LARVICIDAL POTENTIAL OF THE LEAF EXTRACT OF *Datura stramonium* AND *Occimum gratissimum* AGAINST *Culex quinquefasciatus* MOSQUITO SPECIES

BY

CHINTEM, Williams D.G

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BY

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(***B. Tech. (*Hons) *Biochemistry FUTY, 2009***)
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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY ZARIA NIGERIA IN PARTIAL FULFILMENT FOR THE AWARD OF MASTERS OF SCIENCE IN BIOCHEMISTRY

DEPARTMENT OF BIOCHEMISTRY
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OCTOBER, 2013

DECLEARATION

I hereby declare that this thesis entitled "Larvicidal Potential of the Leaf Extract of *Datura stramonium* and *Occimum gratissimum* against *Culex quinquefasciatus* mosquito species" has been written and carried out by me in the Department of Biochemistry Ahmadu Bello University, under the supervision of Prof. H. C. Nzelibe, and Dr. D.B James. And that it is a record of my own research work. It has not been presented before in any previous application for a higher degree.

Chintem Williams D.G.

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Name	Signature	Date

CERTIFICATION

This thesis entitled "Larvicidal Potential of the Leaf Extract of *Datura stramonium* and *Occimum gratissimum* against *Culex quinquefasciatus* mosquito species" meets the regulations governing the award of Master of Science Degree in Biochemistry of the Ahmadu Bello University, Zaria and it is approved for its contribution to scientific knowledge and literary presentation.

Prof. H.C Nzelibe

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DEDICATION

This research work is dedicated to my late grand Mother MA Hanna Samuel Milmi, and to the entire Ngongmu's Family for their abundant support, understanding and love.

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ABSTRACT

The study evaluated the Larvicidal effect of Aqueous, Ethanol, Ethylacetate, n-Hexane and partially purified fraction of two indigenous medicinal plant; *Datura stramonium* and *Occimum gratissimum.* Different concentrations of 6.25, 12.50, 25.00, 50.00, 100.00 and 1,000ppm were prepared from the stock solution of the samples. Third and forth instar larva of *Culex quinquefasciatus* where exposed to these different graded concentrations and mortalities were observed at time intervals. The presence of alkaloid, Tannins and Terpenoids detected in *D. stramonium* Ethanol extract while Sterols, flavonoids, Alkaloids and saponins where present in *O. gratissimum* n-Hexane extract. The effective larval mortality was observed in ethanol extract of *Datura stramonium* and *Occimum gratissimum* n-hexane extract against *Culex. Quinquefasciatus* mosquito larvae (percentage mortality = 96% and 100% at 1000ppm after 24hr whereas LD_{50}=2.565 and 2.515ppm; LD_{90}=17.724 and 30.511ppm, respectively) whereas ethylacetate and aqueous recorded poor mortality. There are strong correlation between mortalities observed in larvae and extract concentration; R^2 = 0.161 and 0.152. On purification using column chromatographic methods, fractions from the crude extracts with the highest larval mortality was found in the DSEE-F1 and OGnHE-F3 displayed high larvicidal activity with percentage mortality of 99% at 100ppm and 100% at 50ppm against *C. quinquefasciatus* respectively, after 24hr respectively. Lethal concentrations were (LC_{50} = 4.390**,** and 1.485ppm respectively. The GCMS result for the larvicidal active fraction revealed the following compounds for DSEE-F1; Glycerin (22.19%), 6-Pentyl-5,6-dihydro-2H-pyran-2-one, 2,2-Dimethylpropanoic acid (26.49%), tridec-2-ynyl ester (12.21%), Hexadecanoic acid (19.16%), Heneicosane (7.59%) and Di-n-octyl phthalate (12.36%) while OGnHE revealed (7) compounds; Thymol (24.34%), Caryophyllene oxide (10.91%), Hexahydrofarnesyl acetone (7.67%), Stearic acid-methyl ester (4.88%), DELTA.8-Tetrahydrocannabinol (6.56%), 2-Ethylhexyl chloroformate (4.28%) and Di-n-octyl phthalate (41.36%). Further purification step of the fraction by pre-TLC showed a single band while the GCMS of revealed presence of Heneicosane and Caryophyllene oxide in fraction one of *D. stramonium* ethanollic extract and *O. gratissimum* n-hexane extract respectively. In conclusion the fractions from the leaf of ethanol extract of *D. stramonium* and *O. gratissimum* n-hexane extract showed potent toxicity against *Cx. quinquefasciatus* larvae. The present work revealed that these compounds could be used as environmentally sound larvicidal agent for mosquito control.

Keywords: Larvicidal activity, *Datura stramonium, Occimum gratissimum, Culex quinquefasciatus,* Chromotographic, GCMS

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ABBREVIATION, GLOSSARRIES AND SYMBOLS

%	=	Percentage
µl	=	Microlitre
mg	=	Milligram
^{0}C	=	Degree Celsius
hr	=	Hour
Da	=	Dalton
kDa	=	killo-Dalton
LC_{50}	=	Lethal Concentration at 50 percent
LC_{90}	=	Lethal Concentration at 90 percent
TLC	=	Thin Layer Chromatography
GCMS	=	Gas Chromatography Mass spectroscopy
DSEE	=	*Datura stramonium* Ethanol Extract
OGnHE	=	*Occimum gratissimum* n-Hexane Extract

1.0 INTRODUCTION

1.1 Background Study

Despite the advances in the techniques used for control of mosquitoes during recent decades, the mosquito continues to pose a serious public health problem. In addition to the persistent irritation the mosquitoes cause human beings and animals simply by virtue of their blood-sucking behaviour and the itching, mosquitoes are also the principal vector of a variety of serious diseases, including malaria, yellow fever, dengue and encephalitis (David and Kubo, 1999; Cheng *et al*., 2004; Hemingway, 2004; Kuo *et al*., 2007). It is estimated that there are about 100 trillion mosquitoes with at least 3,450 different species in the world. They are found from the tropics to the arctic regions. Of all diseases transmitted by the mosquito, malaria is the most deadly and of late it has proved to be the most costly serious problem to manage in countries where it is endemic. Worldwide, approximately 2.7 million human deaths occur each year solely as a result of malaria transmitted by mosquitoes (Nasrin, 2005). Like other true flies, mosquitoes exhibit 'complete metamorphosis. The juvenile form passes through both the larval and pupal stages. From eggs to the new adult, *Anopheles* takes 7-12 days depending on temperature. Female *Anopheles* needs a blood meal for each batch of eggs, 100 or more produced at two or three days intervals. They can feed on nectar or fruit juices but without blood cannot produce eggs (Jacobs-Lorena, 2003). The larvae are anatomically different from the adults, live in different habitats and feed on different type of foods (Clements, 1992). Mosquitoes are a host to a variety of pathogens and parasites including viruses, bacteria, fungi, and nematodes. The blood sucking habits of mosquitoes renders adult mosquitoes prone to acquire parasites or pathogens from one vertebrate host to another, but even so, many aspects of the mosquito's ecology and physiology must be appropriate for it to acquire, harbour, and transmit a particular organism (Haq *et al.*, 2003). Mosquitoes also cause allergic responses to humans that include local skin reaction and systemic reactions such as angioedema and urticaria (Peng *et al.,* 1999).

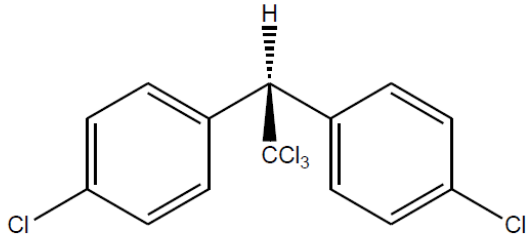
Malaria is a parasitic disease caused by *Plasmodium* species. The parasite is transmitted by the *Anopheles* mosquitoes that flourish in warm climates (Knell, 1991). Mosquitoes of the genus *Anopheles* are common in most temperate and tropical countries provided that there are suitable breeding sites (Clement, 1992). In Nigeria, more than 80% of infections are caused by *P. falciparum*, which is the most dangerous type of infection and can cause death (WHO, 2010). The cumulative human suffering and economic loss caused by malaria is immense and pregnant women suffer severe anaemia and have a high likelihood of delivering infants with low birth

weight. All Kenyan households are affected by the financial hardship caused by malaria. It is estimated that 170 million working days are lost each year because of malaria illness, which in turn affects the country's economy, leading to increased poverty. The level of endemicity of malaria in Kenya varies from region to region with a big variation in the risk which is mainly driven by climate and temperature (including the effects of altitude). It is estimated that 77% of Kenya's population lives in areas where the disease is transmitted. Malaria is endemic along the Lake Victoria region and the Coastal area where transmission takes place for more than six months in a year (Martens *et al.*, 1999). In the areas where malaria is seasonal and in epidemic prone areas all age groups are affected. These are situated in the highlands of the Rift Valley, Nyanza and Western regions (MOH, 2008). Epidemic dengue has become more common since the 1980s and is now second only to malaria as the most important mosquito-borne disease affecting humans. Each year 50 to 100 million cases of dengue fever occur and about 500,000 of them exhibit severe dengue hemorrhagic fever, which has a case fatality rate of about 5% (WHO, 2006). Dengue viruses occur as four antigenically related but distinct serotypes, which cause a broad range of disease, including clinically asymptomatic forms, classic dengue fever (characterised by the sudden onset of fever, headache, retro-orbital pain and myalgia), and the more severe forms such as dengue hemorrhagic fever-dengue shock syndrome. Dengue fever was first described during an epidemic in Philadelphia in 1780, and intermittent pandemics have affected Asia, Africa and the Americas at intervals of 10– 30 years. After World War II, marked changes in human and vector ecology fostered the transmission of multiple dengue serotypes, and the tropical world is currently in the grip of a continuing dengue pandemic (Omena *et al.*, 2007).

Mosquito's are responsible for the spread of many diseases than and several mosquito species belonging to the genera: Anopheles, Culex and Aedes are vectors for the pathogens of diseases such as Malaria, Filariasis, Dengue Haemorrhagic Fever (DHF), Japanese encephalitis and Chikunguinya a viral disease are transmitted by infected female mosquitos (Redwane *et al.*, 2002). Mosquitos are real threat to mankind, and there is increase in the prevalence, particularly in tropical and subtropical zones of the world. Research shows that over 2000 plants were known to have phytochemicals that are capable of killing or repelling mosquitos (Anupam *et at.*, 2011).

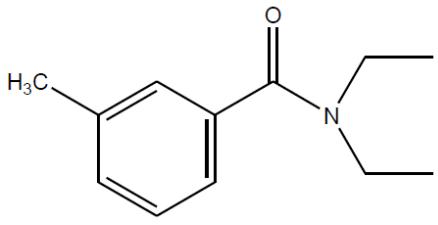
This increase in the geographic distribution of the vector is accompanied by the emergence of the viruses and disease in new areas. There is currently no vaccine or specific therapeutic drug available for dengue; therefore, control focuses on the mosquito. Bed nets are largely ineffective

against this mosquito that normally bites during the day, making source reduction and space spraying the mainstays of control. However, the mosquito breeds in a wide variety of containers, and finding and treating sufficient numbers of them is extremely challenging or impossible for even the most well-funded and organized programs (Haq *et al.*, 2003). Among the complex set of factors influencing dengue transmission, global warming is particularly important because it can create warmer and wetter conditions that increase the risk of infection. Warmer temperatures boost the speed of development of adult mosquitoes, increasing their numbers. Female mosquitoes bite more frequently in hotter temperatures, and warmer winters enable mosquitoes to survive in areas that were formerly too cold. Higher temperatures also shorten the time it takes for the virus inside the mosquito to develop and become infective. This means that the mosquitoes become dangerous to humans more rapidly (Phillips, 2008). Encephalitis is an inflammation of the brain and the causal virus is primarily transmitted by the mosquito, *Culex quenquifaciatus*. The mosquito transmits the virus to birds, horses, mules and occasionally people. Birds serve as the most important host reservoir for the virus in the disease cycle (Dawn *et al.*, 2001). Attempts to control mosquito vector over the past seven decades have included the destruction of breeding grounds for mosquitoes, drainage of stagnant water and indoor residual spraying (IRS) which was developed to routinely bring DDT into contact with the adult female mosquitoes at the most epidemiologically significant point of contact between man and insect.



p,p'-Dichlorodiphenyltrichloroethane (DDT) (

The most common mosquito repellent products available in the market contain DEET (*N*, *N*-Diethyl- 3-methylbenzamide.



N, N-Diethyl-3-methylbenzamide

It is a broad-spectrum repellent that is effective against mosquitoes and other biting insects. However, its allergic reactions and toxicity to man as well as its ability to act as a good solvent for plastics and other synthetic materials, has led to the search for alternative synthetic and natural repellents (Odalo *et al*., 2005).

Long-lasting ITNs were developed in the 1990s using technology to ensure that the insecticide remained on the net for the useful life of the net itself. There has been little innovation in effective treatments since then, despite the seriousness of the health problem. Coils, impregnated mats and aerosols are readily available in the consumer markets, but there is little direct evidence for these impacting on disease transmission and the annual cost of routinely using these interventions is high despite the low cost of the individual coils or sprays (Hemingway, 2009).

The discovery of DDT in 1942 and its first use in Italy in 1944 made the ideal of global eradication of the insect seem possible (Murdock, 2001). Other applications included organophosphates such as temephos and fenthion and insect growth regulators such as diflubenzuron and methoprene generally used for the control of mosquito larvae (Yang *et al*., 2002). Although effective, their repeated use has disrupted natural biological control systems and has led to outbreaks of insect species, which sometimes resulted in the widespread development of resistance, had undesirable effects on non target organisms, and fostered environmental and human health concerns (Sen-Sung *et al*., 2003). Further, certain synthetic pesticides, such as acylamides, phenylureas and phenoxyacetates may break down into more toxic derivatives, while others may persist in nature as recalcitrant environmental pollutants, injurious to humans and wildlife (Munnecke *et al.*, 1982). Thus, there is need to develop non-polluting safe pesticides. An ideal pesticide should have a number of properties, including selective toxicity, and be degradable into smaller harmless molecules such as water, carbon dioxide and ammonia.

Most of the mosquito control programmes target the larval stage in their breeding sites with larvicides, because adulticides may only reduce the adult population temporarily (El Hag *et al.*, 2001). In application of larvicides, mosquito larvae are killed before they disperse to human habitations, and that mosquito larvae, unlike adults (Charlwood and Graves, 1987), cannot change their behaviour to avoid control activities targeted at the larval habitat (Killeen *et al.*, 2002). This can also reduce the overall application of pesticides needed to control mosquito

population (Dharmagadda *et al.,* 2005). This in itself is an advantage that can be exploited to search for new and novel larvicides. Natural products remain the most promising source of novel secondary metabolites.

The plant *Datura stramonium* belonging to the family Solanaceae contain psychoactive substance like Alkaloids, Atropine and scoplaminewhich are classified as deliriant or Anticholinergic (Giannini, 2002). In the Northestern part of Nigeria, the fresh leave, were used as insecticidal and repellant to mosquito. More recently, preparations from the plant have been used as ingredients in in the teatment of asthma. With this exception, however, the plant is generally considered too toxic for medical applications (Sofawora, 1993 and Afolabi *et al*, 2007).

Ocimum gratissimum belongs to the Lamiaceae family. It's essential oil has mosquito repellant, insecticidal properties which where locally used (Burkill, 1995; Mann *et al.,* 2009 and Kéita, *et al.,* 2001). Peasant farmers in northern Nigeria indigenously use various parts of the plants to protect cereals and legumes against pest damage during storage (Mann, 2003; Burkill, 1995). Especially in India whole plant preparations are used to treat stomach ache, sunstroke, headache and influenza. Theseeds have laxative properties and are also used in the treatment of gonorrhoea. The essential oil is applied against fever, inflammations of the throat, ears, eyes, stomach pain, diarrhoea and skin diseases (Afolabi *et al*, 2007).

