mother's best friend in the Asian continent has been advocated as "natural nutrition for the tropics." The leaves can be eaten raw or stored as dried powder for many months without refrigerating without any loss of nutritional value, it is especially promising in the tropics because it is usually in full leaf at the end of the dry season when other foods are typically scarce (Jed, 2005). The flower juices improves the quality and flow of breast milk in nursing mothers and is also useful for urinary problems as it encourages urination. Pods act as de-wormers and in treating malnutrition and diarrhoea. Seeds are used for their antibiotic anti-inflammatory properties to treat rheumatism, arthritis, gout, cramp and boils and they are also used as relaxant for epilepsy.

Plate 2.5: Winged seeds of *Moringa oleifera* (Mag: x2)

e. Fertilizer

The seed cake obtained after oil extraction contains high levels of protein and makes a good fertilizer in agriculture (HDRA, 2002).

f. Living fence

Moringa provide wind protection and shade hence it is used as a living fence (Hausa) tree especially in the arid regions and in the north of Nigeria (Von Maydell, 1986; HDRA, 2002).

g. Alley cropping

It has large tap roots and few lateral roots hence it does not compete for nutrients with crops, its many protein-rich leaves also provide nutrients to neighbouring crops; they are good at reclaiming marginal land (HDRA, 2002).

h. Bio-pesticides/ Natural pesticides

Leaves possess fungicidal properties particularly against *Pythium spp* that causes damping off disease of seedlings (HDRA, 2002); ethanol extracts of the leaves were also found to be effective against plant nematodes *in vivo* and *in vitro* (Kanu, 2005). The seeds possess antimicrobial (Ali *et al*. 2004, Chuang *et.al.* 2007), anti-inflammatory, antispasmodic, diuretic and antitumor properties (Carceres *et. al*. 1992, Guevara *et. al.* 1999).

i. Biofuel, Fuel wood, energy and other uses

The wood is light and is a good fuel for cooking, but it is not suitable for building. The bark can serve for tanning and can be beaten into coarse fibre to make rope or mats.

The wood produces blue dye; chipping from the wood can be used in paper making. The tree also produces viscose resin used in the textile industry and is a potential source of wood for the paper industry (Verma *et.al.,* 1976). The leaves are also used to clean cooking utensils and even walls (HDRA, 2002).

CHAPTER THREE

METHODOLOGY

3.1 Study Design

A laboratory based experimental study design was employed and this was carried out in two phases viz: A larval bioassay to test the larval efficacy of the aqueous extract of *M. oleifera* (AEMOS) on 3rd instar larvae of *Anopheles* mosquito and a toxicity experiment to assess the acute and chronic toxicological effects of the AEMOS on *Poecilia reticulata* commonly referred to as Mosquito or Guppy fish. Complete Randomized Block Design was used for the study.

3.2 Materials and methods

3.2.1 Seed Sampling

The plant, *M. oleifera*(Lam) commonly called *Moringa* or Drumstick tree was used in this study and the plant part used was the seed (Kernel).

The method of seed collection and handling was according to Vyas and Mistry, (1996). Purposive sampling was applied due to the plant's ubiquitous distribution in Nigeria and particularly Ibadan. The seeds used in this study were collected from Akobo area of Ibadan, Oyo State, Nigeria.

3.2.2 Seed Processing

The process carried out on the seed before extraction are summarized in a flow chart below

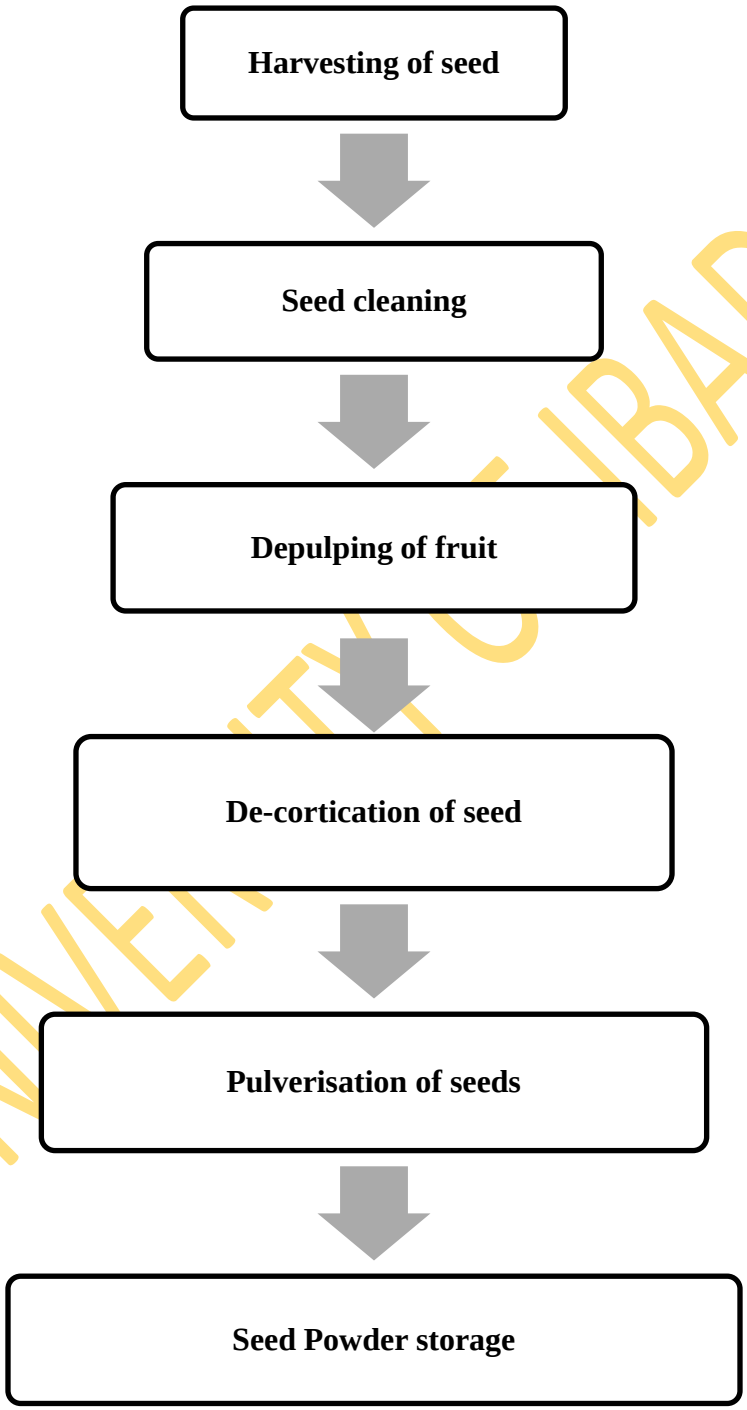


Fig.3.1 The flow chart of *Moringa* seed processing

Harvesting: Fully matured pods were plucked from the *Moringa* trees. The matured pods were greenish brown in colour at the time of collection this was done to avoid breaking up of the pods leading to dispersion of the seeds on the tree once they become fully brown on the tree as the mechanism of dispersion is wind.

Cleaning: The pods were cleaned by hand sorting to separate foreign materials such as dust, sand, stones and left under shade to dry at ambient temperature before depulping was carried out.

Depulping of fruits: The pods were left to dry under shade for 72 hours and were turned over every 12 hours to prevent rotting of the pods due to fermentation of carbohydrate and microbial growth on the pods which will invariably affect the quality of the seeds. After this the pods were depulped to detach the seeds from the pods and the seeds were immediately left to air dry before they were stored in a container to maintain freshness.

De-cortication of seed: The essence of dehulling was to remove the seed coat which might reduce the efficiency of extraction due to absorption of solvent by the shells or pulp. The dehulling process was done manually, by carefully exerting pressure on the seed using pestle and mortar to separate the kernel from the seeds just as is done locally. The resulting sample was winnowed and sieved and handpicked to obtain pure seed kernels which were whitish in colour and soft to touch.

Pulverization of seeds: This was carried out to ensure maximum contact between the solvent and kernel constituents and to increase the surface area of the sample. The seed kernels were pulverized using an electric blender. The particles were pulverized to approximately 0.3-1.25mm to allow for increased surface area penetration of the solvent.

Seed powder storage: the powder was immediately stored in an air tight container until used for extraction, the maximum storage life of the powder used for the study was 7 days. After the 7^{th}day the powder not used were discarded and the process repeated, this was to ensure that there was no deterioration in the quality of the sample used for the study.