1.2 STATEMENT OF RESEARCH PROBLEM

Malaria affects over 3.3 billion people, or half of the world's population, in106 countries and territories. WHO estimates 216 million cases of malaria occurred in 2010. Malaria is the third leading cause of death for children less than five years worldwide, after pneumonia and diarrheal disease. Mosquitos are still the major vectors of encephalitis, filariasis, yellow fever, dengue and chikungunya. In Africa, Malaria still remains the second leading cause of death from infectious

diseases after HIV/AIDS, acounting for 81% of deaths (WHO, 2012). Malaria is a major public health problem in Nigeria and about 97% of the population is at risk and only 3% Nigerians lives in a malaria free highland. It accounts for 300,000 deaths per year in Nigeria comparedwith 215,000 deaths per year from HIV/AIDS. In hospitalizationit account for about 10-30% of and 12-25% of all deaths in children under the ages of five (Enato and Okhamefe, 2004). The use of synthetic insecticides such as organochlorides, organophosphates and carbamates are toxic and adversely affect the environment by contaminating soil, water and air. Adult mosquitos are increasing resistance to systhetic insecticides leading to increase in the number of mosquitos and its breeding places.

But still there is increase in the vector (Mosquito) in urban agglomeration leading to increasing resistance of mosquitos to these insecticides has resulted in the recurrence (WHO, 1996) of these diseases.

1.3 JUSTIFICATION

Mosquito transmitted diseases including Malaria, Filiriasis, Japan encephalitis, Dengue Fever, Dengue Hemorrhagic Fever (DHF), Yellow Fever among others are very persistent to human and other domestic animals. These diseases has impacted adverse effects on human health and well being, death and the economic losses thus the stimulants for the search of new, safe and effective larvicides. Phytochemical active compounds derived from the ubiquitous *Datura stramonium* and *Ocimun gratissimum* could offer unique solutions to unique problems of Malaria bedevilling a relatively developing country like Nigeria.

Much has been reported on the various neurotoxicity and antimicrobial properties of jimson weed and wild basil respectively but little or no report have been published on the active larvicidal constituents isolated from this plants.Terminating the larval stage in the mosquito life

cycle is preferable since the larvae are stationed in their breeding site rather than adult mosquito which is cumbersome to control. Mosquito larvicides like Bacillus sphaericus and Bacillus thuringiensis have been widely andeffectively used in mosquito control programs, but the industrial production of these bacilli is expensive. Due to the widespread of this disease and the vector, there is need for the development of new and potent botanical insecticides from plant source that are effective, environment-friendly, easily biodegradable and affordable.

1.4 Aims and Objectives

1.4.1 General Aim

The aim of this research was to investigate the larvicidal potential of the Leaf Extract of *Datura stramonium* and *Ocimum gratissimum* against *Culex quinquefasciatus* Mosquito species

1.4.2 Specific Objectives

- Carry out qualitative and quantitative phytochemical analysisof *Datura stramonium* and *Ocimum gratissimum*
- To investigate the Larvicidal activity of the aqueous, ethanolic, ethyl acetate and Acetone crude extract against *Culex quinquefasciatus* Mosquito larvae.
- To isolate the fraction(s) with the highest larvicidal activity using Thin Layer and column Chromatographic techniques.
- To partially characterize the larvicidal compound(s) in the bioactive fraction by Fourier Transform Infrared spectroscopy, Gas Chromatography Mass Spectroscopy.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Mosquito

Mosquitos are a family of small, midge-like flies of the Culicidae family,from the Latin *culex.* The word *Mosquito* was formed by "*mosca"* and *ito* from the Spanish meaning little flies (Brown, 1993; Jaeger, 1959). Although a few species are harmless or even useful to humanity, but majority most are a nuisance because they consume blood from living vertebrates, including humans. The females of many species of mosquitos are blood sucking pests, feeding on blood, some of them transmit extremely harmful human and livestock diseases, It is responsible for the world's most serious vector borne diseases, such as Malaria, Encephalitis, Filariasis, Yellow Fever, Dengue and Chikungunya (Reinert *et al*., 2004; Parthiban and David, 2007; Radhika *et al*., 2011). Vector control is primordial and very essential means for controlling transmission of Filariasis, Malaria, Japanese encephalitis and Dengue in human society (Ohkuma *et al*., 2003; Kaushik and Saini, 2009). Some authorities argue accordingly that mosquitos are the most dangerous animals on Earth.

Culex quinquefasciatus *is the vector of Lymphatic Filariasis caused by the Nematode* Wuchereria bancrofti *in the tropics and subtropics. It is also a vector of Avian Malaria, Lymphatic Filariasis and Dengue Fever.*

2.1.1 *The Scientific classification Culex quinquefasciatus*

Kingdom: Animalia

Phylum: Arthropoda

Class: Insecta

Order: Diptera

Family: Culicidae

Genus: *Culex*

Species: ***quinquefasciatus***

Binomial name: ***Culex quinquefasciatus*** Say, 1823

2.1.2 Taxonomy of the Culex

Over 3,500 species of the Culicidae have already been described. They are generally divided into two subfamilies which in turn comprise some 43 genera. More species are discovered and DNA studies recommended rearrangement of the taxonomy of the family. The two main subfamilies are the Anophelinae and Culicinae, with their genera as shown below (Syed and Leal., 2008; Ohkuma *et al*., 2003).

2.1.3 *Subfamilies and genera*

Anophelinae

Anopheles	*Bironella*	*Chagasia*

Culicinae

Aedeomyia	*Aedes*	*Armigeres*	*Ayurakitia*	*Borachinda*
Coquillettidia	*Culex*	*Culiseta*	*Deinocerites*	*Eretmapodites*
Ficalbia	*Galindomyia*	*Haemagogus*	*Heizmannia*	*Hodgesia*
Isostomyia	*Johnbelkinia*	*Kimia*	*Limatus*	*Lutzia*
Malaya	*Mansonia*	*Maorigoeldia*	*Mimomyia*	*Onirion*
Orthopodomyia	*Opifex*	*Psorophora*	*Runchomyia*	*Sabethes*
Shannoniana	*Topomyia*	*Toxorhynchites*	*Trichoprosopon*	*Tripteroides*
Udaya	*Uranotaenia*	*Verrallina*		

In particular, the females of many species of mosquitos are blood sucking pests and dangerous vectors of diseases, whereas some species are not blood sucking, and many of those that do

create a high to low pressure in the blood to obtain blood do transmit disease. Also, in the bloodsucking species, only the females suck blood. Among the mosquitos that transmit diseases, neither all species of mosquitos species transmit the same kinds of diseases, nor do they all transmit the diseases under the same circumstances.

Mosquitos have already been described from various parts of the world, it bite humans routinely acting as vectors for a number of infectious diseases affecting millions of people per year (Hantosh, *et al*., 2012). Others that do not bite humans are the vectors for animal diseases and may become disastrous agents for zoonosis of new diseases when their habitat is disturbed, for instance by sudden deforestation(Wilcox and Ellis., 2006).

Many scientists have suggested complete eradication of mosquitos would not have serious ecological consequences (Fang, 2010).

2.1.4 *Life cycle of C. Quinquefasciatus Mosquito*

Mosquitos undergoes four stages in their life cycle: egg, larva, pupa, and adult. In most species, adult females lay their eggs in stagnant water; some lay eggs near the water's edge; others attach their eggs to aquatic plants. Each species choose the situation and condtion of the water for oviposition and does so according to its own ecological adaptations. Some breed in lakes, temporary puddles, marshes, fresh and salt water up to about one third the concentration of seawater, whereas others acclimatize themselves to the saltiness (Wigglesworth., 1933). Such differences are important because certain ecological preferences keep mosquitos away from most humans, whereas other preferences bring them right into houses at night.

Some species of mosquitos prefer to breed in phytotelmata (natural reservoirs on plants) such as rainwater accumulated in holes in tree trunks, leaf-axils of bromeliads, flowerpot, discarded

bottle or tire are important breeding places for some disease vectors, especially *Aedes* that transmit Dengue and Yellow Fever (Fonseca, 2001).

In contrast, no matter how voracious, mosquitos that breed and feed mainly in remote wetlands and salt marshes may well remain uninfected, and if they do happen to become infected with a relevant pathogen, might seldom encounter humans to infect in turn.The first three stages: egg, larva and pupa are largely aquatic and can easly be used to break the vectors life cycle.

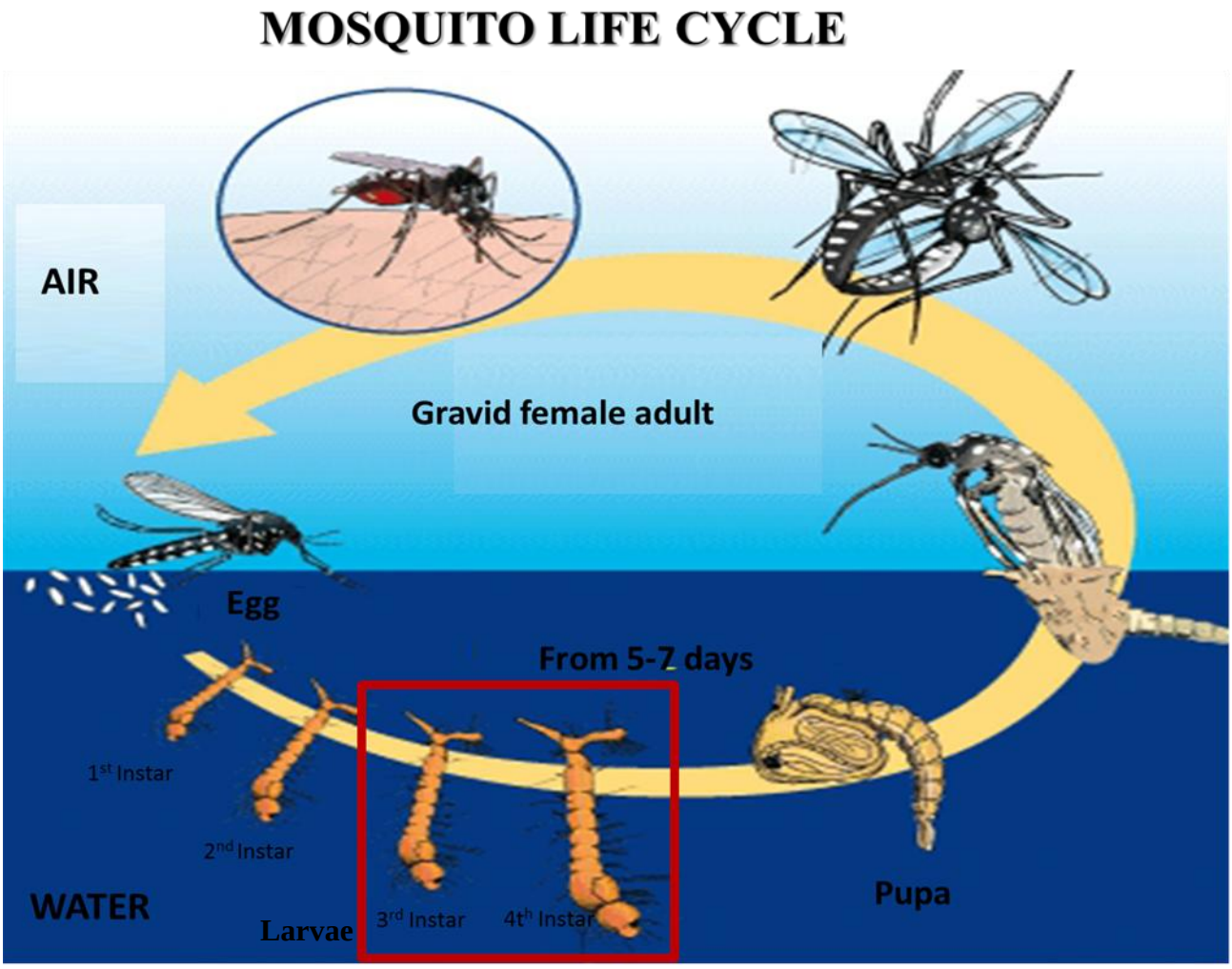


Figure 2.1: Life Cycleof *Culex quinquefaciatus*Mosquito; showing the developmental stages found in the water and Air. The cycle takes approximately 3 weeks, starting by the egg, larvae, Pupae and Adult.

2.1.4.1. *Egg and Ovipoposition*

Mosquito habits of oviposition vary considerably between species, and the morphologies of the eggs vary accordingly (Al-Doghairi 2004, Fonseca *et al*., 2001).

Culex species, members of the genus Culicidae, lay their eggs in arrays (rafts), attached usually to the under-surfaces of water-lily pads. The eggs form layers called rafts that float on the water. This is a common mode of oviposition, and most species of *Culex* are known for the habit, which also occurs in some other genera, such as *Culiseta* and *Uranotaenia* (Spielman, *et al.,* 2001).

2.1.4.2 *Larva*

The mosquito larva has a well-developed head with mouth brushes used for feeding, a large thorax with no legs, and a segmented abdomen.

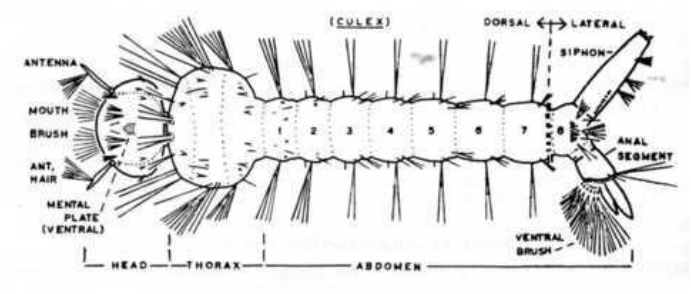


Figure 2.2: A typical representation of *Culex quenquifaciatus* mosquito larvae

Larvae breathe through spiracles located on the eighth abdominal segment, or through a siphon, and therefore must come to the surface frequently. The larvae spend most of their time feeding on algae, bacteria, and other microbes in the surface microlayer. They dive below the surface only when disturbed. Larvae swim either through propulsion with their mouth brushes, or by jerky movements of their entire bodies, giving them the common name of "wigglers" or "wrigglers"(Spielman *et al.,* 2001).

The Larvae develop through four stages called instars, after which they metamorphose into pupae. At the end of each instar, the larvae molt their skins to allow further growth. The Larvae

feed on decaying insects and associated bacteria (Crans, 2001). The larva is the most active stage in the mosquito life cycle.

2.1.4.3 *Pupa*

The Mosquito pupa is comma-shaped. The head and thorax are merged into a cephalothorax, with the abdomen curving around underneath. The pupa can swim actively by flipping its abdomen and it is commonly called a tumbler. However, pupae do not feed during this stage; typically they pass their time hanging from the surface of the water by their respiratory trumpets. If the water is troubled they nimbly swim downwards by flipping their abdomens in much the same way as the larvae do. After a few days or longer, depending on the temperature and other circumstances, the pupa rises to the water surface, the dorsal surface of its cephalothorax splits, and the adult mosquito emerges(Spielman, *et al.*,2001).

2.1.4.4 *Adult*

The period of development from egg to adult varies among species and is strongly influenced by ambient temperature. Some species of mosquitos can develop from egg to adult in as little as five days, but a more typical period of development in tropical conditions could be 40 days or more for most species. The variation of the body size in adult mosquitos depends on the density of the larval population and food supply within the breeding water. Adult mosquitos usually mate within a few days after emerging from the pupal stage.

Males typically live for about a week, feeding on nectar and other sources of sugar while the females obtaining a full blood meal after mating, the female will rest for a few days while the blood digest and eggs are developed. The blood is digested serves as a source of protein for the production of eggs, which gradually fill the abdomen.

This process depends on the temperature, but usually takes two to three days in tropical conditions. Once the eggs are fully developed, the female lays them and resumes host-seeking (Spielman *et al* 2001).

The cycle repeats itself until the female dies. Female mosquito can live longer than a month in captivity, most do not live longer than one to two weeks in nature. Their lifespans depend on temperature, humidity, and their ability to successfully obtain a blood meal while avoiding host defenses and predators. The Length of the adult is within 16mm and weight up to 2.5mg (Zhu, 2006).

2.1.5 Feeding By Adults

Typically, both male and female mosquitos feed on nectar and plant juices, but the mouthparts of the females are adapted for piercing the skin of animal hosts and sucking their blood as ectoparasites. The female needs to obtain nutrients from a blood meal before she can produce eggs. Both plant materials and blood are useful sources of energy in the form of sugars, and blood also supplies more concentrated nutrients, such as lipids, but the most important function of blood meals is to obtain proteins as materials for egg production.

Prior to and during blood feeding, blood-sucking mosquitos inject saliva into the bodies of their source(s) of blood. This saliva serves as an anticoagulant; without it one might expect the female mosquito's proboscis to become clogged with blood clots. The saliva also is the main route by which mosquito physiology offers passenger pathogens access to the hosts' interior. Not surprisingly the salivary glands are a major target to most pathogens, whence they find their way into the host via the stream of saliva.

2.1.6 *Mosquito Saliva*

A recent study suggests mosquito saliva can decrease expression of interferon-α/β during early mosquito-borne virus infection (Taylor, *et al.,* 1980). The contribution of type I interferons (IFN) in recovery from infection with viruses has been demonstrated *in vivo* by the therapeutic and prophylactic effects of administration of IFN-inducers or IFN, and recent research suggests mosquito saliva exacerbates West Nile virus infectionas well as other mosquito-transmitted viruses(Schneider, 2006).

2.1.7 *Egg development and blood digestion*

In species that feed on mammalian or avian blood, hosts whose blood pressure is high, (Billingsley, 1991). When the gut fills up the stomach lining secretes a peritrophic membrane that surrounds the blood. This membrane keeps the blood separate from anything else in the stomach. (Muslu *et al.,* 2011). Once blood is in the stomach, the midgut of the female synthesizes proteolytic enzymes that hydrolyze the blood proteins into free amino acids. These are used as building blocks for the synthesis of egg yolk proteins.

2.1.8 Mosquito as a Disease Vector

Mosquito act as a vector for many disease-causing viruses and parasites. Infected mosquitos carry these organisms from person to person without exhibiting symptoms themselves. Mosquito-borne diseases include:

- The parasitic diseases collectively called malaria, caused by various species of *Plasmodium*, carried by mosquitos of the genus *Anopheles* and some members of *Culicidae*

- Viral diseases, such as Yellow Fever, Dengue Fever and Chikungunya are transmitted mostly by *Aedes aegypti*. Dengue fever is the most common cause of fever in travelers returning from the Caribbean, Central America, and South Central Asia. This disease is spread through the bites of infected mosquitos and cannot be spread person to person. Severe dengue can be fatal, but with good treatment, less than 1% of patients die from dengue.

- Lymphatic filariasis which is the main cause of elephantiasis can be spread *Culex quinquefasciatus* mosquito species and It also is a vector of avian malaria in birds. (Muslu, *et al.*, 2011).
- Tularemia, a bacterial disease caused by *Francisella tularensis*, is variously transmitted, including by biting flies. *Culex* and *Culiseta*, are vectors of tularemia as well as arbovirus infections such as West Nile Virus (Muslu, *et al.*, 2011).

2.2 Malaria

Malaria is an infectious disease caused by the parasite of genius Plasmodium, it affects humans and domestic animals. The four identified species of this parasite causing human malaria are *Plasmodium falciparum, P. vivax, P. ovale and P.malariae.* In Nigeria 98% of all cases of Malaria is due to Plasmodium falciparum. This is the species that is responsible for the severe form of the disease that leads to death. It is transmitted from bites of an infected female *Anopheles* mosquitos to man and *Culex* to avians.

Malaria is highly endemic in Nigeria. It poses a major challenge to the country as it impedes human development. It is both a cause and consequence of underdevelopment and remains one of the leading causes of morbidity and mortality in the country (http://www.rollbackmalaria.org/worldmalariaday, 2009). Malaria accounts for about 63% of all

visits to public health facilities (Out-patient attendances). Thirty percent of hospital admissions are also due to malaria. It is responsible for 29% of childhood death, 25% of infant mortality and 11% of maternal mortality.

The economic loss to Nigeria due to malaria is estimated at N132 Billion annually (Source:Roll Back Malaria) due to cost of treatment. It is a major cause of absenteeism from work and school. It contributes to poverty and results in poor pregnancy outcome.

In Lagos State, malaria is responsible for 70% of outpatient attendance at the secondary healthcare facilities and over 80% of all tracer diseases reported by primary healthcare facilities (HMIS). The most vulnerable groups are under fives, pregnant women, visitors from non-endemic areas, those with sickle cell anaemia, HIV/AIDS.

The burden of the disease has been a major source of concern to Government and development partners. The expression of this challenge has been translated through many instruments: such as Abuja Declaration in year 2000, Millennium Development Goals to reduce disease burden by 2015 and Universal Access to HIV/AIDS, TB and Malaria in 2006. In a Metropolitan area like Lagos State, where peoples behaviour coupled with environmental factors encourage the breeding of mosquitos and thus increase human vector contact which promote the continuous transmission of infection, it is important to position malaria control as a top priority for Government intervention.

2.3 Control Methods used to prevent the spread of disease

- Vector control is aimed at mosquito control or eradication
- Disease prevention, using prophylactic drugs and developing vaccines
- Prevention of mosquito bites, with insecticides, nets and repellents

Since most of such diseases are carried by adult female mosquitos, some scientists have suggested focusing on these to avoid the evolution of resistance (Singh, *et al.*, 2003). Depending on the situation, the most important usually include:

- **Source reduction**
- **Insecticides**
- **Biocontrol** (use of plant extract anduse of predators such as dragonflies)
- **Exclusion** (mosquito nets and window screening)

2.3.1 *Source Reduction*

The elimination of breeding places of mosquitos which includes engineering measures such as filling, leveling and drainage of breeding places, and water management (such as intermittent irrigation). Source reduction can also be done by making water unsuitable for mosquitos to breed, for example, by changing salinity of water. Some specific measures are:

- For *Culex*: abolition of domestic and peridomestic sources of water suitable for breeding, for example removal and disposal of sewage and other waste water
- For *Aedes*: eliminating incidental containers such as discarded tins, crockery, pots, broken bottles, and coconut shells
- For *Anopheles*: abolish breeding places by filling or drainage

The importance of peridomestic control arises largely because most species of mosquitos rarely travel more than a few hundred meters unless the wind is favorable.

2.3.2 *Insecticide*

Insecticides are agents of chemical or biological origin that control insects. Control may result from killing the insect or otherwise preventing it from engaging in behaviors deemed

destructive. Insecticides may be natural or man-made and are applied to target pests in a myriad of formulations and delivery systems e.g sprays, baits, slow-release diffusion, etc (Ware and Whitacre., 2004).

To prevent proliferation of mosquito borne diseases and to improve quality of environment and public health, mosquito control is essential. The major tool in mosquito control operation is the application of insecticides. But this has not been very successful due to human, technical, operational, ecological, and economic factors. In recent years, use of many of the former synthetic insecticides in mosquito control programme has been limited. It is due to lack of novel insecticides, high cost of synthetic insecticides, concern for environmental sustainability, harmful effect on human health, and other non-target populations, their non biodegradable nature, higher rate of biological magnification through ecosystem, and increasing insecticide resistance on a global scale (WHO, 1996 and Brown, 1986).

Thus, the Environmental Protection Act in 1969 has framed a number of rules and regulations to check the application of chemical control agents in nature (Bhatt and Khanal. 2009). This policy has prompted researchers to look for alternative approaches ranging from provision of or promoting the adoption of effective and transparent mosquito management strategies that focus on public education, monitoring and surveillance, source reduction and environment friendly least-toxic larval control. These factors have resulted in an urge to look for environment friendly, cost-effective, biodegradable and target specific insecticides against mosquito species. Considering these, the application of eco-friendly alternatives such as biological control of vectors has become the central focus of the control programmme in lieu of the synthetic chemical insecticides (Shaalan *et al.,* 2005).

One of the most effective alternative approaches under the biological control programme is to explore the floral biodiversity and enter the field of using safer insecticides of botanical origin as

a simple and sustainable method of mosquito control. Further, unlike conventional insecticides which are based on a single active ingredient, plant derived insecticides comprise botanical blends of chemical compounds which act concertedly on both behavourial and physiological processes. Thus there is very little chance of pests developing resistance to such substances. Identifying bio-insecticides that are efficient, as well as being suitable and adaptive to ecological conditions, is imperative for continued effective vector control management such as the use of predetors like dragonflies. Botanicals have widespread insecticidal properties and will obviously work as a new weapon in the arsenal of synthetic insecticides and in future may act as suitable alternative product to fight against mosquito borne diseases.

Roark., (1947) described approximately 1,200 plant species having potential insecticidal value, while Sukumar *et al (1991)* listed and discussed 344 plant species that only exhibited mosquitocidal activity. Shallan *et al* in 2005 reviewed the current state of knowledge on larvicidal plant species, extraction processes, growth and reproduction inhibiting phytochemicals, botanical ovicides, synergistic, additive and antagonistic joint action effects of mixtures, residual capacity, effects on non-target organisms, resistance and screening methodologies, and discussed some promising advances made in phytochemical research.

Applications of phytochemicals in mosquito control were in use since the 1920s Shahi *et al.,* (2010) but the discovery of synthetic insecticides such as DDT in 1939 side tracked the application of phytochemicals in mosquito control programme. Problems due to injudicious and over application of synthetic insecticides in nature, lead to refocus on phytochemicals that are easily biodegradable and have no ill-effects on non-target organisms was appreciated. Since then, the search for new bioactive compounds from the plant kingdom and an effort to determine its structure and commercial production has been initiated. At present phytochemicals make up to 1 per cent of world's pesticide market (Isman., 1997).

2.3.3 *Application of Botanical control as mosquito larvicide*

Human beings have used plant parts, products and secondary metabolites of plant origin in pest control since early historical times. Vector control has been practiced since the early 20th century. During the pre-DDT era, reduction of vector. Some botanical insecticides used in different countries were Chrysanthemum, Pyrethrum, Derris, Quassia, Nicotine, Hellebore, Anabasine, Azadirachtin, d-limonene camphor and Turpentine (Shaalan *et al.,* 2005).

From the early 1950s, DDT and other synthetic organochloride and organophosphate insecticides were extensively used to interrupt transmission of vector borne diseases by reducing densities, human-vector contact and, in particular, the longevity of vector mosquitos. In the mid-1970s, the resurgence of vector borne diseases, along with development of insecticide resistance in vector population, poor human acceptance of indoor house spraying and environmental concerns against the use of insecticides led to a rethinking in vector control strategies; WHO., (2005). As a result, emphasis was given on the application of alternative methods in mosquito control as part of the Integrated Mosquito Management (Rose.,2001).

IMM is a decision-making process for the management of mosquito populations, involving a combination of methods and strategies for long-term maintenance of low levels of vectors. The purpose of IMM is to protect public health from diseases transmitted by mosquitos, maintain healthy environment through proper use and disposal of pesticides and improve the overall quality of life through practical and effective pest control strategies.

The main approaches of IMM include:

I. Source reduction and habitat management by proper sanitation, water management in temporary and permanent water bodies, and channel irrigation. Vegetation management is also necessary to eliminate protection and food for mosquito larvae.

II. Larviciding by application of dipteran specific bacteria, insect growth regulators, surface films and oils, expanded polystyrene beads, phytochemicals, organophosphates and organochlorides

III. Adulticiding by application of synthetic pyrethroids, organophosphates and synthetic or

IV. Plant derived repellents, insecticide impregnated bed nets, genetic manipulations of vector species, etc

V. Use of mosquito density assessment in adult and larval condition and disease surveillance

VI. Application of biological control methods by using entomophagous bacteria, fungi, microsporidians, predators and parasites (Anupam, *et al*., 2012)

Of the above avenues of IMM, larviciding approach is the more proactive, pro-environment, target specific and safer approach than controlling adult mosquitos. Application of larvicide from botanical origin was extensively studied as an essential part of IMM, and various mosquito control agents such as ocimenone, rotenone, capllin, quassin, thymol, eugenol, neolignans, arborine and goniothalamin were developed (Shaalan *et al.,* 2005).

2.4 **Nature of active ingredients responsible for larval toxicity**

The plant world comprises a rich untapped pool of phytochemicals that may be widely used in place of synthetic insecticides in mosquito control programme. Kishore *et al.*, (2011), reviewed the efficacy of phytochemicals against mosquito larvae according to their chemical nature and described the mosquito larvicidal potentiality of several plant derived secondary materials, such as, alkanes, alkenes, alkynes and simple aromatics, lactones, essential oils and fatty acids, terpenes, alkaloids, steroids, isoflavonoids, pterocarpans and lignans.

2.5 Mode of action of phytochemicals in target insectbody

Generally, the active toxic ingredients of plant extracts are secondary metabolites that are evolved to protect plants from herbivores. The insects feed onthese secondary metabolites potentially encounteringtoxic substances with relatively non-specific effectson a wide range of molecular targets. These targetsrange from proteins (enzymes, receptors, signalingmolecules, ion-channels and structural proteins), nucleic acids, biomembranes and other cellularcomponents (Rattan., 2010). This in turn, affects insect physiologyin many different ways and at various receptor sites,the principal and major effect is the abnormality in the nervoussystem (such as, in neurotransmitter synthesis, storage,release, binding, and re-uptake, receptor activationand function, enzymes involved in signal transduction pathway.

According to Rattan., (2010) the mechanism of action of plant secondary metabolites on insect body include physiological disruptions, such as inhibition of acetylecholinestrase (by essential oils), GABA-gated chloride channel (by thymol), sodium and potassium ion exchange disruption (by pyrethrin) and inhibition of cellular respiration (by rotenone). Such disruption also includes the blockage of calcium channels (by ryanodine), of nerve cell membrane action (by sabadilla), of octopamine receptors (thymol), hormonal balance disruption, mitotic poisoning (byazadirachtin), disruption of the molecular events of morphogenesis and alteration in the behavior andmemory of cholinergic system (by essential oil), *etc* (Minjas and Sarda, 1986).

The most important activity is the inhibition of acetylcholinerase activity (AChE) as it is a key enzyme responsible for terminating the nerve impulse transmission through synaptic pathway. AChE hasbeen observed to be organophosphorus and carbamateresistant, and it is well-known that the alteration in AChE is one of the main resistance mechanisms in insect pests; (Senthilnathan, 2008).

2.6 Mode of Action of Insecticides/Larvicide

Insecticides and Larvicide generally target the nervous system, growth and development, or energy production of the vector (Amy, 2006). The control and management of disease vector are identified as

i. The Nervous System
ii. Growth and Development
iii. Energy Production
iv. Metabolism

2.6.2 The Nervous System

The nervous system functions as a fast acting means of transmitting important information throughout the body (Ghosh, 2012). This system has two components:

a) The *peripheral nervous system* to receive and transmit incoming signals (taste, smell,sight, sound, and touch) and to transmit outgoing signals to the muscles and other organs, effectively telling them how to respond, and
b) The *central nervous system (CNS)* to interpret the signals and coordinate the body's responses and movements.

Both humans and insects have many different neurotransmitters that work at different sites throughout the nervous system. Some neurotransmitters are *excitatory* e.g, *acetylcholine (ACh)*, and some are *inhibitory* e.g. *gamma-aminobutyric acid (GABA*) are important targets of some insecticides.

ACh can either excite or inhibit its target neurons-depending on the particular neuron and the specific receptors at the site, ACh can cause particular neurons to fire continuing the nerve impulse transmission, or it can cause the nerve impulse to stop at that particular site.

Incontrast, GABA is an inhibitory neurotransmitter; when GABA is activated at a synapse, the nerve impulse stops. Some insecticides interfere with the normal action of these neurotransmitters. Other insecticides attacking the nervous system work by other means. The most common mechanisms are explained below.

2.6.2.1 Cholinesterase Inhibition

a) Organophosphate and carbamate insecticides are known as cholinesterase inhibitors. They bind to the enzyme that is normally responsible for breaking down ACh after it has carried its message across the synapse. When an insect has been poisoned by a cholinesterase inhibitor, the cholinesterase is not available to help break down the ACh, and the neurotransmitter continues to cause the neuron to "fire," or send its electrical charge. This causes overstimulation of the nervous system, and the insect dies.