3.2.3 Extraction procedures

Distilled water was added to the powdered seed in the proportion of 1 seed (approx. 200 mg) per 10 ml of distilled water (Gerdes, 1997, Ferreira *et.al.*, 2009). The whole mixture was then stirred for 60 minutes at room temperature (25˚C) using a magnetic stirrer and then filtered through Whatman No. 1 paper. Soluble solids concentration of the AEMOS was taken into consideration and was calculated for the mass present in the water extract to give the yield in weight of soluble solids per weight of powdered seeds.

This was done just before use in the laboratory to ensure purity and no bio-degradation of active ingredients before the toxicity test and to limit possible fermentation.

3.3 Experimental Animals

3.3.1 Mosquito larvae breeding

Anopheles gambiae s.s., the most notorious vector of malaria in Africa and the principal vector of Lymphatic filariasis in sub- Saharan Africa (Okumu *et. al*., 2007, WHO, 1997) was used in this study to assess the larvicidal effectiveness of the water extract of *M. oleifera* seeds.

3rd instar larvae of *A. gambiae s.s.*, were obtained from an established colony reared in the insectary of the Molecular Entomology and Vector Control unit, Public Health Division, Nigeria Institute of Medical Research, Yaba, Lagos, under greenhouse conditions (25-30^{o}C, Relative humidity 60-70%) following standard operating procedures for mosquito maintenance (WHO, 1975) and modified by Adebayo *et. al.* (1999).

The female adult *A. gambiae s.s*,(Kisumu) from already established colony in the laboratory were fed with blood meal from exposed skin of experimental animals (Guinea pigs) in a netted cage (37x30x28 cm) at ambient temperature overnight in a dark room. Also moistened filter papers were gently placed on moistened cotton wool and mounted on petri dishes in the cage to facilitate oviposition of mosquito. After 24 hours, the moistened filter papers were filled with batches of brown-black coloured eggs laid singly on the papers. The filter papers containing the eggs were then carefully transferred into bowls of water in the insectary. Within 48 hours, the eggs had hatched to larvae and were seen floating parallel to the water surface and examined to confirm that they were *Anopheles* species. The larvae obtained were fed *ad libtum* with baby fish

meal (approximately 0.015g) which was evenly spread across the water surface daily in the bowls; the bowls were covered with plastic mosquito net to prevent intrusion of predators of the larvae and escape of emerged adult. The culture medium was maintained according to the standard maintenance procedure (WHO, 1975) until used for the bioassay i.e. third instar stages.

3.3.2 Sampling of *Poecilia reticulata* (Mosquito/Guppy fish)

P. reticulata, commonly called mosquito or guppy fish was used for the toxicity test and was obtained from the open drains of the Nigeria institute of Medical Research, Yaba, Lagos. The fishes were left to acclimatize for 8 weeks and were kept in well aerated holding tanks under standard conditions of light (12h with alternate day and night cycles) and temperatures 27 ± 2^{o}C , with access to commercial fish feed diet. The investigational protocol was in accordance with international standard on the care and use of experimental animals (EEC, 1986, Ferreira *et. al.*, 2009).

Plate 3.1: Acclimatisation of the mosquito fishes in the laboratory. (Mag: x5)

Plate 3.2: The guppy fishes just before the toxicity assay. (Mag: x50)

3.4 Data Collection

3.4.1 Evaluation of AEMOS on larvae of *A. gambiae s.s.* (Larval Bioassay)

The larvicidal effect of the aqueous extract of *M. oleifera* (AEMOS) on 3rd instars larvae of *A. gambiae s.s.* was carried out under greenhouse conditions. Preliminary tests were carried out to determine the range of lethal concentrations and the most susceptible instar stage of the larvae. All the concentrations above 8700 µg/mL gave rise to 100% mortality within 24 hours. Subsequently, further trials were carried out at lower concentrations to properly monitor the effect of the extract on the larvae. Five aliquots from the stock solution were prepared by serial dilution method (1160, 1450, 2900, 5800 and 8700 µg/mL).

Three independent experiments were carried out in four replicates each. Distilled water was used as negative control and 20 larvae per treatment were used (WHO, 1996 & WHO, 2005). The mosquito larvae were treated with the extracts according to the methodology described by WHO (1981). Twenty larvae of *A. gambiae s.s.* were introduced in different test concentrations of the extract along with control containing distilled water without any test solution. After adding the larvae, the glass dishes were kept in the laboratory, four replicates were maintained for each concentration and mortality observed and recorded 24 hourly for 5 days at room temperature. Larvae were considered dead if they were immobile and unable to reach the water surface after removal into clean water and a further observation for 24 hours.

3.4.2 Toxicity Evaluation on *P. reticulata*, (Guppy fish)

Twenty guppies per treatment were exposed to different concentrations of the AEMOS; 3 aliquots from the stock solution were prepared by serial dilution method (10, 20 and 30 mg/L) with control containing distilled water without any test solution. Treatment was introduced randomly, two independent tests in triplicates was setup for the experiment and the total volume of medium used was 200ml per treatment. The behavioural conditions and mortality in each setup was observed so as to define the response of the test organism to aqueous extract *Moringa* seed.

Parameters such as behavioural responses, increased respiration, loss of orientation, discoloration, motility and mortality were monitored and recorded hourly for the first six hours and there after three hourly for the rest 24 hour period (Ferreira *et. al.*, (2009), Ghosh (1984), Paget & Barnes (1964) and Turner (1965).

The investigational protocol was carried out in controlled environmental conditions in well aerated holding tanks under standard conditions of light (12h with alternate day and night cycles) and temperatures 27 ± 2^{o}C , with access to commercial fish feed diet so as to define the response of the test organism to the *Moringa* seed extract and it was in accordance with international standard on the care and use of experimental animals (EEC Directive, (1986); American Public Health Association (1987); Ferreira *et. al.*, (2009)**).**

Plate 3.3: Larval bioassay setup in the laboratory

Plate 3.4: Larval bioassay replicates arrangement

Plate 3.5: Toxicity assay on *P. reticulata* (guppy fish) in the laboratory

Plate 3.6: Aqueous extract of *M. oleifera* seed (AEMOS) prior to use

3.5 Data Analyses

3.5.1 Larvicidal Bioassay Statistical Analyses

Data were analysed using both descriptive and inferential statistics, the control mortality was corrected using Abbott's formula (WHO, 2003) and Log-Probit analysis was carried out to determine the median (LC_{50}) and 90% lethal concentrations (LC_{90}) values, their 95% confidence intervals were obtained by the method (Finney, 1971). Regression analysis was carried out to compare and determine the strength of relation between the doses administered and mortality observed in the mosquito population used in the study. Statistica 7 program and SPSS Software version 15 were used for the analyses at p=0.05

3.5.2 Toxicological analysis (Mosquito fish assay)

The LC_{50} and LC_{90} values and their 95% confidence intervals were obtained using the Log-Probit analysis method (Finney, 1971) at p=0.05 level of significance. Data were also analysed using descriptive statistics and inferential statistics. Regression analysis was carried out to compare and determine the strength of relation between the doses administered and mortality observed during the study. SPSS Software version 15 was used for the analysis.

CHAPTER FOUR

RESULTS

This chapter presents the results of the aqueous extraction process, the physical characteristics of the extract and the results obtained from the larvicidal bioassay on *A. gambiae* s.s., the behavioural responses of *P. reticulata* as well as the result obtained from the acute toxicity assay on mosquito fishes at different levels of concentration and over a range of exposure period.

4.1 The Soluble solids yield

M. oleifera seed has important medicinal properties and value and is an effective antimicrobial agent whose insecticidal properties and toxicological effects on non-target organisms are being considered in our current investigation. Table 4.1 shows the physical characteristics of the aqueous extract of *M. oleifera* seed powder with respect to weight, colour, and smell. Soluble solids concentration of the aqueous extract of *M. oleifera* was taken into consideration in the experiment and was calculated for the mass present in the extract. The yield of 37%w/w (i.e. 37mg of soluble solids in 100mg of powdered seeds) was obtained from the extract. This served as the basis for the different concentrations used throughout the study.