Like insects, humans also use ACh as a neurotransmitter and cholinesterase to break it down, and cholinesterase poisoning in humans can be very severe. Upon each exposure to an organophosphate or carbamate insecticide, more cholinesterase becomes bound and is unavailable to do its job. Although cholinesterase inhibition by carbamates is somewhat reversible but organophosphate poisoning is not reversible.

This means the insecticide does not release the bound cholinesterase. Fortunately, the body continually produces cholinesterase, although it may take several weeks to again reach the desirable circulating level. Applicators using cholinesterase inhibiting pesticides regularly should consider having their cholinesterase level monitored. A simple blood test performed in the person and at intervals throughout the application season predicts whether an applicator is being exposed to too much organophosphate or carbamate.

2.6.2.2 Acetylcholine Receptor Stimulation

Neonicotinoid insecticides act as agonists of the acetylcholine receptor. That is, they mimic the action of the neurotransmitter, acetylcholine (ACh). Although cholinesterase is not affected by these insecticides, the nerve is continually stimulated by the neonicotinoid itself, and the end result is similar to that caused by cholinesterase inhibitors-overstimulation of the nervous system leads to poisoning and death. Fortunately, the neonicotinoids are a closer mimic for the insect's ACh than for human ACh, giving this class of insecticides more specificity for insects and less ability to poison humans.

Spinosad is also an acetylcholine receptor agonist. The exact mechanism of spinosad is somewhat different than that of the neonicotinoid class, but the end result is the same.

2.6.1.3 *Chloride Channel Regulation*

Avermectins are derived from a soil microorganism and belong to a group called the macrolactones. Avermectins bind to the chloride channel. This channel normally blocks reactions in some nerves, preventing excessive stimulation of the central nervous system (CNS). Avermectins activate the chloride channel, causing an inhibitory effect, which, when excessive, results in the insect's death.

Organochlorine insecticides

cyclodiene affect the chloride channel by inhibiting the GABA receptor. The GABA receptor has an inhibitory function at its site. When a cyclodiene insecticide binds to the GABA molecule, the neurotransmitter can no longer close the chloride channel for which it acts as a gate. Thus there is nothing to stop the electrical charge from continuing down the neuron. The end result is overstimulation of the nervous system.

Bifenazate affects the GABA-gated chloride channel as an agonist. That is, it causes the gate to have the same action as GABA would cause, which closes the gate. Nerve impulses are then unable to travel down the chloride channel.

2.6.2.4 Sodium Channel Modulators

Pyrethrins are naturally-occurring compounds derived from members of the chrysanthemum family. While they have a quick knock-down effect against insects, they are unstable in the environment, so may not last long enough to kill the pest.

Pyrethroids are synthetic versions of pyrethrins, specifically designed to be more stable in the environment (although still lasting only days or weeks), and thus provide longer-lasting control.

Pyrethrins and pyrethroids act on tiny channels through which sodium is pumped to cause excitation of neurons. They prevent the sodium channels from closing, resulting in continual nerve impulse transmission, tremors, and eventually, death.

Pyrethrins and pyrethroids are well-known irritants of humans' respiratory systems as well as of the skin and eyes. Applicators that have an allergic reaction to these insecticides must either increase the amount of personal protective equipment worn during handling, or stop working with this class of insecticides.

2.6.3 Growth and Development

Unlike humans, insects must shed their skin in order to grow and to develop into their next life stage. Insects' skin is a hard *exoskeleton*, also called the *cuticle*, which provides both protection and structure(Ghosh, 2012).

Molting is necessary not only for the insect to grow, but also for the insect to reach the adult stage so that it can reproduce.

Hormones play various roles in molting. Disruption of, or interference with, any of these hormones inactivates the molting process. Some insecticides target the insect's growth and development processes through interfering with hormones, and others through blocking theproduction of a structural component of the exoskeleton.

a) Chitin Synthesis Inhibitors (CSIs) Chitin is an important component of the insect's cuticle. Some insecticides, called **chitin synthesis inhibitors**, block the production of chitin. An insect poisoned with a CSI cannot make chitin and so cannot molt. Because molting must take place for the insect to reach the adult stage, a CSI poisoned insect also cannot reproduce. Eventually, the insect dies.

Because humans do not make chitin, CSIs are not considered toxic to humans. However, CSIs are very toxic to any organism that has an exoskeleton, such as crustaceans (shellfish), and should be used with great care, if at all, in areas where they could contaminate the environment.

b) Insect Growth Regulators or IGRs: attack the insect's endocrine system, which produces the hormones needed for growth and for development into an adult form. Insects poisoned with IGRs cannot molt or reproduce, and eventually they die. Many of the currently available IGRs mimic a special protein called *juvenile hormone*. In a normal insect, juvenile hormone is circulated throughout the insect's body and "tells" the insect to stay in its current stage. After a certain amount of time, the insect stops producing juvenile hormone, and the insect *metamorphoses*, or changes, into its next life stage. When an insect is poisoned by an IGR that mimics juvenile hormone, the insect doesn't receive the signal to metamorphose because, even though the insect may have stopped

producing juvenile hormone, the IGR is still circulating throughout its body and sending the signal to stay in the current stage.

c) *Prothoracicotropic hormone (PTTH)* is another insect development hormone. The insecticide **azadirachtin**, which is derived from neem oil, interferes with synthesis of PTTH. Besides its ability to kill through interfering with growth and development, azadirachtin also acts as a feeding deterrent. Humans do not make or use the hormones insects use in molting. Because of this, IGRs are considered to have little human toxicity.

d) *Nonspecific Growth Regulators:* The exact mode of action of the **mite growth regulator** hexythiazox is not well understood. Hexythiazox kills the eggs before the mites hatch and also some immature mites. Adult mites are not killed, although adults exposed to residues may lay eggs that are not viable.

2.6.4 Energy Production

All organisms generate energy from the food they take in. As organisms digest the nutrients in the food they consume, they store the energy from those nutrients in molecules known as *Adenosine Triphosphate* (*ATP)*. The energy stored in the ATP molecules can then be used to do work such as thinking, moving, growing, or synthesizing chemicals and structures that the body needs. Some insecticides inhibit or disrupt energy production. Initially, the insect can mobilize enough stored energy to continue its basic functions. While it can eat and digest food in the initial stages after being poisoned, it cannot produce more energy from the food. Eventually, the insect "runs out of steam," stops eating and even moving, and dies. Two main processes in energy production: *Electron Transport Chain* and *Oxidative Phosphorylation*, which are normally linked together, are described below.

a) Electron Transport Inhibition: Electron transport is an important process in the production of energy in plants and animals. When this process is disrupted, oxidative phosphorylation is inhibited, and energy (ATP) cannot be stored for later use.

b) Oxidative Phosphorylation Disruption: is the process through which ATP is synthesized in plants and animals. **Organotin miticides** inhibit oxidative phosphorylation directly, while **pyrroles** work by uncoupling oxidative phosphorylation from electron transport. The end result for both groups is that the cell is unable to produce ATP for energy.

2.6.4 *Metabolism*

Some insecticides block feeding. Different classes of insecticides work through different mechanisms (Amy, 2006), as described below.

a) *Bacillus thuringiensis (Bt)* is a microbe that produces a crystal with a toxic effect against some insects. When Bt is eaten by a larva, it attacks the lining of the insect's midgut and causes it to stop feeding and ultimately to die. Different strains, or varieties, of Bt produce slightly different crystals which have selective toxicity against various insects.

b) *Azadirachtin* acts as both a feeding deterrent and a growth regulator. In this case, feeding is affected through azadirachtin's interference with *phagostimulants*, which play a role in normal feeding behavior of insects and related arthropods.

c) *Cryolite*, an inorganic insecticide, is a non-specific feeding blocker. Its exact mechanism of action is not yet well understood.

2.7 Scope for isolation of toxic larvicidal active compounds

Several studies have documented on the efficacy of plant extracts as the reservoir pool of bioactive toxic agents against mosquito larvae. But only a few have been commercially produced

and extensively used in vector control programmes. The major problem in the failure in laboratory to land movements of bioactive toxic phytochemicals are poor characterization and inefficiency in determining the structure of active toxic compound(s) responsible for larvicidal activity (Ghosh, 2012; Fonseca, *et al*., 2001).

To achieve the production of a green biopesticide, the following steps are recommended during research design with phytochemicals:-

1. Screening of floral biodiversity in search of crude plant extracts having mosquito larvicidal potentiality
2. Preparation of plant solvent extracts starting from non-polar to polar chemicals and determination of the most effective solvent extract
3. Evaporation of the liquid solvent to obtain solid residue and determination of the lethal concentration (LC_{50}/LC_{100} values)
4. Phytochemical analysis of the solid residue and application of column chromatography and thin layer chromatography to purify and isolate toxic phytochemical with larvicidal potentiality
5. Determination of the structure of active principle by Infra Red (IR) spectroscopic, nuclear magnetic resonance (NMR) and Gas Chromatography-Mass Spectroscopy (GC-MS) analysis
6. Study of the effect of active ingredient on non target organisms; and
7. Field evaluation of the active principle before its recommendation in vector control programme andcommercial production (Ghosh, 2012).

2.8 Variation of larvicidal potentiality

The efficacy of phytochemicals against mosquito larvae can vary significantly depending on plant species, plant parts used, age of plant parts (young, mature or senescent), solvent used during extraction as well as upon the available vector species. Sukumar *et al.*,(*1991*) have described the existence of variations in the level of effectiveness of phytochemical compounds on target mosquito species *vis-a-vis* plant parts from which these were extracted, responses in species and their developmental stages against the specified extract, solvent of extraction, geographical origin of the plant, photosensitivity of some of the compounds in the extract, effect on growth and reproduction (Patil, *et al*., 2012). Changes in the larvicidal efficacy of the plant extracts occurred due to geographical origin of the plant (in *Citrus* sp, *Jatropha* sp, *Ocimum sanctum, Momordica charantia*, *Piper* sp and *Azadirechta indica*; response in the different mosquito species (in *Curcuma domestica*, *Withania somnifera*, *Jatropha curcas*, *Piper retrofractum*, *Cestrum diurnum*, *Citrullus vulgaris*, and *Tridax procumbens* due to variation in the species of plant examined (in *Euphorbia* sp, *Phyllanthus* sp, *Curcuma* sp, *Solanum* sp, *Ocimum* sp, *Eucalyptus* sp, *Plumbago* sp, *Vitex* sp, *Piper* sp, *Annona* sp and *Cleome* sp and between plant parts used to study the larvicidal efficacy (in *Euphorbia tirucalli*, *Solanum xanthocarpum*, *Azadirechta indica*, *Solanum villosum*, *Annona squamosa*, *Withania somnifera* and *Ocimum sanctum.*

However, variation of the larvicidal potential of the same plant changed with the solvents used as evidenced in case of *Citrullus colocynthis*, *Azadirechta indica*, *Annona squamosa* and *Solanum nigrum* (Anupam., 2011, Ghosh, 2012).

2.9 Extraction of Active Biochemicals from Plants

It has been shown that the extraction of active biochemicals from plants depends upon the polarity of the solvents used. Polar solvent will extract polar molecules and non-polar solvents extract non-polar molecules. This was achieved by using mainly eleven solvent systems ranging from hexane/ petroleum ether, the most non polar (polarity index of 0.1 that mainly extracts essential oil) to that of water, the most polar (polarity index of 10.2) that extracts biochemical with higher molecular weights such as proteins, glycans, *etc*. Chloroform or ethyl acetate is moderately polar (polarity index of 4.1) that mainly extracts steroids, alkaloids, *etc*. It has been found that solvents with minimum polarity have been used such as hexane or petroleum ether or that with maximum polarity such as aqueous/ steam distillation (Ghosh, 2012). However, those biochemicals that were extracted using moderately polar solvents were also seen to give good results as reported by a few bioassay. Thus, different solvent types can significantly affect the potency of extracted plant compounds and there is difference in the chemo-profile of the plant species. The lowest LC_{50} value was reported in *Solenostemma argel* against *Cx. Pipiens (Al-Doghari et al., 2004)*

2.10 *Datura stramonium* (Jimsonweed)

Datura stramonium Linn. - Named by Carl Linnaeus as published in Species Plantarum in 1753. The genus was derived from ancient hindu word for plant, dhatura. The species name is from New Latin, stramonium, meaning thorn apple. Stramonium is originally from Greek, strychnos (nightshade) and manikos (mad) (Maibam, *et al.*, 2011)

2.10.1 Scientific Name of D. stramonium

Kingdom:	*Plantae*
(unranked):	Angiosperms
(unranked):	Eudicots
(unranked):	Asterids
Order:	Solanales
Family:	Solanaceae
Genus:	*Datura*
Species:	*D. stramonium*

2.10.2 Description

Jimsonweed is an annual herb which grows up to 5 feet tall. It has a pale green stem with spreading branches. Leaves are ovate with green or purplish coloration, coarsely serrated along edges, and 3 to 8 inches long. Flowers are white or purple with a 5-pointed corolla up to four inches long and set on short stalks in the axils of branches. Seeds are contained in a hard, spiny capsule, about 2 inches in diameter, which splits lengthwise into four parts when ripe.

Plate I: Image of Daturastramonium *leaf*

Date capture: 19/02/2013

2.10.3 Historic use of D. stramonium

Historic use of Jimson weed and various other species of *Datura* has occurred for many purposes throughout time. In Europe the plant was used for witch craft, in salves or ointments. Throughout most European countries the seeds were used to brew beer (Shaman Australia Ethno botanicals). The weed was dried and smoked; the users were left on a high which consisted of hallucinations and total relaxation. Jimson weed was thought to cure those with deafness, soothe insomniacs, and release the heat of those with a fever. *D. stramonium* is thought to be one of two plants identified in 4,000-year-old rock paintings throughout the Pecos river region of Texas and northern Mexico, used by the Huichol Indians along with peyote to commune with the spirit world (Boyd and Dering, 2000).

Hernandez (1959), reported that the decoction of leaves to the body for fever or administered as a suppository. The fruit and leaves were considered good for pain in the chest. If too much was taken, it was believed to cause insanity. In northwestern New Spain, the Opata rubbed a leaf of Taguaro on the painful area for spleen disease. They believed it also matured tumors and abscesses (Nentuig, 1977). An ointment of the ground seeds and suet is rubbed on boils, pimples, and swellings; the powdered leaves are applied to hemorroids; and hot baths containing the plant give relief to colds and diarrhea (Curtain, 1947).

2.10.4 Medicinal Uses

D. stramonium is now used to treat asthma, and gastrointestinal problems, also aches, abscesses, arthritis, boils, headaches, hemorroids, rattlesnake bites, sprains, swellings, and tumors (Sandoval,1998). It acts as a sedative in large doses and as a stimulant and deleriant in high ones. *Datura* is an anodyne, antibiotic, antispasmodic and narcotic. Relieving the pains of rheumatism and sciatica when applied as an ointment, and easing spasms of Parkinson’s disease are unproven accounts of the effects of Jimson weed.

Most of the plant is used for medicinal reasons. Eating the seeds rapidly gets the plant to the nervous system, but also increases the risk of lethal overdose. The leaves can be dried and smoked to relax the bronchiole muscles of the throat, and leaves are used also to line beds of those with insomnia. Annette Sandoval in Homegrown Healing recommends using the fresh leaves, flowers, or seeds.

2.10.5 Active Compounds of D. stramonium

D. stramonium contains hyoscine, as well as atropine, hyoscyamine, apohyoscine, and meteloidine. Thus it is poisonous and hallucinogenic as well as acting as a pain killer (Duke, 1985, Ivancheva *et al*., 2006).

2.10.6 Non-Medicinal Uses D. stramonium

D. stramonium (or *innoxia*) has been found to rapidly clear 2,4,6-trinitrotoluene (TNT) from munition waste sites, and to transform it via nitroreduction i.e it properties as remediators of explosives (Lucero *et al.*, 1999).

2.10.6 Phytochemicals of D. stramonium

The medicinal value of a plant depends on its bioactive phytochemical constituents that produce definite physiological action in the body. The most important bioactive phytochemical constituents include presence of alkaloids, tannins, saponins, glycosides and flavonoids

(Aderotimi *et al*., 2006). The major tropane alkaloids such as hyoscyamine and scopolamine and several minor tropane alkaloids have been identified in *Datura* species. Typical examples of minor alkaloids in *D. stramonium* are tigloidin, aposcopolamine, apoatropin, hyoscyamine N-

oxide and scopolamine N-oxide17-20. 6a-ditigloyloxytropane and 7-hydroxyhyoscyamine are reported for the first time in this species (Das, 2012).

Distribution of hyoscyamine and scopolamine in *D. stromonium* was studied. The production of hycyamine and scopolamine in *D. stromonium* has been investigated in the different plant parts, at different stages of their life cycle. The maximum contents were found in the stems and leaves of young plants, hyocyamine being always the predominate component. These compounds were included in many pharmacopieas because of their anticholinergic activities (Goldfrank, and Flommenbaum., 2006). *D. stromonium* contain variety of alkaloids including atropine, hyoscamine and scopolamine (Ivancheva *et al*., 2006).

Sixty-four tropane alkaloids have been detected from *D. stramonium*. Two new tropane alkaloids, 3-phenylacetoxy-6, 7-epoxynortropane and 7-hydroxyapoatropine were tentatively identified. The alkaloids scopoline, 3-(hydroxyacetoxy) tropane, 3-hydroxy-6-(2-methylbutyryloxy) tropane, 3, 7-dihydroxy-6-tigloyloxytropane, 3-tigloyloxy-6-propionyloxytropane, 3 phenylacetoxy-6,7-epoxytropane, 3-phenylacetoxy-6-hydroxytropane, aponor scopolamine, 3a, 6a-ditigloyloxytropane and 7-hydroxyhyoscyamine are reported for the first time for this species.

Other alkaloids found in *D. Stramonium* reported by Strahil., (2006) include; Hygrine, 3á, 6a-Ditigloyloxy-7-hydroxytropane, 6-Hydroxyhyoscyamine, Pseudotropine, 3á-Tigloyloxytropane, Hydroxy-6-tigloyloxytropane, Phenylacetoxytropane, 3-Acetoxy-6-isobutyryloxytropan, 3-(2-Phenylpropionyloxy) tropane, Littorine, 6-Hydroxyapoatropine, 3-Tropoyloxy-6-acetoxytropane, Tropine,3-Acetoxytropane,3-Hydroxy-6-acetoxytropane,3-Hydroxy-6-methylbutyryloxytropane, 3-Tigloloxy-6-isobutyryloxytropane, Aponorscopolamine, 7-Hydroxyhyoscyamine, Meteloidine,

3a, 6â-Ditigloyloxytropane. The secondary metabolites identified in the plant materials showed antimicrobial activity (Banso and Adeyemo 2006).

However, the tropane alkaloids which are responsible for both the medicinal and hallucinogenic properties are fatally toxic in only slightly higher amounts than the medicinal dosage, and careless use often results in hospitalizations and deaths.

2.10.7 Anticholinergic activity of D. stramonium

The alkaloids found in *D. stramonium*, are organic esters used clinically as anticholinergic agents. Jimson weed has been reported as a drug of abuse and has been involved in the accidental poisoning of humans and animals (Maibam, *et al.,* 2011). Symptoms of acute Jimson weed poisoning included dryness of the mouth and extreme thirst, dryness of the skin, pupil dilation and impaired vision, urinary retention, rapid heartbeat, confusion, restlessness, hallucinations, and loss of consciousness. The anticholinergic syndrome results from the inhibition of central and peripheral muscarinic neurotransmission (Boumba., 2005).

2.10.8 ***Acaricidal, repellent and oviposition deterrent properties of D. stramonium***

The ethanol extracts obtained from both leaf and seed in *D. stromonium* (Solanaceae) were investigated for acaricidal, repellent and oviposition deterrent properties against adult two-spotted spider mites (*T. urticae* Koch) (Acari: Tetranychidae) under laboratory conditions. Leaf and seed extracts, which were applied in 167.25 and 145.75 g/L concentrations, respectively (using a Petri leaf disc-spray tower method), caused 98% and 25% mortality among spider mite adults after 48hrs. These results suggest that *D. stramonium* extracts could be used to manage the two-spotted spider mite (Kurnal and Yalcin, 2009).

2.10.9 Larvicidal and mosquito repellent activities of D. stramonium

Ethanolic extracts of leaves of *D. stromonium* were evaluated for larvicidal and mosquito repellent activities against *Aedes aegypti, Anopheles stephensi* and *Culex quinquefasciatus*. The LD_{50} values for larvicidal activity were found to be 86.25, 16.07 and 6.25 mg/L against *Aedes aegypti, Anopheles stephensi* and *Culex quinquefasciatus* respectively. The ethanolic leaves extract of *D. stromonium* provided complete protection time (mosquito repellency) of 2.7, 71.7 and 117.7 min against *Aedes aegypti, Anopheles stephensi* and *Culex quinquefasciatus* at higher concentration (Swathi *et al*., 2012)

2.11 The *Ocimum gratissimum*plant

Ocimum gratissimum is a herbaceous plant which belongs to the Labiatae family. The plant is indigenous to tropical areas especially India and it is also in West Africa. In Nigeria, it is found in the Savannah and coastal areas. It is cultivated in Ceylon, South Sea Islands, and also within Nepal, Bengal, Chittagong and Deccan (Nadkarni., 1999). It is known by various names in different parts of the world. In India it is known by its several vernacular names, the most commonly used ones being Vriddhutulsi (Sanskrit), Ram tulsi (Hindi), Nimma tulasi (Kannada). In the southern part of Nigeria, the plant is called "effinrin-nla" by the Yoruba speaking tribe. It is called "Ahuji" by the Igbo, while in the Northern part of Nigeria, the Hausas call it "Daidoya" (Effraim., 2012).

2.11.1 Scientific classification O. gratissimum

Kingdom: Plantae

(unranked): Angiosperms

(unranked): Eudicots

(unranked): Asterids

Order: Lamiales

Family: Lamiaceae

Genus: *Ocimum*

Species: ***O. gratissimum***

2.11.2 Morphology and Microscopy

O. gratissimum is a shrub up to 1.9m in height with stems that are branched. The leaves measure up to 10 x 5 cm, and are ovate to ovate-lanceolate, sub-acuminate to acuminate at apex, cuneate and decurrent at base with a coarsely crenate, serrate margin, pubscent and dotted on both the

sides (Effraim, 2012). The leaves show the presence of covering and glandular trichomes. Stomata are rare or absent on the upper surface while they are present on the lower surface. Ordinary trichomes are few, while the long ones up to 6-celled are present on the margins mostly; the short ones which are 2 celled, are mostly found on the lamina. Petioles are up to 6cm long and racemes up to 18 cm long. The peduncles are densely pubescent. Calyx is up to 5mm long, campanulate and 5-7 mm long, greenish-white to greenish-yellow in colour. Nutlets are mucilaginous when they are wet (Bhat, 2003).

Plate II: Image *Ocimum gratissimum Leaf*
Date captured: 20/March/2013

2.11.3 Pharmacology of extracts and essential oils of Ocimum gratissimum

The essential oil of *Ocimum gratissimum* contains Eugenol and shows some evidence of antibacterial activity (Oboh *et al*., 2009 andSilva *et al*., 2010). Leaf extract of *O. gratissimum* showed antidiabetic properties in streptozocin-induced in diabetic rats (Mohammed *et al*., 2008). A test on guinea pigs found evidence that the essential oil relaxes the muscles of the small intestine, consistent with the traditional use of the plant to treat gastrointestinal disorders (Mohammed *et al*., 2008). Antitumor and anti-cancer effects have been reported in *invitro* experiments (Socorro *et al.,* 2002). A study on rats also found evidence that a leaf extract of the plant prevented diarrhea. A study on goats also found that the essential oil has anthelmintic activity (Pessoa *et al*, 2002). The ethanolic extract of *Ocimum gratissimum* produced a significant and sustained increase in the sexual activity of normal male mice, without any adverse effects. Thus, the resultant aphrodisiac effectiveness of the extract lends support to the claims for its traditional usage in sexual disorder (Pande and Pathak, 2009). The essential oil has potential for use as a food preservative (Nguefack., 2009) and is toxic to *Leishmania* (Oliveira, 2009.

2.11.4 Traditional Uses of O. gratissimum

O. gratissimum has been used extensively in the traditional system of medicine in many countries. In the North east of Brazil, it is used for medicinal, condiment and culinary purpose. The flowers and the leaves of this plant are rich in essential oils so it is used in preparation of teas and infusion (Rabeloe, 2003). In the coastal areas of Nigeria, the plant is used in the treatment of pilepsy, high fever and diarrhoe (Effraim, 2012). In the Savannah areas decoctions of the leaves are used to treat mental illness (Akinmoladun., 2007).

It is also used in the treatment of fungal infections, fever, cold and catarrh (Ijeh, *et al.*, 2005). Brazilian tropical forest inhabitants use a decoction of *O. gratissimum* roots as a sedative for children (Cristiana *et al.*, 2006). People of Kenyan and sub Saharan African communities' use this plant for various purposes like viz., the leaves are rubbed between the palms and sniffed as a treatment for blocked nostrils, they are also used for abdominal pains, sore eyes, ear infections, coughs, barrenness, fever, convulsions, and tooth gargle, regulation of menstruation and as a cure for pro-lapse of the rectum (Matasyoh *et al*., 2007).

In India, the whole plant has been used for the treatment of sunstroke, headache, and influenza, diaphoretic, antipyretic and for its anti-inflammatory activity (Prajapati *et al*., 2003).

In Nigeria, the plant is commonly used in folk medicine to treat different diseases such as upper respiratory tract infections, diarrhoea, headache, diseases of the eye, skin diseases, pneumonia, mcough, fever and conjunctivitis. Infusion of *O. gratissimum* leaves is used as pulmonary antisepticum, anti-tussivum and antispasmodicum (Ngassoum*et al*., 2003

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

Silica gel, for column 60-230 mesh, Silica gel 60 F_{254} plates (Merck) TLC, One pipette delivering 100-1000µl, Disposable tips (100µl, 500µl) for measuring aliquots of dilute solutions, Five 1ml pipettes, three droppers with rubber suction bulbs, Two wire loops, 4 nylon netting cage 75x35x35cm, 120ml disposable bowls, measuring cylinder and a strainer was used to transfer test larvae into test cups or vessels.

3.1.1 Collection and identification of plants

The plant leaves *Datura stramonium*was harvested from the North Easthern Region Mambilla plateau of Nigeria while *Ocimum gratissimum* was harvested around Samaru-Zaria Metropolis. The plants were identified at the Herbarium unit, Department of Biological Science, Ahamadu Ballo University, Zaria.

3.1.2 *Chemicals*

All chemicals used were of analytical grade and was purchased from sigma Altrich Company limited, except otherwise stated.

3.2 METHOD

3.2.1 Preparation of extracts

The plant leaves were washed and dried in shade at room temperature and ground to fine powder in an electric grinder. Extracts of distil water, ethanol, ethyl acetate and n-hexane were prepared by mixing 50g of dried powder with 500ml of each solvent with constant

stirring on a magnetic stirrer. The suspension of the dried powder in water was left for 24hr, filtered through Whatman No.1 filter paper then filtrate was concentrated using rotary evaporator and stored in amber coloured air tight bottle at room temperature till use.

3.2.2 Harvesting of Mosquito Larvae

Culex spp. mosquito larvae was collected around A.B.U. water works, Main Campus. The larvae was identified and authenticated at the entomology research laboratory of Department of Biological Sciences A.B.U. Zaria. The larvae were reared in plastic and enamel trays in tap water. They were maintained in a growth chamber and at room temperature. The larvae were fed a diet of brewer's yeast and biscuits (Bhat and Kempraj, 2012). Pupae was transferred from the trays to a cup containing tap water and placed in screened cages where adults emerged. Adults of *Culex spp*. were reared in wooden or plastic cages. Adults were continuously provided with 10% sucrose solution soaked on a cotton pad, placed at the middle of the cage. After mating day, adult females was deprived of sugar for 12hr then provided with a mouse placed in restrained position overnight for blood feeding. A petri dish with moisten filter paper was provided in the cage for oviposition and was maintained at the same environmental condition. The third and fourth instars larvae of the second generation were used for the Bioassay (Senthilnathan, 2007).

3.3 EXPERIMENTAL DESIGN

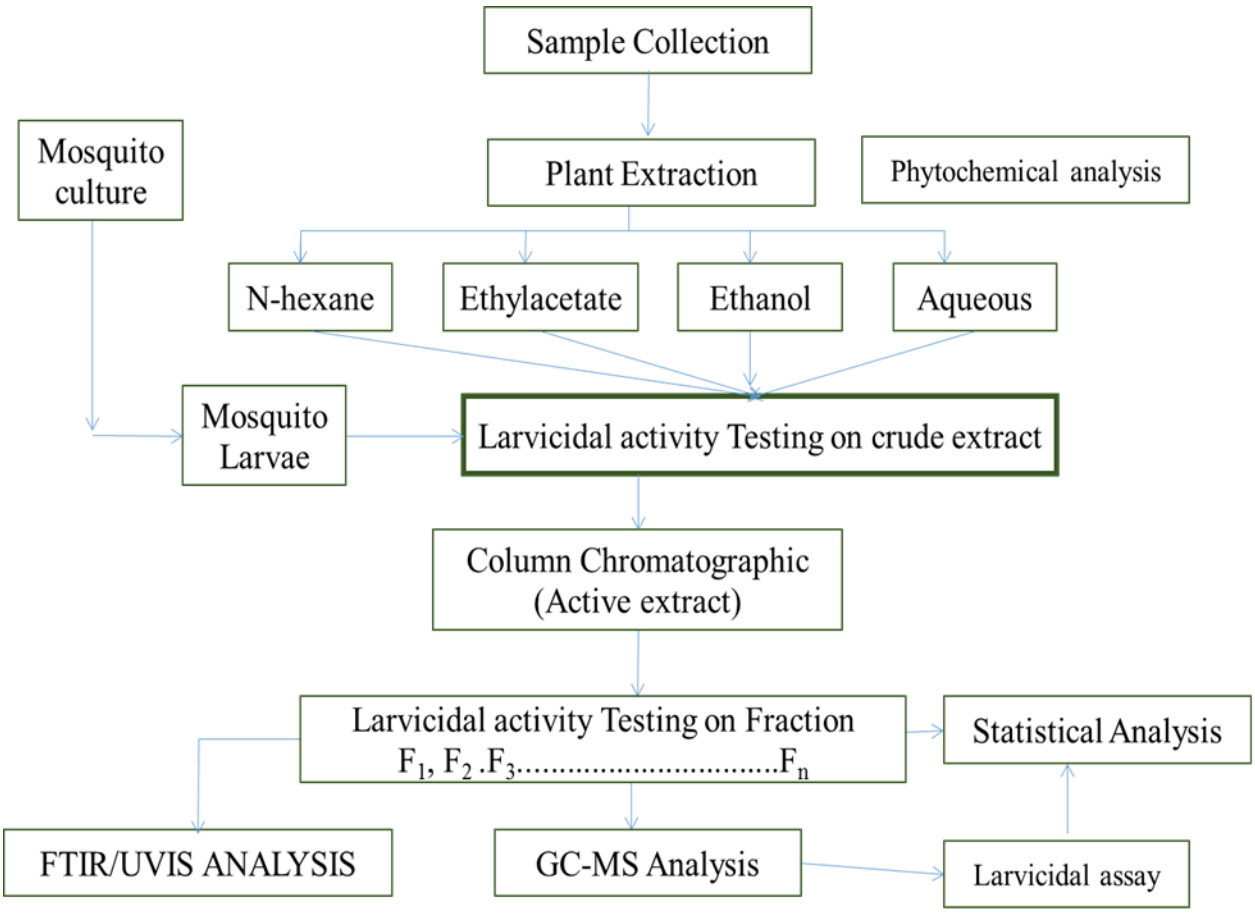


Figure 3: Work Flow experimental design

3.4 Phytochemical Analysis

Phytochemical analysis: Alkaloid, Tanins, Flavonoids, Terpenoids, Anthraquinone, Saponins, Cardiac glycocide and sterols were carried out on the two plant extracts using a standard procedure for identification of phytochemical constituents as described by Harborne (1973), Trease and Evans, (1989) and Sofowora (1993).