Table 4.1: Physical characteristics of the aqueous extract of *M. oleifera* seed powder

Parameters	Seed Powder	Aqueous Extract
Soluble solids (w/w)	100mg	37mg
Colour	White	Translucent
Smell	Nutty smell	Mild
Taste	Aspartame sweetness	Bland
Appearance	Fine Powder	Clear

4.2 Larvicidal Properties/ Results

4.2.1 Larvicidal activity of the aqueous extract of *Moringa oleifera* seed (AEMOS) on *Anopheles gambiae* s.s.

Assessments of the efficacy of different phytochemicals obtained from various plants have been carried out by researchers on different species of mosquito in the field of vector control (Promisiri *et. al.*2006; Okumu *et. al.*, 2007 and Ferreira *et. al.*, 2009). The result of our experiment showed that the aqueous extract of *M. oleifera* seeds was lethal to 3rd instar stage of *A. gambiae s.s.* larvae. Table 4.2 shows the mean mortality (%) of larvae of *A. gambiae s.s.* exposed to different concentrations of the aqueous extract (AEMOS) over 120 hours exposure time. A closer look reveals that significant differences exist in the mortality levels over the exposure duration across the different concentrations ($p<0.05$). The crude AEMOS at 8700µg/ml caused 94.67±5.03% mortality of 3rd instar larvae within 24 hours and lethal effects were still observed in lower concentrations as exposure time increased. Mortality pattern was not the same at each treatment level over the period of exposure as mortality for the AEMOS increased as the concentration and exposure time increased showing a progression in larval death in a dose dependent manner over the period of exposure (Fig4.1).

The mosquito larvae exposed to the different concentrations showed behavioural effects such as inability to swim to the top within 45 minute of exposure, reduction in wriggling rate of the larvae. Generally, all the concentrations of the AEMOS used (1160- 8700 µg/ml) showed reduction in the wriggling rate of the larvae and the larvicidal effect was due to the action of the AEMOS which increased with increase in concentration (Fig 4.1). In the control experiment, however, larvae activities were not different from normal and they floated normally and no mortality was observed throughout the period of the study.

Table 4.2: Mean mortality (%) of 3rd instar larvae of *A. gambiae s.s.* exposed to aqueous extract of *M. oleifera* seed (AEMOS)

Treatments	Mean Mortality (%)			
Concentration (µg/ml)	24 hours	48 hours	72 hours	96 hours
8700	94.67±5.03	97.67±2.52	99.33±1.15	99.33±1.15
5800	89.00±1.00	92.33±2.08	94.67±1.15	97.00±2.65
2900	59.00±5.20	72.67±4.04	85.33±4.62	90.33±3.25
1450	23.33±5.77	33.00±0.00	35.33±0.58	39.00±1.00
1160	12.67±5.51	13.67±5.51	13.67±5.51	15.00±7.81

All concentrations measured in µg/ml.
Values are means ± SD of three independent experiments performed in quadruplicates at 95% confidence intervals.
Five concentrations were tested in each experiment with 20 mosquitoes per replicate (i.e. n=480 mosquitoes/ independent experiment).
AEMOS is expressed as soluble solids based on the proportion of 1 seed: 10 ml distilled water.
Data was analysed using STATISTICA 7 Program.

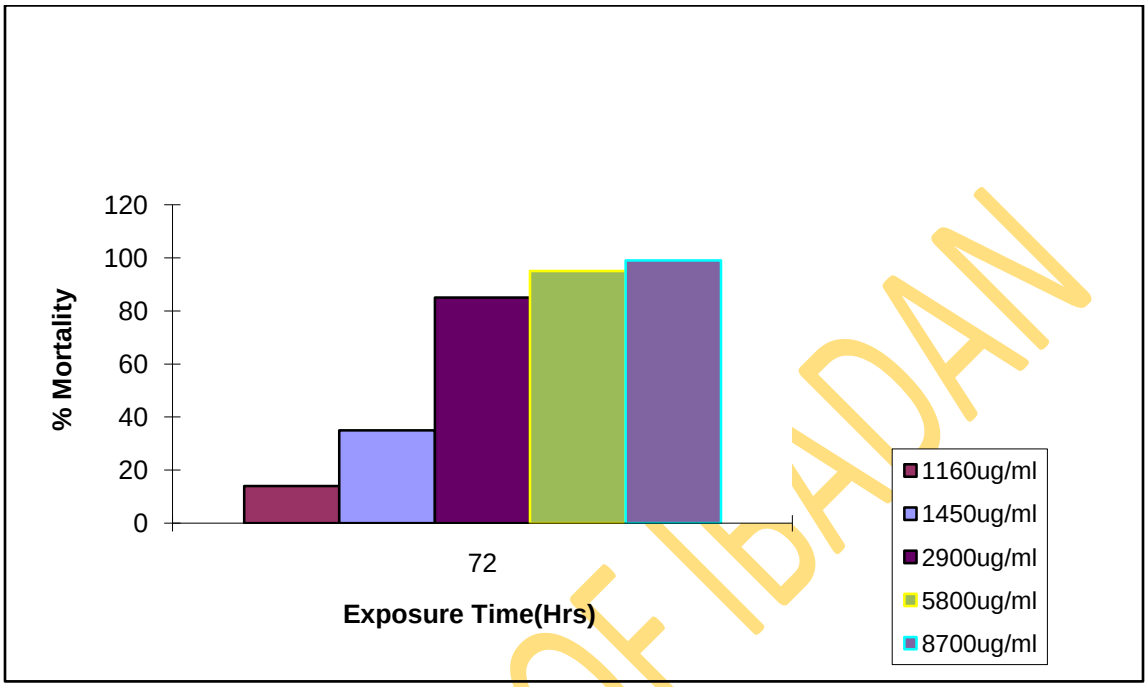


Fig 4.1: Larvicidal activity of aqueous extract of *M. oleifera* on larvae of *A. gambiae s.s.* at 72 hours of exposure.

4.2.2 Dose Response relationship of AEMOS and *A. gambiae s.s.*

All the concentrations were lethal to the larvae but with different degree of effectiveness as shown in Fig 4.2, while at the different concentrations the exposed larvae responded to the treatments in a dose dependent manner. The effect of the AEMOS on larval activity was evident on introduction of the extract and even hours after exposure. Moribund larvae sank to the bottom of the test solution but when touched with a pin or dropping pipette, they responded with little body movement to move away from the area of disturbance. Larval mortality increased across the treatment levels as the concentration increased giving a sigmoid-like curve upon analysis. This pointed to the fact that mortality was caused by the introduction of the extract while zero percent mortality was observed in the control. All dead and moribund larvae were removed from the solution as soon as sighted using picking pins.

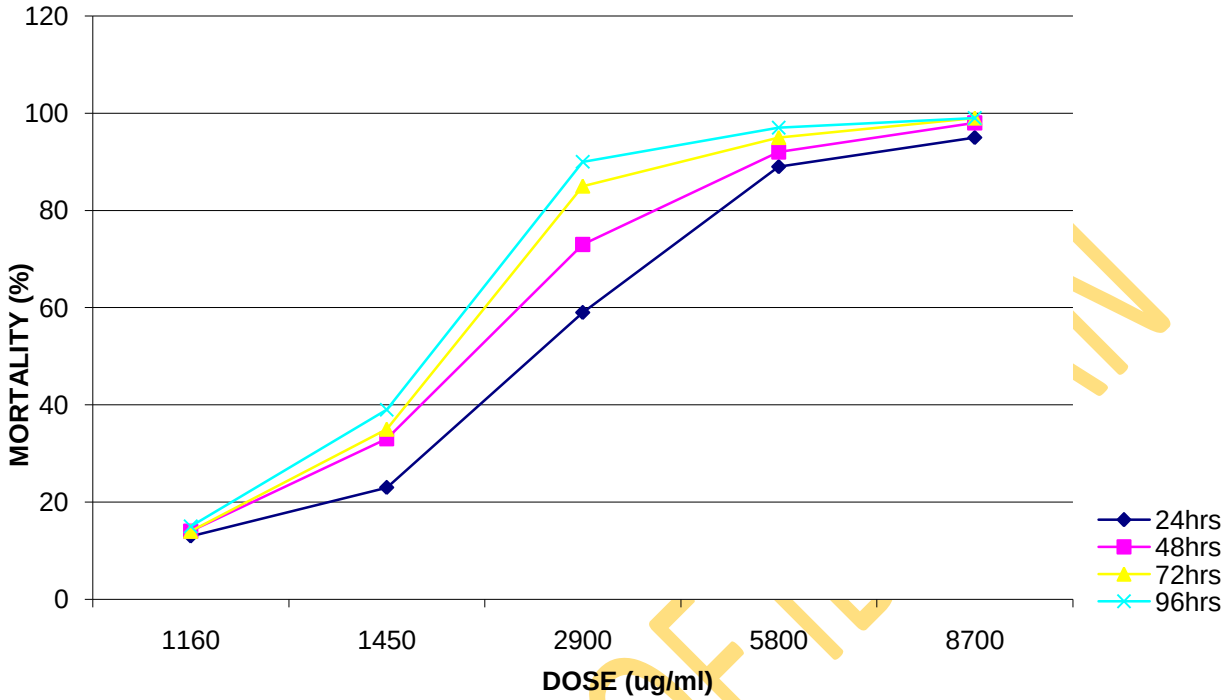


Fig 4.2: The larvicidal effectiveness of aqueous extract of *M. oleifera* seed at different concentrations within 96-hours of exposure.