3.4.1 *Test for Saponins*

About 2g of the powdered sample was boiled in 20ml of distilled water in a water bath and filtered. About 10ml of the filtrate was mixed with 5ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of emulsification.

3.4.2 *Test for tannins*

About 0.5g of the dried powdered sample was boiled in 20ml of water in a test tube then filtered and 1ml of 0.1% ferric chloride was added and observed for the brownish green or a blue-black colouration.

3.4.3 *Test for flavonoids*

Dilute ammonia solution (5ml) was added to a portion of aqueous filtrate followed by addition of concentrated sulfuric acid. A yellow colouration observed indicate the presence of flavonoids.

3.4.4 *Test for sterols*

About 2ml of acetic anhydride was added to 0.5g of Ethanolic extract of the sample with 2ml sulfuric acid. The colour change from violet to blue or green indicates the presence of sterols.

3.4.5 *Test for Terpenoids*

Using Salkowski's test, 5ml of the extract was mixed in 2ml of chloroform and 3ml of concentrated sulfuric acid was carefully added to form a layer. A reddish brown colouration of the interface formed will show a positive result for the presence of Terpenoids.

3.4.6 *Test for Anthracenes*

The test for Anthracenes of 5ml aqueous extract was shaken with equal volume of chloroform and allowed to form a chloroform layer. 10ml of ammonia was added, shaken vigorously and then allowed to separate

3.4.7 *Test for cardiac glycosides*

The test for cardiac glycosides was carried out using the Keller-Kiliani test. About 5ml of extract was treated with 2ml of glacial acetic acid containing 1 drop of ferric chloride solution. This was underplayed with 1ml of concentrated sulfuric acid. A brown ring of interface indicates a deoxysugar characteristic of cardenolides. A violet ring may appear above and below the brown ring, formed just gradually throughout thin layer. This indicates the presence of cardiac glycosides.

3.4.8 *Test for alkaloids*

About 1 ml of the aqueous plant extract was treated with 2ml of picric acid solution. Formation of orange colouration indicates the presence of alkaloids.

3.5 Preparation of Stock Solutions

A Stock solution of 20ml of 1% was prepared by weighing 200mg of the crude extract and adding 20ml distil water which is referred to as the stock solution. The solution was kept in a screw-cap vial, with aluminum foil over the mouth of the vial prior to use.

3.6 Preparation of Test Concentrations For Bioassay

The stock solution is then serially diluted according to WHO guidelines (2005). Triplicate of test volumes were prepared into final concentration of 250ml. Six concentrations of aqueous extract, n-hexane, ethyl acetate and Ethanolic extract in DMSO at 6.25ppm, 12.5ppm, 25ppm, 50ppn, 100ppm and 1000ppm in boiled and cooled tap water (chlorine-free water). When making a series of concentrations, the lowest concentration should be prepared first. Small volumes of dilutions was transferred to test cups by means of pipettes with disposable tips. The addition of small volumes of solution to 100 ml, 200 ml or greater volumes of water will not cause noticeable variability in the final concentration.

3.7.0 Determination of larvicidal Bioassay

3.7.1 Bioassay was performed according to WHO guidelines (2005). After making test concentration, $3r^{d}$ and 4^{th} instars larvae was introduced into each plastic bowel (500 ml capacity). Small, unhealthy or damaged larvae was removed (Hashmat *et al.*, 2012).

3.7.2 The healthy mosquito larvae was exposed to a wide range of test concentrations and a control to find out the activity range of the materials under test by determination of the larva mortality. After determining the mortality of larvae in this wide range of concentrations, mortality in 24hours was determine at LC_{50} and LC_{90} values.

3.7.3 Batches of 25 third and fourth instars larvae were transferred by means of strainers, screen loops or droppers to small disposable test cups, each containing 100ml of water. Small, unhealthy or damaged larvae should be removed and replaced.

3.7.4 The depth of the water in the cups or vessels should remain between 5 cm and 10 cm to avoid undue mortality as aresult of lack of oxygen.

3.7.5 The appropriate volume of dilution of 1.0ml, 0.5ml and 0.1ml of aliqout is added to 100ml water in the cups to obtain the desired target dosage, starting with the lowest concentration.

3.7.6 Triplicates was set up for each concentration and an equal number of controls are set up simultaneously with tap water, to which 1 ml of respective organic solvents was added.

3.7.7 Each test should be run three times on different days. For long exposures, larval food should be added to each test cup, particularly if high mortality is noted in control.

3.7.8 The test containers were maintained held at 25–28^{o}c and preferably a photoperiod of 12hr light followed by 12 h dark (12L: 12D).

3.7.9 After 24hr exposure, larval mortality was recorded. For slow-acting insecticides, 48h reading may be required.

3.7.10 Moribund larvae are counted and added to dead larvae for calculating percentage mortality.

3.7.11 The mortalities of treated groups should be corrected according to Abbott's formula (Abbott 1952).

$$\text{Mortality (\%)} = \frac{\text{Number of dead larvae}}{\text{Number of larvae introduced}} * 100$$

$$\text{Corrected \% mortality} = \left(1 - \frac{\text{n in Co before treatment X n in T after treatment}}{\text{n in Co after treatment X n in T before treatment}}\right) \times 100$$

Where: n = Insect population, T = treated, Co = control

3.8 Thin Layer Chromatography (TLC)

TLC was carried out to determine the number of components in the metabolite. A chromatographic plate coated with silica gel was used. The dark brown metabolite was dissolved in 95% ethanol and n-Hexane for *D. stramonium* and *O. gratissimum* respectively and applied several times to the plate using a micro haematocrit capillary tube until the quantity loaded was adjudged sufficient for the experiment. The plate was placed in a chromatographic tank and eluted with a mixture of n-Hexane / Ethylacetate (6:4) and (7:3) for *D. stramonium* and *O gratissimum* respectively. Thereafter the plate was removed, dried in air and developed in 20% sulphuric acid in Alcohol.

3.9 Partial purification by Chromatographic Fractionation

The 1.0g crude extract of *D. stramonium* Ethanol extract (DSEE) and *O gratissimum* n-hexane extract (OGnHE) each were partially purified using column chromatochraphy to identify the compound(s) responsible for the Larvicidal activity. A total of 38 and 42 fractions respectively were collected, each having a volume of approximately 50ml/20min flowrate. Each of the fractions was spotted on TLC plate. The developed TLC plate was allowed to dry and was viewed under UV radiation (λ=254 nm and 365 nm). The plate was further sprayed with 20% sulfuric in alcohol solutions and dried for 15 minutes at 110°C (Ejele *et al*., 2012). These two procedures enable the fractions to be pooled together into seven (7) and six (6) main fractions respectively each were based on the pattern and R_f values of the spots on the TLC plate.The combined yield of fractions with the same R_f value and combined yield as % of total extract fractionated is shown in Appendix 5 and 6. These intermediate pooled fractions were further subjected to second phase of Larvicidal bioassay of both plants DSEE and OGnHE respectively.

3.10 CHARACTERIZATION OF LARVICIDAL COMPOUND(S) IN THE BIOACTIVE FRACTION BY UVIS, FTIR, GCMS SPECTROSCOPY

3.10.1 UV-Visible Spectroscopy

3.10.2 Fourier Transform Infra-Red spectroscopyanalysis

The FTIR analysis of the fraction with the highest larvicidal property was carried out to determine functional group(s) present using FTIR spectrophotometer (Shidmazu model), available at the National Research Institute for Chemical Technology (NARICT) Laboratory Zaria.

3.11 Identification of active component by Gas Chromatography/Mass Spectroscopy

For the identification of metabolites showing larvicidal potentials, the samples were subjected to GC-MS analysis. The samples (1µl) was injected into a RTX-5 column (60mX 0.25 mm i.d., film thickness 0.25µm) of GC-MS (model GC-MS-QP-2010 plus, Shimadzu Make). Helium was used as carrier gas at a constant column flow rate 1.58 ml/min at 108 kpa inlet pressure. Temperature programming was maintained from 100°C to 200°C with constant rise of 5°C/min and then held isothermal at 200°C for 6 min; further the temperature was increased by 10 °C/min up to 290°C and again held isothermal at 290°C for 10min. The injector and ion source temperatures were 270 °C and 250°C, respectively. The active fractions (DSEE-F1) and (OGnHE-F3) (2mg/ml) dissolve in ethanol and n-hexane (HPLC grade, Merck, India) respectively were injected. Mass spectra were taken at 70eV; a scan interval of 0.5s and fragments from 40 to 800Dalton. The final confirmation of constituents was made by computer matching of the mass spectra of peaks with the National Institute Science and Technology (NIST) libraries 2005

mass spectral database at the National Research Institute for chemical Technology (NARICT), Zaria Kaduna State, Nigeria.

3.12 Statistical analysis

The analysis of Probit regression (Finney, 1971), Mortality rates of larvae and activity expression (Log Probit Analysis) of lethal concentration (LC_{50} and LC_{90}) was compared across the plant species, the different solvent extract and the purified fraction of both plants investigated. Data from mortality was analyzed by ANOVA (Debella, *et al* 2007. P-value of less than 0.05 was statistically significant.

CHAPTER FOUR

4.0 RESULT

4.1. Determination of phytochemicals composition of *D. stramonium*and *O. gratissimum*leaf extract

The percentage yield was significantly high ($p<0.05$) in aqueous extract for *D. stramonium* (2.02±0.03)% and in ethanol extract for *O. grattissimum* (10.08± 0.32)% respectively. The least for *D. stramonium* was obtain in n-hexane ($p>0.05$) while Ethylacetate for *O. gratissimum* shown in (Appendix 1).

4.1.1 Phytochemicals screening of the Aqueous, Ethanol, Ethylacetate and N-Hexane extract of *D. Stramonium* and *O. Gratissimum* extracts

The Qualitative Determination of some phytochemicals present in the Aqueous, Ethanol, Ethylacetate and N-Hexane leave extracts of *D. Stramonium* and *O. Gratissimum* are presented in Table 4.1.1. The result shows that for *D. stramonium*, Sterol was present in all extracts while, C. glycoside and saponins were absent in all the extracts. Alkaloid, tannins and Flavonoids where present in all solvent except in hexane extract, while terpenoids was present in all solvent except in Ethylacetate extract. While anthraquinones were present only in Aqueous and Ethanol extracts of *D. Stramonium* on the oher hand *O. Gratissimum* indicated presence of Alkaloid, Flavonoids, Terpenoids and Sterol in all extracts while, C. glycoside and anthraquinones were completely absent in all the solvent extracts.Tannins was found to be absent in Aqueous, Ethylacetate and hexaneextracts. Saponins was only absent in Aqueous extract.

Table 4.1.1: Phytochemicals screening of theAqueous, Ethanol, Ethylacetate and N-Hexane extract of *D. stramonium*and *O. Gratissimum*leaf extract.

Phytochemicals	***D. stramonium***				***O. gratissimum***			
	AQ	ETOH	ETAC	Hex	AQ	ETOH	ETAC	Hex
Alkaloid	+	+	+	-	+	+	+	+
Tanins	+	+	+	-	-	-	+	-
Flavonoids	+	+	+	-	+	+	+	+
Terpenoids	+	+	-	+	+	+	+	+
Anthraquinone	+	+	-	-	-	-	-	-
Saponins	-	-	-	-	-	±	±	±
C. glycocide	-	-	-	-	-	-	-	-
Sterols	+	+	+	+	+	+	±	+

+ = Positive; ± = Trace; - = Negative

4.1.2 The Quantitative Determination of Phytochemical composition of *D. stramonium* and *O. gratissimum*

The Quantitative determination of Phytochemical composition in *D. stramonium*and *O. gratissimum*is presented in Table 4.1.2. The result shows significantly high amounts of Alkaloid, Tannins and Terpenoids as 2.24±0.22, 0.73± 0.10 and 0.61± 0.01 in 100mg of ($P<0.05$) respectively in *D. stramonium* in decreasing order. While for *O. gratissimum* also significantly shows high amounts of Alkaloid, Flavonoids and Steroids were found to be 1.28±0.15, 1.14±0.42 and 0.98±0.34 in mg/g of respectively in ($P<0.05$). There was no significant amount of Flavonoids, Steriods and Glycoside in *D. stramonium*ethanol leaf ($P>0.05$) while *O. Gratissimum* had considerable significance of Glycosides, Terpenoids and saponin (0.39±0.07, 0.23±0.04 and 0.22±0.10) in mg/g correspondingy.

Table 4.1.2: Quantitative Determination of Phytochemical composition in Aqueous, Ethanol, Ethylacetate and N-Hexane extract of DSEE and OGnHE

Phytochemical	DSEE	OGnHE
Alkaliods (mg/100 g)	2.24± 0.22^{c}	1.28± 0.15^{c}
Saponin (mg/100 g)	0.12 ± 0.14^{a}	0.22±0.10^{a}
Flavonoids (mg/100g)	0.04± 0.78^{a}	0.98±0.34^{b}
Steriods (mg/100g)	0.02 ± 0.01^{a}	1.14±0.42^{c}
Tannins (mg/100g)	0.73± 0.10^{a}	0.18±0.01^{a}
Glycosides (mg/100g)	0.02± 0.01^{a}	0.39±0.07^{b}
Terpenoids (mg/100g)	0.61± 0.01^{b}	0.23±0.04^{a}

Values represent Mean±SD of N=4. Values in the same column with different superscript across the raw differ significantly (0.05). DSEE= *D. stramonium* Ethanol extract, OGnHE=*O. Gratissimum* N-Hexane extract

4.2 Phase 1 Larvicidal Bioassay of *D. stramonium* and *O. gratissimum* leaves Extracts against *Culex quenquifaciatus* larvae

Larvicidal bioassay of crude extracts and partially purified fraction of two medicinal plants, *Datura stramonium* and *Ocimum gratissimum* were assayed for their larval toxicity against the early third and fourth instar larvae of *Culex quinquefasciatus* mosquito. Mortality was observed and recorded after 12, 24 and 48hours of exposure respectively.

4.2.1 Percentage mortality and Lethal Concentration of different Solvent extracts of *D. stramonium* leaves against *C. quenquifaciatus* Mosquito Larvae

The Percentage mortality and median lethal concentration of crude aqueous, ethanol, ethyl acetate and n-hexane extract of *D. stramonium* leaves against *Culex quenquifaciatus* mosquito larvae is shown in Table 4.2.1. Ethanol leave extract registered significant mortality ($p<0.05$) of about 99 per cent while the other solvent extract show mortality of less than 60 per cent in the larvicidal bioassays. Ethanol extract presented 99 percent mortality at 1000ppm after 48 hours and 91 Percent mortality at 25ppm after 24hr of treatment was considered effective concentration.

While the LC_{50} of the ethanol extract was the lowest at 2.57ppm and LC_{90} of 17.72ppm followed by Aqueous extract with LC_{50} 8.191ppm and LC_{90} of 66.127ppm. On the other hand Ethyl acetate and hexane extract showed poor mortality in larvicidal activity with relative LC_{50} of 14.637ppm and 26.49ppm respectively.

Table 4.2.1: Percentage mortality and lethal concentrationofdifferent solvent extract of*D. stramonium* leave against *Culex quenquifaciatus*mosquitolarvae at 12hr, 24hr and 48hr.