4.2.3 Pattern of effectiveness of AEMOS on *A. gambiae s.s.*

The trend of effectiveness of the AEMOS as a larvicide at different concentrations is shown in Fig.4.3. The graph shows that at 24 hours the rate of larval mortality increased as the dose increased across the different treatment levels with the lowest mortality (12.7%) observed for the least concentration (1160 µg/ml) while at the highest concentration it was over 94% mortality.

In relation to exposure time, the effect of the larvicide at higher concentrations (2900 µg/ml) at 48 hours was more than twice that recorded at lower concentrations ((1450,1160 µg/ml).(Fig. 4.3). The optimum effect,(94.7%) was observed in 24 hours in the highest concentration(8700 µg/ml) whereas lower concentrations(5800, 2900 µg/ml) attained this only after 48 hours of exposure and the lowest concentration (1160 µg/ml) was only able to produce 15% larvicidal effect at the end of the experiment. The result of the ANOVA for comparing mortality across the different treatments is shown in Tables 4.3 and 4.4. The result indicated that mortality records over the exposure period were significantly different among the treatment groups. ($p< 0.05$). It was also observed that at higher concentrations there was complete inhibition of pupation during the periods of exposure while at lower concentrations some of the larvae in spite of being 3rd instar larvae lived as long as 7 days before they either pupated or died.

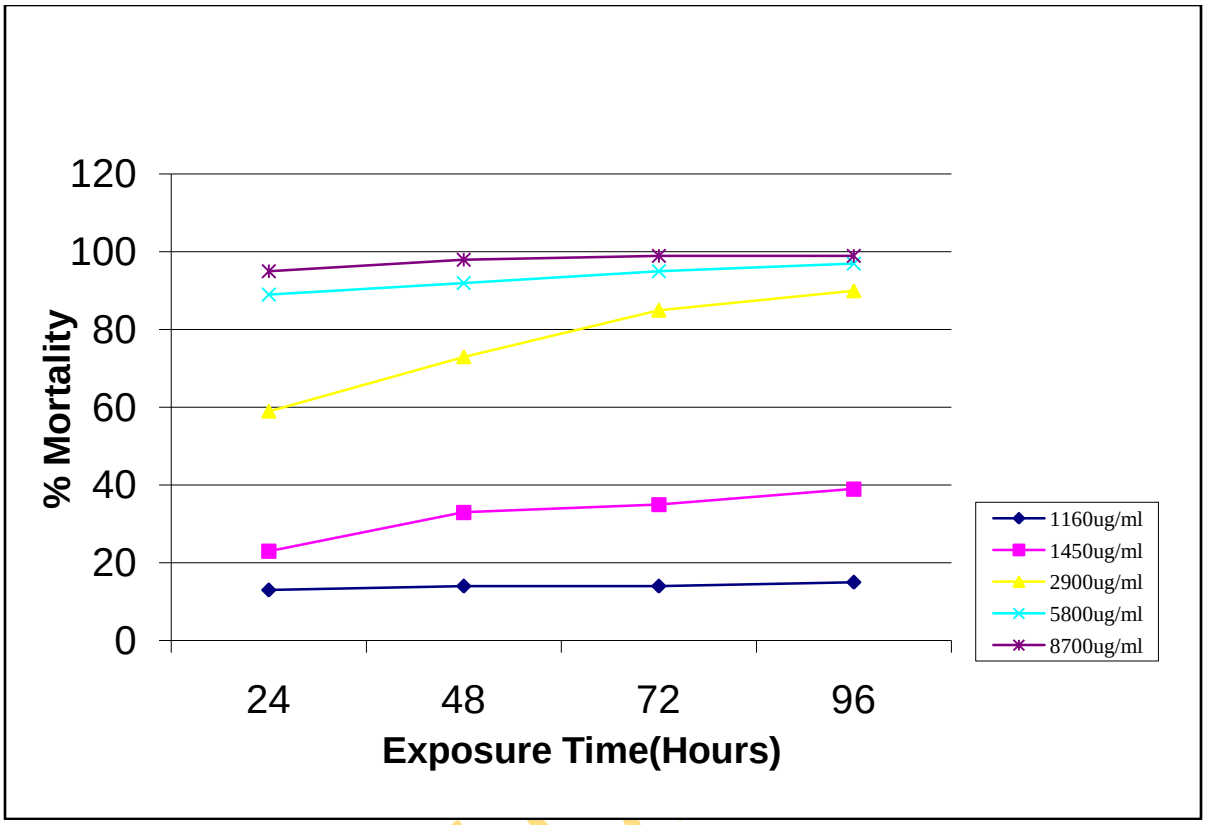


Fig 4.3: Pattern of Dose-Response relationship at different Exposure periods

Table 4.3 Significance of AEMOS treatments to *Anopheles gambiae* larvae mortality

Source of Variation	SS	df	MS	F	P-value
Between Treatments	22571.3	4	5642.83	111.19	0.00
Within treatments	761.25	15	50.75		
Total	23332.55	19			

Table 4.4: Significance of exposure time to *Anopheles gambiae* larvae mortality in AEMOS

Source of Variation	SS	df	MS	F	P-value
Exposure time	22637.52	4	5659.38	200.18	0.00
Treatments	437.16	3	145.72	5.15	0.02
Error	23413.93	12	28.27		

4.2.4 Lethal concentrations determination of the AEMOS.

The result of the Regression analysis (Fig.4.4) shows that there was a high linear relationship between mortality of the mosquito larvae and the concentrations of the AEMOS based on the coefficient of Determination values (r^2=0.87) with a linear equation of (Y=0.01x+12.41). The median anti-larval potency (LC_{50}) of the extract at 24 hours was 2505.8µg/ml while the lethal concentration that results in 90% mortality of the population (LC_{90}) was 6293.4µg/ml, as projected by logarithm of the concentration in base 2. The median anti-larval potency (LC_{50}) of the extract at 96 hours was 1754.7µg/ml while the corresponding LC_{90} was 3396.7µg/ml, as projected by logarithm of the concentration in base 2.

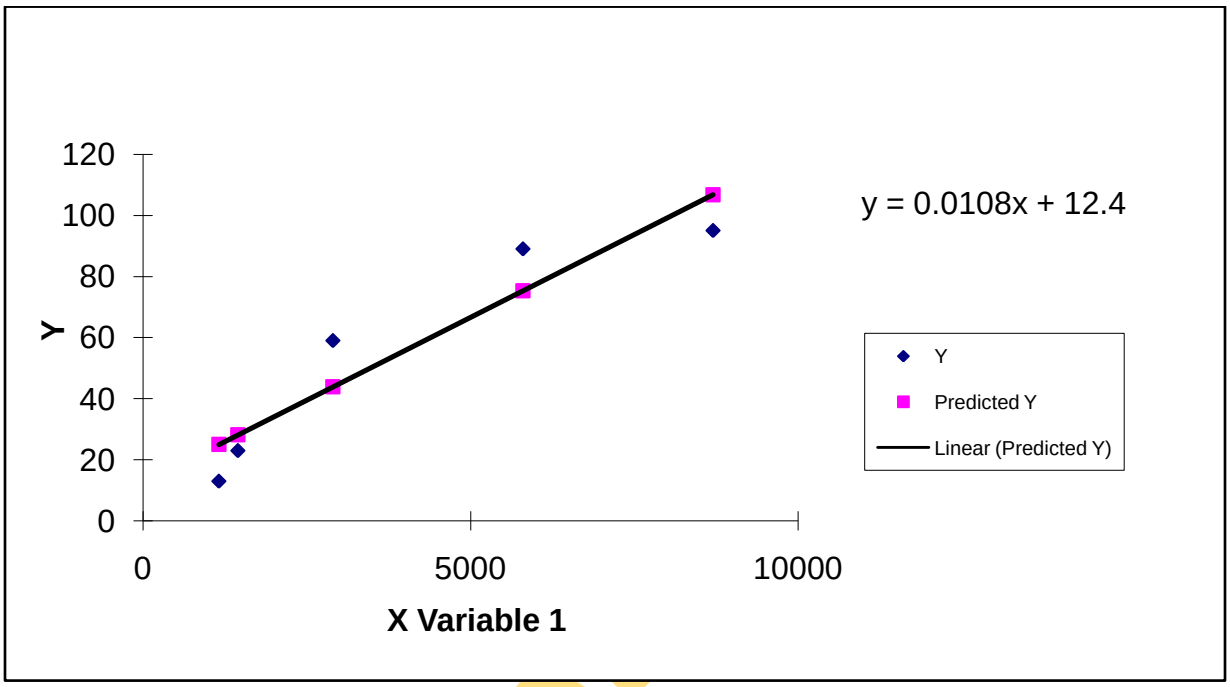


Fig 4.4: Regression Analysis showing the line fit plot of larval mortality on concentration

Table 4.5: Lethal concentrations of AEMOS on larvae of *A. gambiae* s.s. after 24-hour exposure

Time (Hrs)	96[1]	24[1]
LC_{50}[2]	1754.7 (1248.3-2427.4)	2505.8 (2271.6-2760.7)
LC_{90}	3396.7(2448.8-8634.7)	6293.4(5455.2-7528.6)

[1] Hours after exposure
[2] LC values were determined by Probit Analysis (Finney, 1971)
All concentrations were in µg/ml with 95% confidence intervals in brackets.

4.3 Toxicity assay involving *P. reticulata* (mosquito fish)

4.3.1 Observed Behavioural/ Definitive Characteristics

The behavioural responses observed in the toxicity assay shows that the guppies exhibited variations in their tolerance to aqueous extracts of *M. oleifera.* Table 4.5 shows that upon addition of the toxicant, there were different toxicity effects and the treatment had varying degree of effect on the fishes throughout the period of observation. All the treatment levels induced erratic movements and swimming in the fishes as soon as the extract was added into the medium. This effect was observed to reduce as the exposure time increased in all the treatment levels (Table 4.5).

The effect of the dosages on yawning (air gulping) mechanism of the fishes was observed to increase in the first hour of exposure to the extract and this increased with exposure time (Table 4.5). Reduced activity (dullness) was not observed in all the media after exposure to the treatment but this was noticed in the higher doses (20 and 30 mg/L) after 18 hours exposure to the extract (Table 4.5). Loss of reflex was also observed within the first hour of exposure to the treatment and continued in the higher concentrations till 24 hours of exposure in the medium before there was a gradual restoration of reflex. This indicates that as the concentration increased the propensity to loss in reflex increased in the fishes (Table 4.5). Discolouration of fishes was observed at the highest concentration (30mg/L) after 24 hours exposure to the extract. All other treatment levels showed no discoloration on the fishes throughout the experiment (Table 4.5).

Table 4.6: Behavioural Pattern of *P. reticulata* during a 24-Hour toxicity testing

Exposure Time/ Behaviour Concentration(mg/L)	1 Hr. 0 10 20 30	6 Hrs. 10 20 30	12 Hrs. 10 20 30	18 Hrs. 10 20 30	24 Hrs. 10 20 30
Erratic Behaviour	+ + + +	+ + +	- - +	- - -	- - -
Yawning (Air gulping)	- + + +	- - -	- - -	- - -	- - -
Reduced Activity (Dullness)	- - - -	- - -	- + +	- - +	+ + +
Loss of Reflex	- + + +	- + +	- - +	+ + -	- + +
Discolouration	- - - -	- - -	- - -	- - -	- - +

Key: + =Present

- =Absent

The effect of the doses on the swimming ability of the fishes within the 24-hour exposure period showed that erratic swimming increased as the concentration increased, (Fig.4.5) and at the highest concentration (30mg/L) this was observed to be highest at 12 hours of exposure to the treatment.

The effect of exposure time on the guppies in terms of loss of reflex was directly proportional, (Fig. 4.6). In essence, loss of reflex increased as the exposure time increased with the highest observed at 18 hours of exposure to the treatment. The effect of the different concentrations on loss of reflex was also described (Fig 4.7) and it shows that as the concentration increased the propensity to loss in reflex increased in the fishes, hence, the fishes exposed to the higher concentrations exhibited more reflex loss tendencies within the exposure time.

4.3.2 Acute toxicity evaluation

The lethal concentration (LC_{50}) of the AEMOS on *P. reticulata* after 24 hour exposure using Probit analysis was, 36.4(34.2-39.4) mg/L, as projected by logarithm of the concentration in base 2.

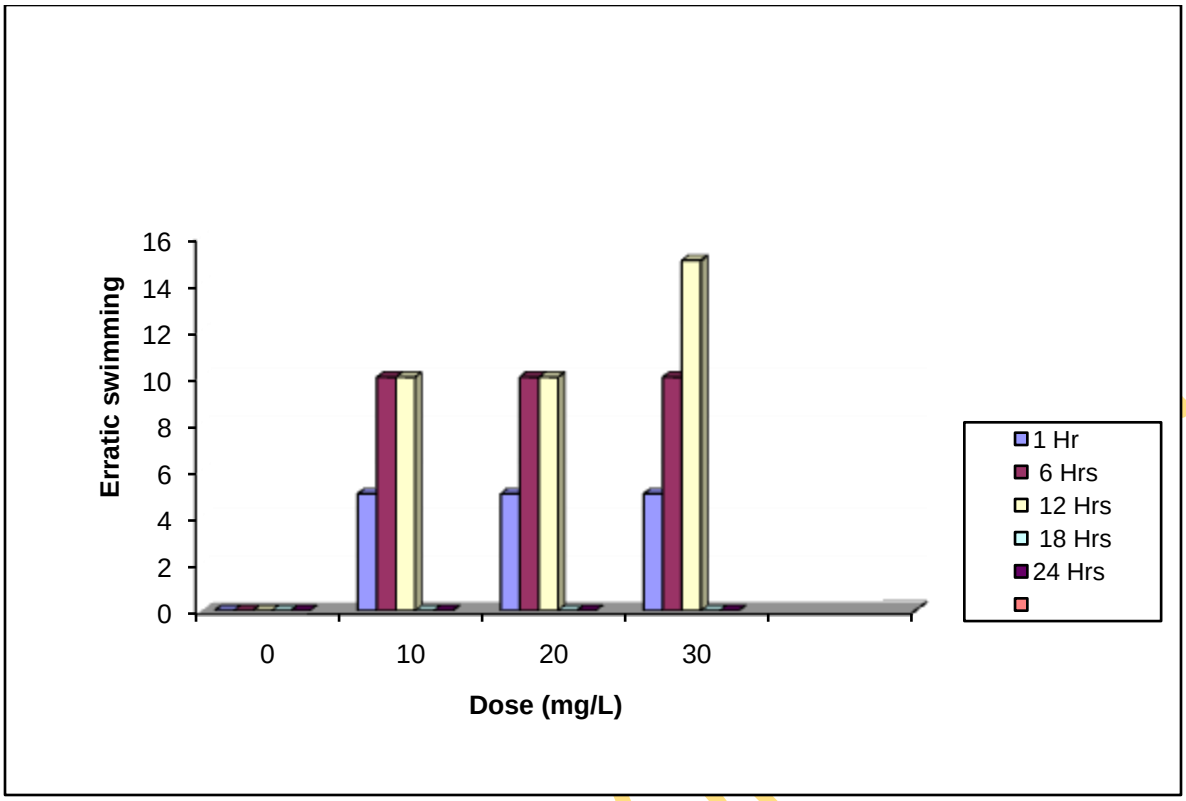


Fig.4.5: Effect of treatment doses on fish swimming ability within 24-hours exposure time