EXTRACT	Percent mortality (%)							Lethal conc (ppm)		
(r)	Conc. (ppm)	6.25	12.5	25.0	50.0	100	1000	LC^{a}_{50} (95% FL)	LC^{b}_{90} (95% FL)	R^2
Aqueous	12hrs	---	---	8	9	16	24	8.19	66.13	0.17
	24hrs	---	4	36	40	48	52			
	48hrs	4	12	56	56	60	60			
Ethanol	12hrs	8	28	84	36	48	60	2.57	17.72	0.16
	24hrs	24	40	91	91	94	98			
	48hrs	32	52	96	96	96	99			
Ethyl acetate	12hrs	---	1	4	5	4	13	14.64	63.09	0.83
	24hrs	---	5	4	5	11	23			
	48hrs	---	5	8	9	21	39			
n-Hexane	12hrs	---	1	4	5	4	23	26.49	167.68	0.84
	24hrs	---	5	4	5	11	13			
	48hrs	1	5	8	9	21	39			

(R), No. of replicates @ 25 larvae/replicate at each concentration; aLC_{50}, lethal concentration forkilling 50 per cent of thetreated larvae; bLC_{90},concentration for killing 90 per cent of the treated larvae.

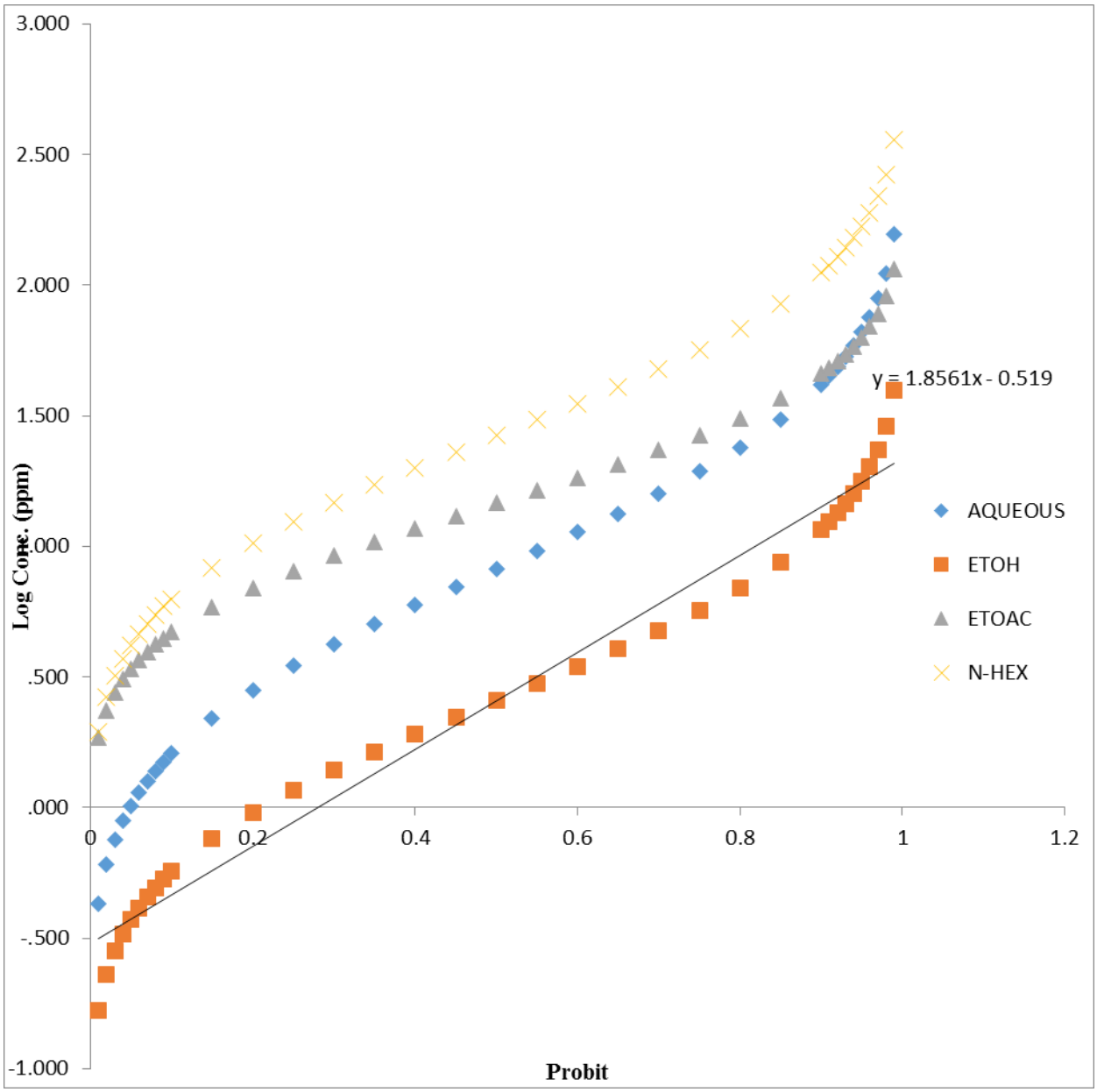


Figure 3: A plot showing the log conc (ppm) of against probit of the *Datura stramonium* Ethanol extract was used to estimate the LC50 and LC90 against mosquito larvae

4.2.2 Percentage mortality and Lethal Concentration of different Solvent extracts of *O. gratissimum* leaves against *C. quenquifaciatus* Mosquito larvae

Percentage mortality and Lethal Concentration of crude aqueous, ethanol, ethyl acetate and n-hexane extract of *O. gratissimum* leaves against *Culex quenquifaciatus* mosquito larvae is shown in Table 4.2.2. The result showed that N-hexane extract presented 100 per cent mortality at 1000ppm after 48 hours and 96% mortality at 25ppm after 24hr of treatment which has indicated to be the best solvent for extraction bio-larvicidal. The LC_{50} of the n-hexane extract was the lowest at 2.52ppm and LC_{90} of about 30.51ppm followed by Ethanol extract with LC_{50} of 3.29ppm and LC_{90} of 55.25ppm. On the other hand aqueous extract had the highest LC_{50} at 36.173ppm with very poor mortality. Hexane extract had low LC_{50} with a relatively more toxic chatactristic and was considered to be the best solvent system for the extraction of Larvicidal active principle.

Table 4.2.2: Percentage mortality and Lethal Concentration of different solvent extracts of *O. gratissimum* leave against *Culex quenquifaciatus* mosquito larvae after 12hr, 24hr and 48hr.

EXTRACT	Percent mortality (%)							Lethal conc (ppm)		
(r)	Conc. (ppm)	6.25	12.5	25.0	50.0	100	1000	LC^{a}_{50} (95% FL)	LC^{b}_{90} (95% FL)	R^2
Aqueous	12hrs	---	---	---	---	---	8	36.17	320.	0.449
	24hrs	---	---	---	20	9	13			
	48hrs	---	---	---	27	45	49			
Ethanol	12hrs	---	---	8	19	16	52	3.59	55.25	0.080
	24hrs	36	47	79	80	80	80			
	48hrs	36	75	78	80	80	80			
Ethyl acetate	12hrs	---	---	5	15	16	40	7.68^{b}	27.38	0.915
	24hrs	4	4	8	16	16	44			
	48hrs	4	4	8	16	16	44			
n-Hexane	12hrs	---	32	40	12	32	59	2.52^{c}	30.51	0.152
	24hrs	48	72	96	83	80	95			
	48hrs	64	83	99	99	95	100			

Values represent Mean±SD of N=3 and r=25 larvae. Values in the same column with different superscript across the raw differs significantly (0.05). An LC_{50}, lethal concentration for killing 50 percent of the treated larvae; bLC_{90},concentration for killing 90 percent of the treated larvae.

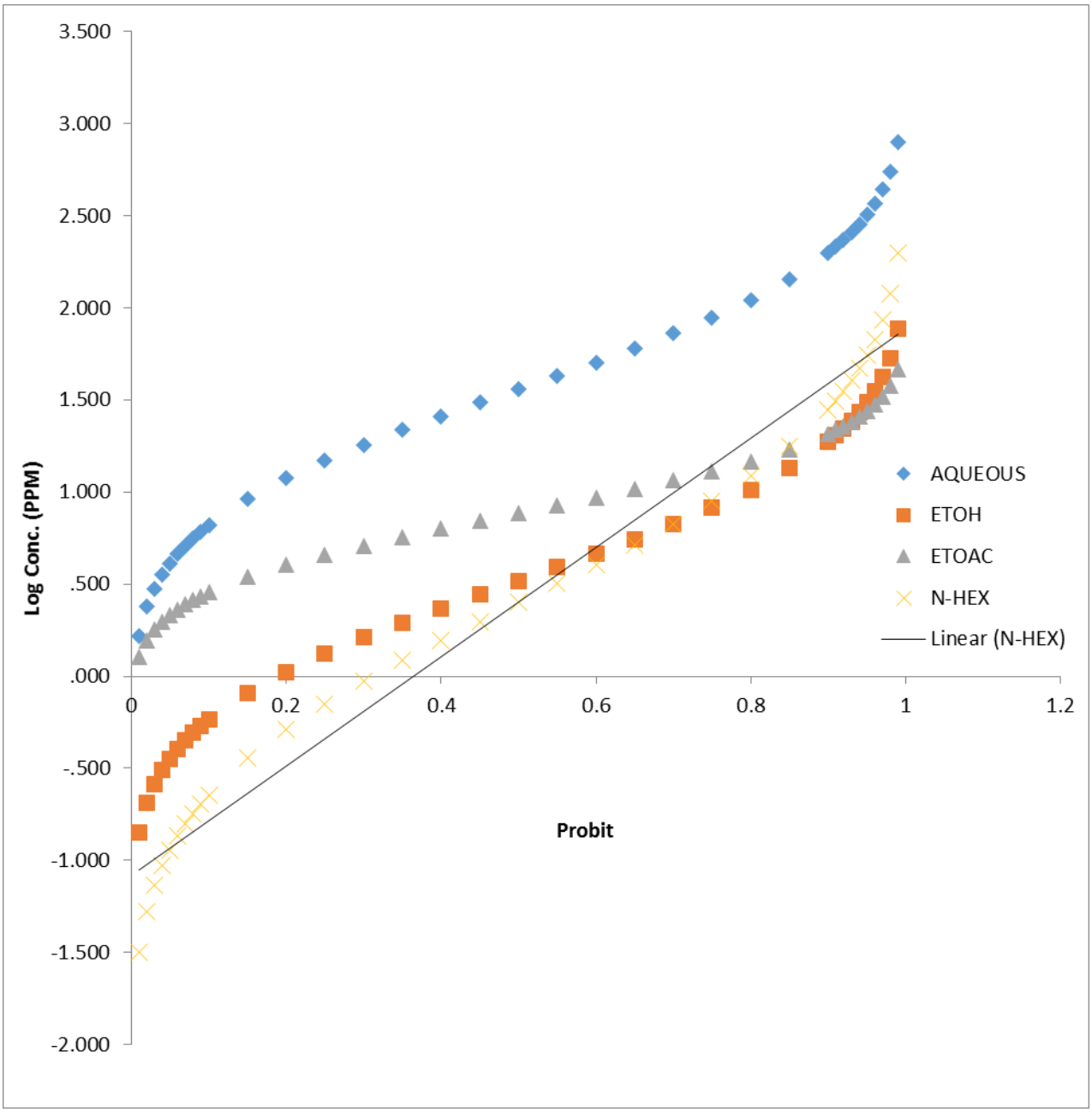


Figure 4: A plot showing the log conc (ppm) againstProbit of the *Ocimum grattissimum* solvent extract was used to estimate the LC_{50} and LC_{90} against mosquito larvae.

4.3 Partial Purification fraction of *D. Stramonium* Ethanol (DSEE) and *O. gratissimum* n-Hexane (OGnHE) by TLC and column chromatographic techniques

4.3.1 Thin Layer Chromatography (TLC)

Figure 4.1 showed that *D. stramonium* ethanolic extract expressed seven (7) bands with distinctive characteristic while *O. gratissimum* n-hexane extract expressed six (6) characteristic bands. It revealed the range of R_f value is between 0.77and 0.17 for D stramonium while the range of R_f value of O gratissimum is between 0.86 and 0.27