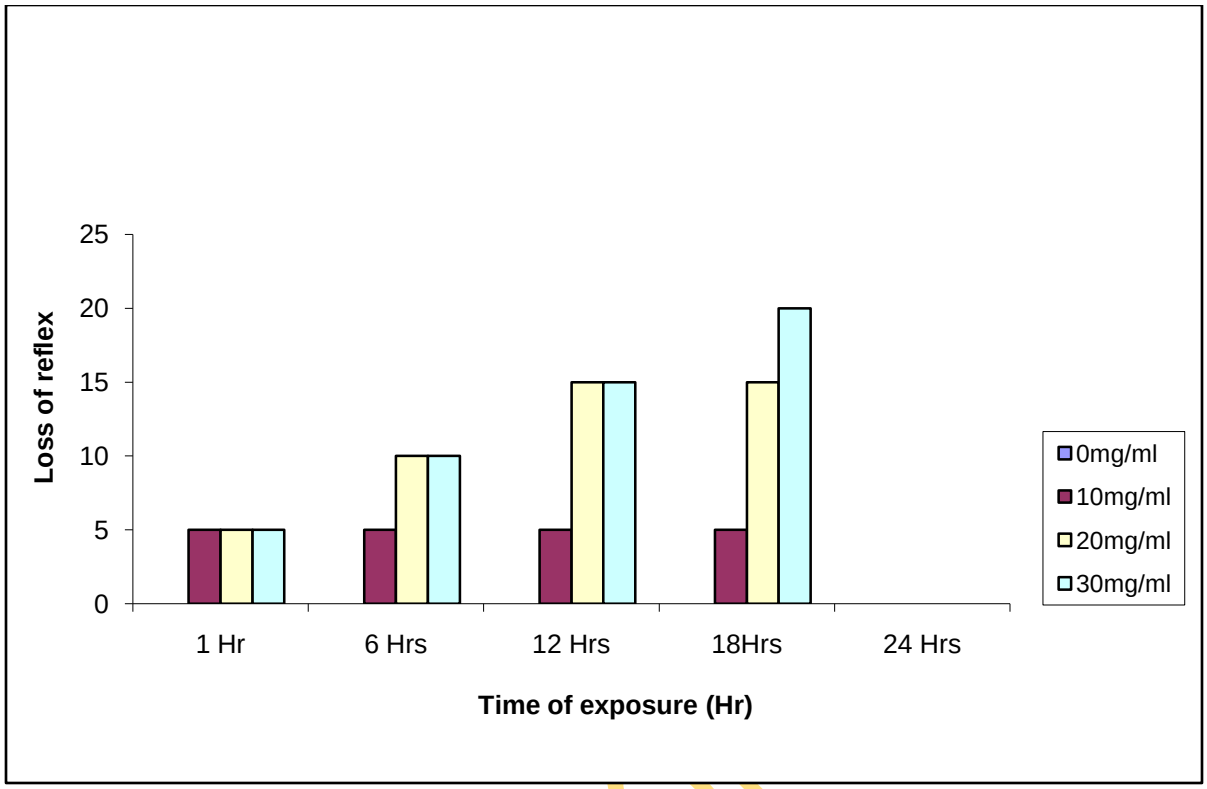


Fig 4.6: Effect of exposure time on loss of reflex in guppies

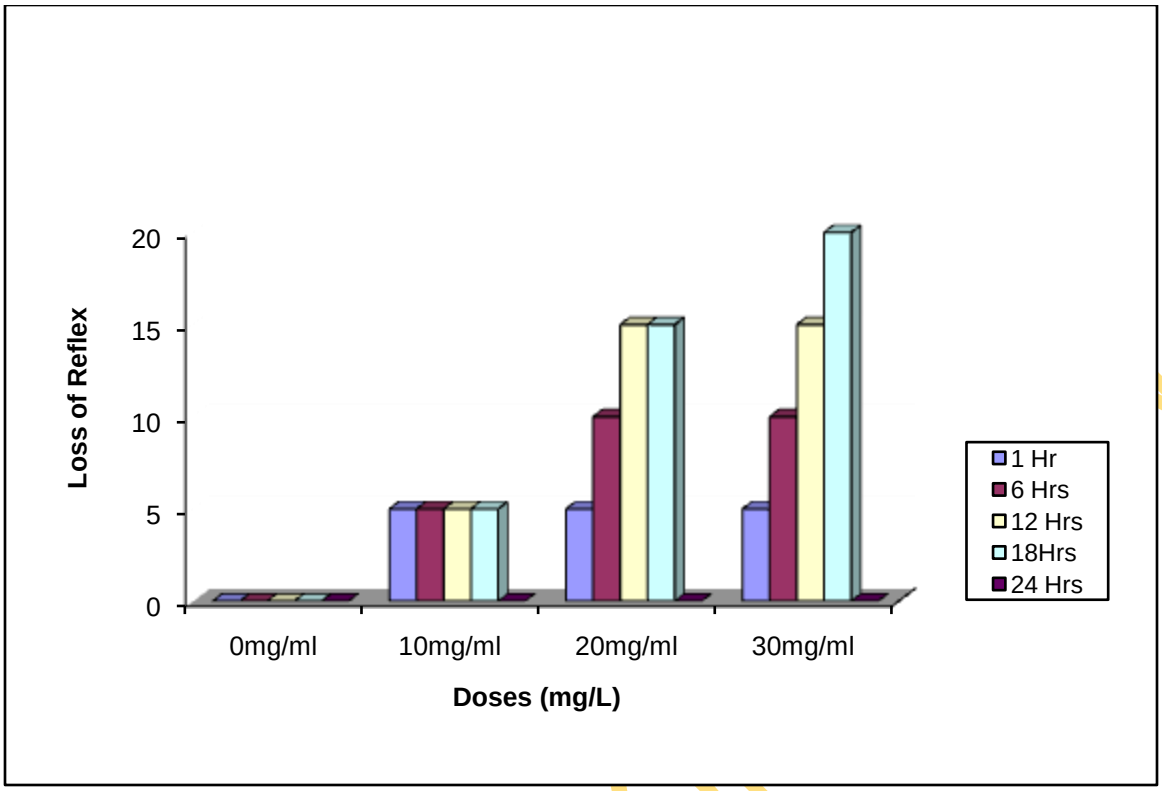


Fig. 4.7: Effect of treatment doses on loss of reflex in mosquito fishes.

CHAPTER FIVE

DISCUSSION

Control of *Anopheles* mosquito is essential as it is the major and primary vector of malaria, filariasis, and many other arthropod-vector related diseases in sub-Saharan Africa; and they constitute an intolerable biting nuisance (Collins and Paskewitz, 1995; Okumu *et. al,* 2007). Recently, there have been concerted efforts at promoting botanicals as environment friendly pesticides, microbial sprays, and insect growth regulators amidst other control measures (Senthil and Kalaivani, 2005, Cetin *et.al.* 2004). The diversity of plant species is huge and phytochemicals obtained from them are important sources of safe and biodegradable chemicals which can be screened for mosquito and insecticidal activities and tested for mammalian toxicity (Mittal and Subbarao, 2003). A survey of literature on control of different species of mosquitoes reveal that assessment of the efficacy of different phyto-chemicals obtained from various plants have been carried out by a number of researchers in the field of vector control (Sukumar *et. al*.,1991, Nwankwo *et. al.,* 2011, Njom and Eze, 2011).

Biotechnologists and entomologists agree that efficiency of mosquito control should be with selectivity for the specific target organism. In this study, evaluation of the aqueous extract of *M. oleifera* seed against third instar larvae of *A. gambiae s.s.* in the laboratory showed that it was larvicidal to the mosquito species and with very minimal toxic reactions observed in the non-target animal, *P. reticulata.*

5.1 Efficacy of the aqueous extract of *Moringa oleifera* seed (AEMOS)

The Aqueous extract of *M. oleifera* seed (AEMOS) was observed to have slow action on the mosquito larvae, especially in the lower concentrations, the AEMOS is a natural product and little quantity of it will still yield the desired result over time. In previous studies 32.1ppm of de-oiled neem seed extract gave 85% mortality of *Culex quinquefaciatus* after 12 days of exposure (Singh, 1984). Okumu *et.al* (2007) also observed that the action of neem oil formulation was slow and increased the mosquito larval period. The implication then is that if at lower concentrations the effect will still be produced, then it makes it a potentially cost-effective larvicide as little of it can achieve much in terms of mosquito control. As can be deduced from

the response pattern, an increase in concentration of the AEMOS is directly proportional to larvicidal effectiveness over the exposure period (Fig 4.3).

With respect to time, several factors are responsible for the *Anopheles* mortality observed in the different concentrations. The main effect is possibly due to tracheal flooding and chemical toxicity. Hence it is important that further studies be carried out to clearly determine and ascertain that the mechanism of toxicity of the product affects the midgut epithelium and secondarily the gastric cacea and the malphigian tubules in the *A. gambiae s.s.* mosquito. It is also pertinent to suggest that the histopathological effect on the larvae may differ qualitatively according to concentrations assayed and the duration of the treatment on the *A. gambiae s.s.*complex

Based on findings, it is likely that treating of the 3rd instars would result in more efficient control while giving enough time in days or hours to act on the mosquitoes' breeding medium, thus producing positive outcomes unlike most synthetic chemicals which though may have quick knockdown effects on the mosquito population but with great environmental consequences including environmental imbalance and resistance in the long run.

5.2 Susceptibility of *A. gambiae s.s.* to AEMOS.

The result of the larval susceptibility test revealed that all the concentrations induced mortality within 24 hours of the exposure indicating that the larvae were susceptible to the AEMOS. The high *Anopheles* larval mortality observed across the concentrations within 24 hours indicates its high toxicity to the larvae when compared to mortality in the control. The effectiveness was further proved by Abbotts' (1925) formula all the concentrations of AEMOS evaluated in the experiment showed varying degrees of effectiveness on larval mortality on the mosquito larvae and optimum effect was based on the period of exposure.

This finding agrees with previous studies that have been carried out globally, Okumu *et.al* (2007) reported that neem oil formulation was toxic to third instar larvae of *A. gambiae* while the effectiveness of the extracts of *Piper nigrum* Linn., against *Culex pipiens, Aedes aegypti* and

Aedes togoi have been established to cause behavioural changes and larval mortality (Park *et. al*., 2002). Leaf extract of *Lantana camara* and *Catharanthus roseus* were found to be highly toxic to *Aedes aegypti* even at very low doses (Remia & Logaswamy, 2010), *M. oleifera* extract in combination with *Trichoderma* soil sprinkle (a bio-control agent) was highly effective against *Sclerotium rolfsii* , the causative agent of the damping off and stem rot disease in cowpea with more than 94 % and 70 % disease control in the green house and field respectively (Adandonon *et.al*. 2006).

Ajayi (2008) found that methanol extract of *M. oleifera* root was larvicidal to Anopheline lavae; an evaluation of the activity of *Pinus longifolia* (Pine) oil against mosquito showed that it had both larvicidal and repellent activity against various species of mosquitoes (Ansari *et.al.* 2005) while aqueous extract of *Solanum villosum* berry were found to be highly lavicidal on the dengue vector *Stegomyia aegypti* (Chowdhury *et. al.*, 2008).

The study confirmed that the Moringa extract is an effective larvicide since the control had minimal effect (i.e. less than 20 %mortality) on mosquitoes according to WHO standard for testing potential larvicide effectiveness (WHO, 2003) and it is certain that the larvicidal effects observed were due to the AEMOS.

5.3 Bioefficacy of larvicide component of AEMOS.

Several works have been carried out to demonstrate the potency and bio efficacy of plant based larvicides which are non-toxic to man and domestic animals to serve as basis for the development of safer agents in the control of mosquito larvae. Generally, various researchers have also intensified the use of bio-control agents for integrated management of specific diseases in recent times (Ponmurugan and Baby, 2006) and new control methodologies aim at reducing mosquito breeding sites and biting activity by using a combination of chemical–biological control methods that suit several advocated bio-control methods to reduce the population of mosquito and to reduce the man–vector contact (Service, 1980).

This study found that the aqueous extract of *M. oleifera* seeds enhanced high larvicidal activity against the third instar larvae of *A. gambiae* s.s. Early reports on the use of plant extracts against

mosquito larvae shows that chemicals from plant extracts have larvicidal effects on mosquitoes among other effects. Campbell *et.al*., (1933) reported that extract from the Russian weed, *Anabasis aphylla* was larvicidal to *Culex sp* larvae. Ajayi (2008) screened 48 medicinal plants in Nigeria for their antimicrobial activity and 23 of these plants (47.9 %) caused over 70% mortality of the test organism including Anopheline and Culicine larvae. In a similar work done by Nath *et. al*., (2006) it was indicated that root extract of *M. oleifera* showed larvicidal activity against *Aedes albopictus* and *Culex quinquefasciatus* at higher doses.

The report of this work has relevance to the study of Prabhu *et. al*., (2011) who evaluated the larvicidal and pupicidal potential of the seed extract of *M. oleifera* against the malarial vector *A. stephensi* and found that the plant extract exhibited larvicidal activities on different instars and pupation of the mosquito species.

5.4 Prolongation of Developmental period

The mode of action of the extract as it impacted on the development of the larvae was studied and it was found that there was delay in the development of the larvae to the pupal stage after exposure of the third instar larvae to all the concentrations of the extract and this was especially noted in the lower concentrations.

The benefits of larval prolongation is that mosquito larvae numbers are reduced due to the longer periods needed for new generations to complete the mosquito life cycle (Harvetz & Curtins, 1967) and many studies have drawn attention to the effects of plant extracts on growth retardation and elongation of developmental periods on mosquito species. Okumu *et. al.*, 2007 found that exposure of *A. gambiae* larvae to *Azadirachta indica* oil formulation resulted in prolonged larval periods, significant reductions in growth indices and pupation. Mohtar *et. al*., (1999) reported that a methanol-aqueous extract of *Nerium indicum* leaf at 100mg/L had elongation effect on the preimagio period for all the larval instars of *Aedes aegypti* treated compared to the control. Promisiri *et. al*., (2006) posited that there was delay in the development of *Aedes aegypti* larvae to the pupal stage after exposure to three medicinal plants, *Mammea siamensis, Anethum graveolens and Annona muricata.* Mwangi and Mukiama, (1988) also observed that a fraction of *Melia volkensi* fruit kernel extract had growth inhibition activity at

low concentration on mosquito larvae. However Ferreira *et. al.,* (2009) found that water extract of *M. oleifera* seeds did not demonstrate capacity to prevent egg hatching on *Aedes aegypti.*

The effect on prolongation of developmental period reported in this study may be due to the presence of low juvenile hormone levels in the larvae or else it may be due to chemical compounds in the plant extract suppressing the presence of ecdysone; preventing normal pupation and preventing movement to the next developmental stage thus preventing adult emergence from occurring with the resultant effect of reducing the mosquito population.

5.5 Dose response effect of AEMOS on *A. gambiae s.s.* larvae.

The larvicidal activity of the aqueous extract used in this study against *A. gambiae s.s.* was observed to increase as the dose increased hence there was an increasing progression toward larvae death over the period of exposure of the larvae to the treatments in a dose dependent manner. Studies on plant derived insecticides show that some of these have dose dependent mode of action against target organisms and the trend from this experiment supports the work of Okumu *et. al.,* 2007 which showed that the larvicidal activity of neem oil formulation against *A. gambiae* was observed to decrease as the dose decreased. This was same in the investigation of the efficacy of two botanicals against *Aedes aegypti.* (Remia & Logaswamy, 2010).

Studies with water extract of *M. oleifera* seeds showed a 24hour-LC_{50} value of 1260 ug/ml against 3rd instar larvae of *Aedes aegypti* (Ferreira *et. al.,*2009), also methanolic extract of *M. oleifera* seeds were found larvicidal against 3rd instar larvae of *A. stephensis* with LC_{50} and LC_{90}values of 72.5ppm and 139.8ppm respectively (Prabhu *et.al.*, 2011), Nath *et. al.*, (2006), indicated that root extract of *M. oleifera* showed an LC_{90} of 498.2 and 486.60 ppm respectively against *Aedes albopictus* and *Culex quinquefasciatus.* In contrast, the present study showed 96 hour-LC_{50} value of AEMOS at 1754.7µg/ml and a 24 hour- LC_{50} value of 2505.8µg/ml on *A. gambiae s.s.* Larvicidal activity may vary depending on the mosquito species and geographical location where plant was sourced. This invariably will determine the level of susceptibility of the mosquito species to the extract and also the weight of soluble solids content present in the plant extract respectively.

5.6 Effect of AEMOS on *P. reticulata (g*uppy fish) behaviour

The induction of erratic swimming on the fishes upon addition of the extract shows that the extract is a toxicant but this behavioural effect reduced with exposure time and this is so because of the biodegradable component of the extract which enabled the fishes to overcome the effect within hours of exposure to the product.

Air gulping ability of the fishes was also affected as the fishes had to reach for air by swimming to the top of the water medium for air; this was initiated due to excitation induced by the addition of the extract in the medium. This effect was also reduced with exposure time, showing that the effect was transitional in nature as it was not observed again throughout the duration of the study and the behaviour of the fishes in the treatment medium were comparable to what was obtainable in the control medium all through the study. This report agrees with the work of many researchers who worked on the toxicity of different chemicals and their effects on fishes (Balza *et al*.1989, Muniyan and Veeraraghavan 1999, Ayuba and Ofojekwu 2002, Chung-Min Liao *et al.* 2003 and Ayotunde *et. al*., 2011.). Promisiri *et. al*., 2006 reported that *Mammea siamensis, Anethum graveolens* and *Annona muricata* demonstrated no or very low toxicity to guppies at concentrations active to mosquito larvae.

Reduced activity as a behavioural parameter was not observed from the onset of the experiment as the fishes were in an excited state for about 12 hours after exposure and the reduced activity observed later after about 18 hours of exposure was possibly due to fatigue from excitation resulting from the introduction of the toxicant at a higher dose. This they overcame after 24 hours and the behaviour of the fishes were comparable in all the treatment groups including control. This observation is at variance with the observation of Adedapo *et. al.*, (2009), who observed that the behavioural changes noted in rats following aqueous extract of *M. oleifera* administration was slight dullness at the onset of administration and the animals later became active after some hours of exposure to the doses.

The guppies exposed to high doses experienced continued loss of reflex till 24 hours after exposure unlike in the lower doses where loss of reflex was transient. This effect may be due to

the higher dosage of extract in the medium which resulted in prolongation of the effect on the exposed guppies in terms of locomotive ability.

Discoloration is another parameter used to indicate toxicant exposure in fishes. In this study it was observed that at the lower concentrations there was no discoloration while in the higher concentrations discoloration became obvious after 24 hours of exposure. This indicates that at higher doses the extract may lead to discoloration of the fishes but at the level of the lower doses there may be no discoloration effect on the non-target aquatic animals residing in the aquatic medium in the environment.

These behavioural effects are good pointers and indicators of the effect of this aqueous extract on the non- target aquatic organisms in the natural environment but suffice to say that the dosages used in the toxicity study of the guppies are way higher than the dosages found to be larvicidal to the *Anopheles* larvae in the larval bioassay carried out in this study. The highest concentration found to be effectively larvicidal to the *A. gambiae s.s.* was 8700ug/ml and this served as the basis for the determination of doses for the guppy toxicity tests in accordance with toxicity test regulation (i.e. 10-fold of the doses found larvicidal to larvae).

In essence, this implies that in the real application of the aqueous extract to water bodies as a bio-larvicide for the control of the malarial vector, *A. gambiae* s.s., there may never be a time when the dosage administered will be as much as to cause adverse behavioural effects in terms of discoloration as observed in this study. A critical observation shows that there was a relationship between the dosage and exposure time on the behaviour of the guppy fishes in the medium throughout the study and in the words of Choochote *et. al*. 1999, not only can medicinal plant extract be effective but they may also greatly reduce the risk of adverse ecological effects and they do not induce insecticide resistance in mosquito and since these chemicals are taken from medicinal plants, they are expected to have low human toxicity and a high degree of biodegradation.

5.7 Acute Toxicty test on *Poecilia reticulata*

The acute toxicity assay in the present study showed that the aqueous extract had a 24 hour-LC_{50} value of 36.4µg/ml; this showed that the extract had lethal effect on the guppies at higher concentrations. This report agrees with the work of many authors (Balza *et al*.1989, Muniyan and Veeraraghavan 1999, Ayuba and Ofojekwu 2002 and Chung-Min Liao *et al.* 2003 and Ayotunde *et. al*., 2011.) who worked on the toxicity of different chemicals to freshwater fishes. Promisiri *et. al*., 2006 reported that *Mammea siamensis*, *Anethum graveolens* and *Annona muricata* demonstrated no or very low toxicity to guppies at concentrations active to mosquito larvae. Some plant extracts though have potential as larvicides have also been found to be lethal to guppies and non-target animals. *Kaemferia galanga and Stemona tuberosa* extracts were found to be toxic at concentrations of 50µg/ml (Promisiri *et. al*., 2006) while *M. oleifera* seed extract at 200mg/L were found to have toxicity and mutagenic effects on guppies, protozoan and bacteria (Ndabigengesere *et. al*., 1995) and this is far higher than the dose found to be larvicidal to mosquitoes in this study.

M. oleifera seed extracts have earlier been reported to be non-toxic and recommended for coagulation of raw water when compared to inorganic or synthetic organic coagulants being as efficient as aluminium salts (Olsen, 1987, Olayemi & Alabi, 1994, Ndabigengesere & Narasich, 1998 and Schwarz, 2000) and dramatically decreasing clay and bacteria contents (Madsen *et. al*., 1987, Brom *et. al*., 2002, GhebreMicheal *et. al*., 2005) in developing countries. The report from this study provides clue(s) to what could be expected from a more in-depth investigation of *Moringa* based extracts on the malarial vector *A. gambiae s.s.*

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This study shows that the aqueous extract of *M. oleifera* seed was very effective on *A. gambiae* s.s. to minimize its role in malaria transmission as larval mortalities were observed with the use of the respective concentration doses within the exposure periods. In line with the objectives of this study and from the perspective of environmental health, the *A. gambiae* s.s. were effectively controlled by direct mortality or inhibition of growth, thus, forcing them to remain at the non-infective stage of their lifecycle without causing any adverse effect on the environment and directly or indirectly safeguarding health of the public.

Also, higher concentrations (20 mg/L, 30 mg/L) of the extract of *Moringa* used in this study exerted corresponding toxic effects and death of guppies (*P. reticulata*) tested for the purpose of assessing the selective and acute toxicological impact of the plant extract on ecology.

The toxic effects on behaviour observed in this experiment showed that the extract is rich in toxic ingredients which may exert some toxicologically related effects on the target organisms with minimal effects on the non-target organism. Thus, *Moringa* extract based on their activity may be used to control the malaria vector, *A. gambiae* s.s. and will not be toxic to non-target organism if used within the dosages lethal to the mosquito larvae.

Relatively this study shows that the aqueous extract of *Moringa* could be useful for larvicidal purposes in Anopheline control. Appropriate bio-technology measures could be adapted to improve its yield and activity in future because according to Essien *et al.,* (1983), higher plants constitute a major area of resources for mankind especially in the third world and hence need to fully harness these resources. More so, the study of Burkhill,(1994) also emphasizes that the efficient use of plant resources for larviciding purposes serves as alternative to synthetic chemicals in case of insecticide resistance which could naturally occur in addition to higher cost and environmental pollution.

6.2 Recommendations

Based on the findings of this study and the potentials of the *Moringa* plant, the following are recommended:

1. **Public enlightenment campaign and awareness**

   There is a need to embark on focused and aggressive public awareness campaign to educate the Nigerian public on the dangers of continued use of synthetic chemicals and the need to go back to the abandoned indigenous techniques and technologies including the use of natural products that are compatible with the environment and non- toxic to man and his environment. This will also include public sensitisations and mobilization through involvement of the media (Print and mass media), Political support through policy and advocacy, mass education on the importance of the *Moringa* tree, to promote its cultivation in Nigeria, so that every household can have access to the many potentials of the tree.

2. **Mass and sustainable production of *Moringa* seeds.**

   The production of *Moringa* seeds should not be limited to large scale industries, individuals and communities can be encouraged to cultivate the plant to ensure availability, affordability and sustainability. The local production of the seed can also serve as a source of income to the unemployed. The technology employed in this study ensures that even individuals in the community can locally produce the extract after observing the various steps highlighted in the methodology for seed processing and extraction. The advantage is that environmental management of the communities will now be made easy through community level approach and participation and as individuals take care of their immediate environment the cumulative result will be a cleaner society, free from insect vectors and vector-borne diseases.

3. **Production of *Moringa*-based larvicide**

   Moringa-based products have been shown to exhibit a wide range of effects that are potentially useful for vector control in agriculture, Pharmacy and medicine but presently none of these have been made at commercial quantities in Nigeria for household or community use. Manufacturing of these products for home use can be stimulated through local businesses and unlike other natural products such as *Bti (Bacillus thuringensis)* will not require importation from outside Nigeria but rather if sustained can be an avenue for

economic revenue generation through exportation to other countries. These products could also be incorporated as part of ingredients to provide chemical framework for locally available insecticides for synergistic effects in the formulation of new products.

4. Field application

The result of this study is recommended for field trials, little quantity of the extract can go a long way in achieving the desired result. Appropriate dispersing equipment and dilution factors should be considered to ensure effectiveness on application sites. The concentrations of extract prepared will determine its effectiveness; also exposure time has effect on the larvicidal activity of the product. It should be noted that only the quantity of extract needed at a time should be prepared to ensure that its biodegradation does not occur.

5. Integrated Vector Management

The incorporation of *Moringa*-based larvicide into the integrated vector management programme will complement the available traditional methods of mosquito control. This will lead to a more efficient control of mosquito population and disease transmission reduction, thus, minimizing the prevalence of malaria and its economic impacts in Nigeria.

6.3 Future Research

Future research should aim at the following:

i. *Moringa* seed extraction using organic solvents to compare quality and quantity of yield.
ii. Determination of active ingredients of the extract
iii. Study on the shelf life and stability of the active ingredients at different temperatures
iv. Characterization of the extract
v. Field trials of Moringa as a larvicide
vi. Extensive research on toxicity effects of the extract on non- target animals.

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