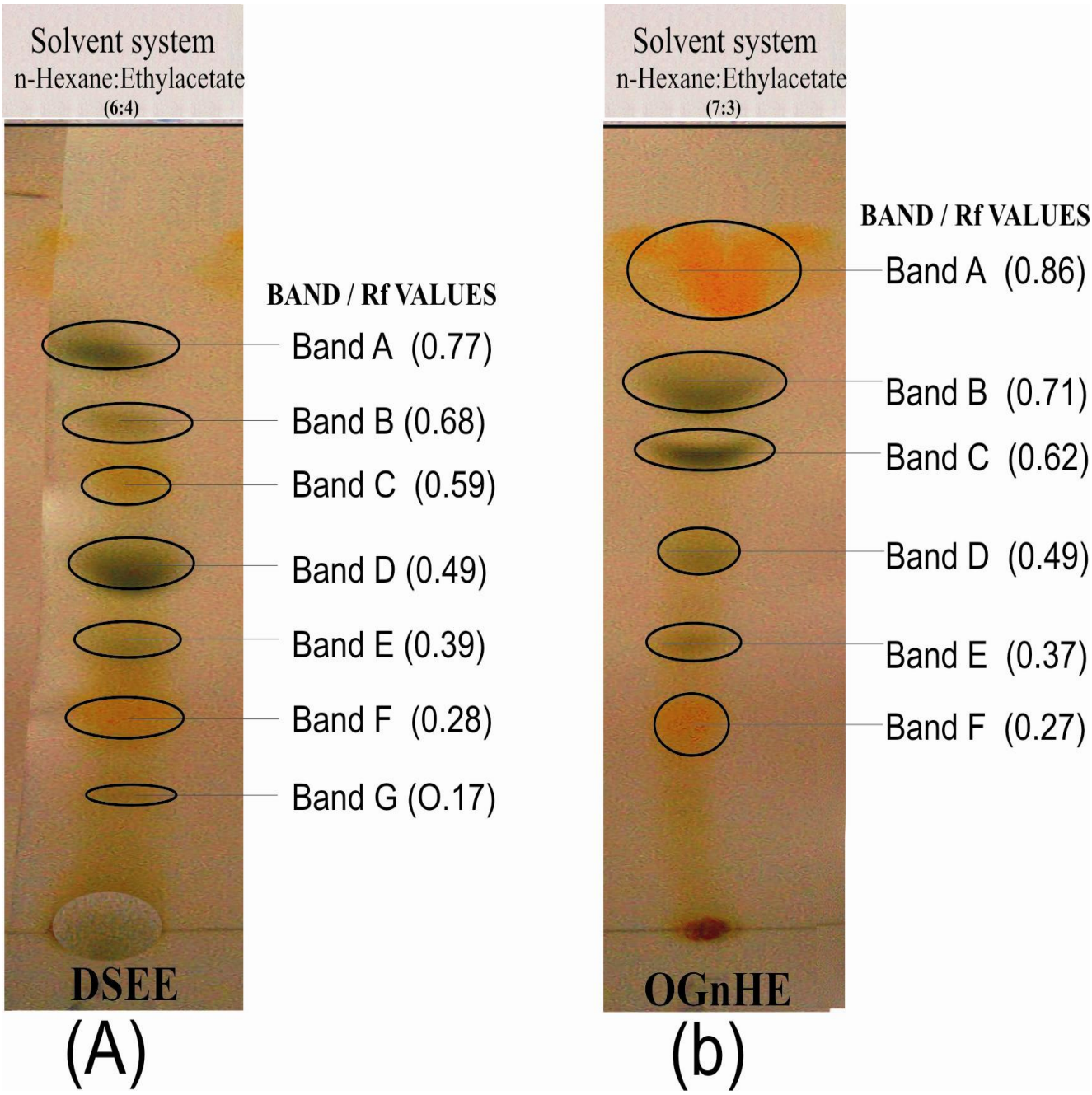
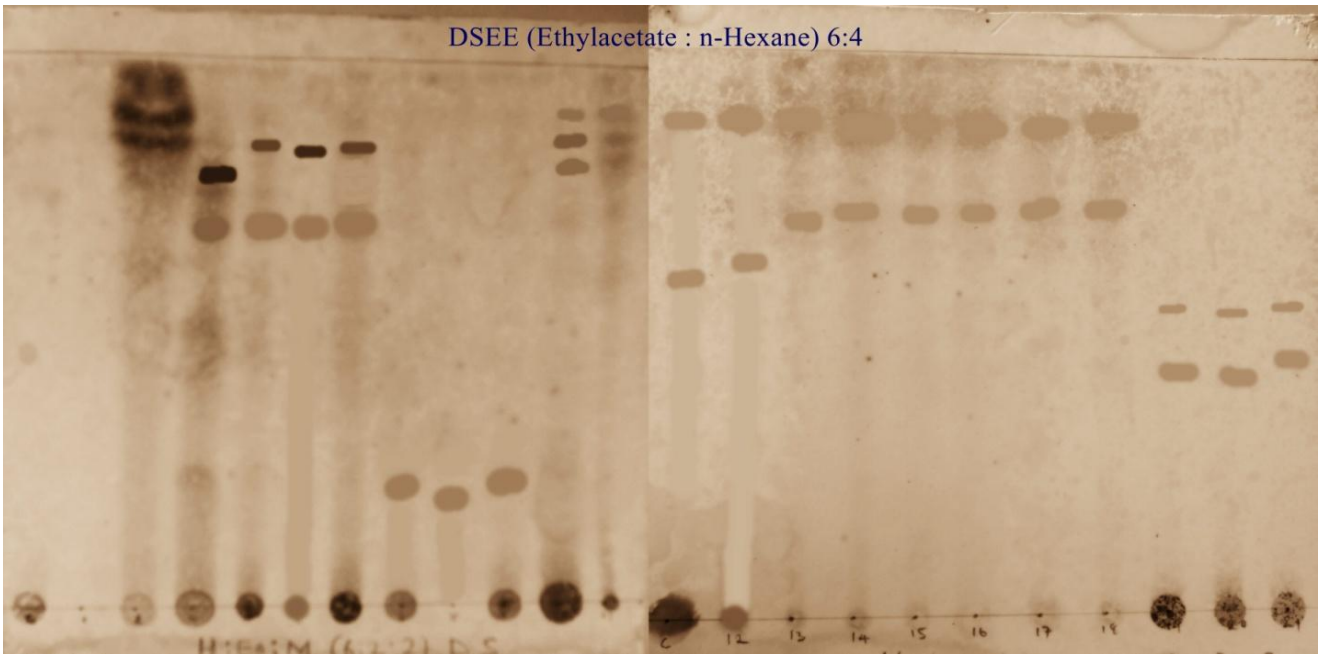
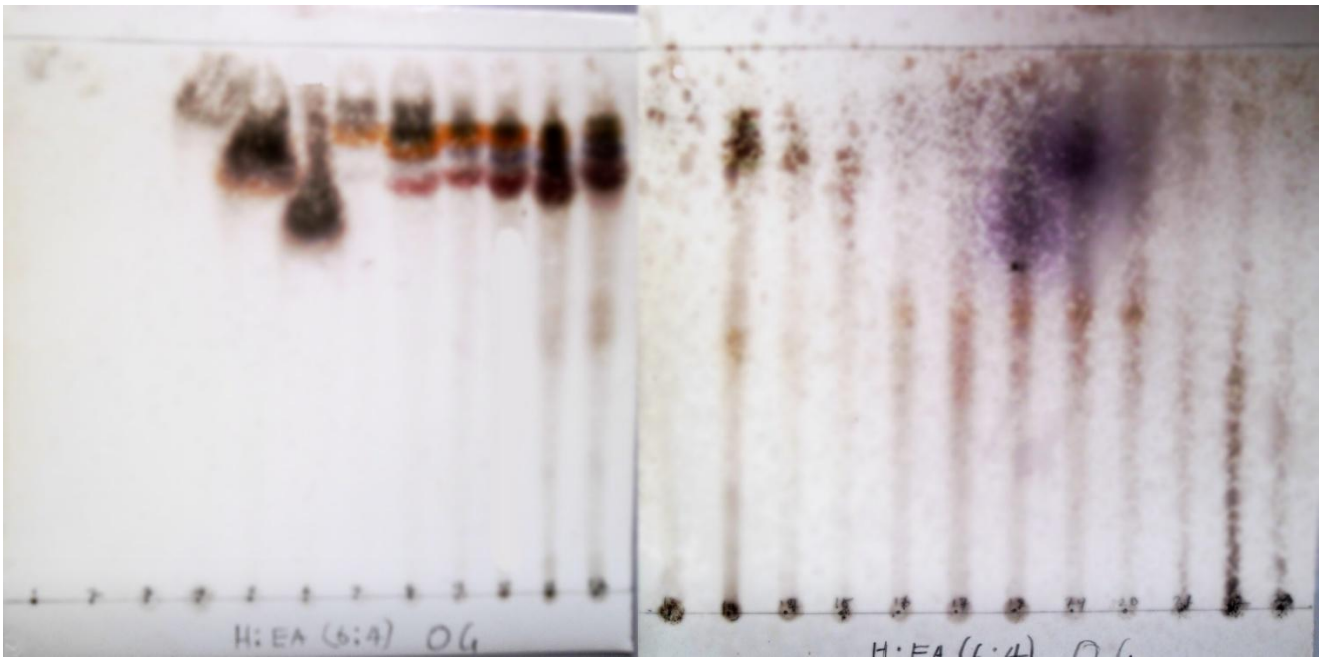


Figure 4.1: TLC separation and Retention factor (Rf) values of the various seperating bands in the DSEE and OGnHE leave using hexane:Ethylacetate (6:4 and 7:3) respectively as mobile phase, visualized with 20% sulfuric acid. (a) DSEE-*Datura stramonium* Ethanol extract (b) OGnHE-*Ocimum gratissimum*n-Hexane extract.



(a). Chromatogram of *Datura stramonium* Ethanol Extract (DSEE)- fractions



(b). Chromatogram of *Ocimum gratissimum*n-Hexane extract (OGnHE)-Fraction

Figure 4.2: Chromatogram of DSEE and OGnHE fraction profile

4.4 Phase 2 Larvicidal Bioassay of isolated fractions for DSEE and OGnHE against Culex quenquifaciatus mosquito larvae at 12hrand 24hr

Laboratory toxicity evaluation of the different pooled fractions (appendix 5 and 6) of DSEE-*Datura stramonium* Ethanol Extract and OGnHE- *Ocimum gratissimum*n-Hexane extract against *Cx. quinquefasciatus* larvae after 12hr and 24hr. Thin layer chromatography of DSEE showed seven different spots, suggesting that it could contain as many as seven different compounds while OGnHE revealed six spots, suggesting the possibility of six bioactive compounds (Figure 4.1).

4.4.1 Larvicidal activity of DSEE-*Datura stramonium* fractions against laboratory reared larvae of *Cx. Quinquefasciatus.*

The toxicity of DSEE fractions indicated that DSEE-F1 and DSEE-F2 showed percentage mortality of greater than 50%. DSEE-Fraction 1 had the maximum percentage mortality of 99% at 100ppm after 24hr against *Cx. quinquefasciatus* larvae as in Table 4.4.1 with lower LC_{50} values of 4.39ppm compared to the control. Moderate activity was found in DSEE-F3 and DSEE-F4 while the DSEE-F5, DSEE-F6 and DSEE-F7 showed very low activity even at higher concentrations. On the contrary DSEE-F5 had the least percentage mortality of 23% with $LC_{50}$and LC_{90} of 19.56ppm and 31.14ppm respectively at 100ppm after 24h.

The DSEE-F1 had the highest percentage mortality of 93% at 25ppm after 24h with LC_{50} of 4.39ppm. The maximum mortality was found at the concentration of 100 ppm at same time as above.

Table 4.4.1: Larvicidal activity of DSEE-*Datura stramonium*partially purified Fraction against *Culex quenquifaciatus* after 12hrs and 24 hours

FRACTION	Rf value	Percent mortality						Lethal concentration (ppm)	
r		Conc(ppm)	6.25	12.5	25.0	50.0	100	LC_{50}	LC_{90}
CONTROL	-	12hrs	40	68	95	100	100	2.04^{a} (0.61-3.47)	5.42 (3.27-23.80)
		24hrs	44	80	100	100	100		
DSEE-F1	0.69	12hrs	33	46	67	67	73	4.39^{b} (0.58-5.96)	6.96 (4.05-58.36)
		24hrs	38	60	93	93	99		
DSEE-F2	0.77	12hrs	13	27	27	33	47	9.02^{c} (5.30-20.49)	98.61 (27.34-122.30)
		24hrs	20	33	33	40	53		
DSEE-F3	0.83	12hrs	---	---	13	20	33	13.15^{e} (4.42-7.33)	19.391 (6.82-23.40)
		24hrs	---	---	20	33	40		
DSEE-F4	0.38	12hrs	---	6	27	30	40	11.48^{d} (4.10-7.70)	18.41 (7.98-40.02)
		24hrs	---	13	27	33	40		
DSEE-F5	0.4	12hrs	---	---	6	13	26	16.23^{f} (5.00-20.34)	21.27 (7.48-55.83)
		24hrs	---	---	10	26	33		
DSEE-F6	0.44	12hrs	---	---	3	13	27	16.16^{f} (4.99-27.73)	21.81 (7.67-63.08)
		24hrs	---	---	13	23	27		
DSEE-F7	0.54	12hrs	---	---	---	20	10	19.56^{g} (5.30-30.49)	31.14 (7.34-132.13)
		24hrs	---	---	---	27	23		

Values represent Mean±SD of N=3 and r=25 larvae. Values in the same column with different superscript across the raw differs significantly (0.05). An LC_{50}, lethal concentration for killing 50 percent of the treated larvae; bLC_{90},concentration for killing 90 percent of the treated larvae, Control=Dichorvos.

4.4.2 Larvicidal activity of OGnHE-*Ocimum gratissimum* fractions against laboratory reared larvae of *Cx. Quinquefasciatus.*

Laboratory toxicity evaluation of thecombinedfractions of OGnHE indicated that OGnHE-F3 showed maximum larvicidal activity of 100% at 50ppm after 24hr against *Cx. quinquefasciatus* larvae as shown in Table 4.4.2, with lower $LC_{50}$and LC_{90}values. Moderate activity was found in OGnHE-F4, OGnHE-F5, OGnHE-F6, OGnHE-F7 and OGnHE-F2 of (33, 33, 40, 47 and 60)% respectively while OGnHE-F1 showed the least activity even at higher concentrations of 100ppm.

The $LC_{50}$and LC_{90} values were found for OGnHE-F3 was (1.485ppm and 4.28ppm) respectively while OGnHE-F1 had the least percentage mortality of 20% with LC_{50} and LC_{90} of 17.51ppm and 83.28ppm respectively at concentration 100ppm after 24h. The other fractions; OGnHE-F4, OGnHE-F5, OGnHE-F6, OGnHE-F7 and OGnHE-F2 had LC_{50} of 7.390ppm, 4.342ppm, 5.067ppm, 5.295ppm and 3.79ppm respectively. The OGnHE-F3 had the highest percentage mortality of 100% at 50ppm after 24h and there was no significant diference between the OGnHE-F3 and the control group at $p<0.05$.

Table 4.4.2: Larvicidal activity of OGnHE-*Ocimum gratissimum* partially purified Fraction against Culex quenquifaciatus mosquito larvae after 12hrs and 24 hours

FRACTIONs	Rf value	Percent mortality						Lethal concentration (ppm)	
r		Conc.(ppm)	6.25	12.5	25.0	50.0	100	LC^{a}_{50} (95% FL)	LC^{b}_{90} (95% FL)
CONTROL	-	12hrs	40	68	95	100	100	2.04^{a} (0.61-3.47)	5.42 (3.27-23.80)
		24hrs	44	80	100	100	100		
OGnHE-F1	0.69	12hrs	---	--	7	7	13	17.51^{f} (7.6-18.10)	83.279 (17.4-331.9)
		24hrs	---	--	7	7	20		
OGnHE-F2	0.77	12hrs	---	20	20	40	46	3.79^{b} (3.20-4.86)	9.148 (6.50-92.7)
		24hrs	---	26	33	40	60		
OGnHE-F3	0.83	12hrs	20	66	87	87	100	1.49^{a} (1.03-2.89)	4.283 (3.20-7.51)
		24hrs	26	80	93	100	100		
OGnHE-F4	0.38	12hrs	---	13	20	20	23	7.39^{e} (5.0-29.31)	29.920 (12.46-97.62)
		24hrs	---	13	13	20	33		
OGnHE-F5	0.4	12hrs	---	6	20	53	27	4.34^{c} (3.43-6.74)	15.243 (8.80-64.53)
		24hrs	---	20	33	60	33		
OGnHE-F6	0.44	12hrs	---	6	20	40	40	5.07^{d} (4.07-8.05)	13.541 (8.38-48.79)
		24hrs	---	7	26	40	40		

Values represent Mean±SD of N=3 and r=25 larvae. Values in the same column with different superscript across the raw differs significantly (0.05). An LC_{50}, lethal concentration for killing 50 percent of the treated larvae; bLC_{90},concentration for killing 90 percent of the treated larvae, Control=Dichorvos.

4.5 Characterization of the Larvicidal Compound(s) in the Bioactive Fraction by FTIR and GC-MS Spectroscopy.

Table 4.5.1 Characterization of bioactive fraction DSEE and OGnHE by FTIR

The Determination of functional group(s) of Datura stramonium and Ocimum gratissimum fraction was carried out using Fourier Transform Infrared Spectroscopy, it revealed the present of six functional groups for DSEE-F1 active fraction and eight peaks for OGnHE-F3 active fraction as shown in table Table 4.5.1a and 4.5.1a.

In Table 4.5.1a, the peaks at around 442.68 and 1104.28 indicate the presence of C-X bond of weak intensity haloalkanes, flouroalkane and its derivative group present. The peak at around 1433.16 indicates C-C Aromatic compounds presence with medium intensity. The peak at around 1733.1 indicates the presence of C=O ie (Aldehydes, Saturated aliphatic compounds) bond of strong intensity group. The peak at around 3433.41 indicates the presence of O-H ie (Alcohols and Phenols) bond of weak intensity, spectrum shown in appendix 8.0.

On the other hand in Table 4.5.2a, the peaks at 728.15, 809.17, 1379.15 and 1460.16 indicates the presence of C-H ie (Aromatic, Alkyl group and Methylene) bond of strong intensity. While the peaks at 1611.58 and 1727.31 indicate the presence of C=O bond of strong intensity, Carboxylic acids derivatives, Aldehydes and Saturated Aliphatic group present. Peak 2442.09 indicates the presence of O-H (Phenol or Alcohol) bond of strong intensity, spectrum shown in Appendix 9.0.

Table 4.5.2a Fourier Transform Infrared Spectroscopy of *Datura stramonium* ethanol extract (DSEE-F1) of active larvicidal fraction against *Culex quenquifaciatus* mosquito larvae

Peak s/n	Absorption peak(cm^{-})	Intensity	Bond	Functional group
1.	442.68	19.709	C-X	Iodoalkane
2.	1104.28	65.993	C-X	Flouroalkane
3.	1433.16	70.048	C-C	Aromatics
4.	1733.1	70.32	C=O	Aldehydes, Saturated aliphatic
5.	2930.9	65.314	C-O	Alkyl
6.	3433.41	65.623	O-H	Alcohols, Phenols

DSEE-F1 = *Datura stramonium* ethanol bioactive fraction No. 1

Table 4.5.2b Fourier Transform Infrared Spectroscopy of *Ocimum gratissimum* n-hexane extract (OGnHE-F3) of active larvicidal fraction against *Culex quenquifaciatus* mosquito larvae

s/n	Absorption peak (cm^{-})	Intensity	Bond	Functional group
1.	728.15	87.656	C-H	Aromatic
2.	809.17	87.369	C-H	Aromatic
3.	1379.15	78.248	C-H	Alkyl group
4.	1460.16	73.577	C-H	Methylene
5.	1611.58	89.078	C=O	Carboxylic acids/Derivatives
6.	1727.31	71.24	C=O	Aldehyde, Saturated Aliphatic
7.	2442.09	85.547	O-H	Phenol/Alcohol

OGnHE-F3 = *O. gratssimum*n-Hexane bioactive fraction Number 3

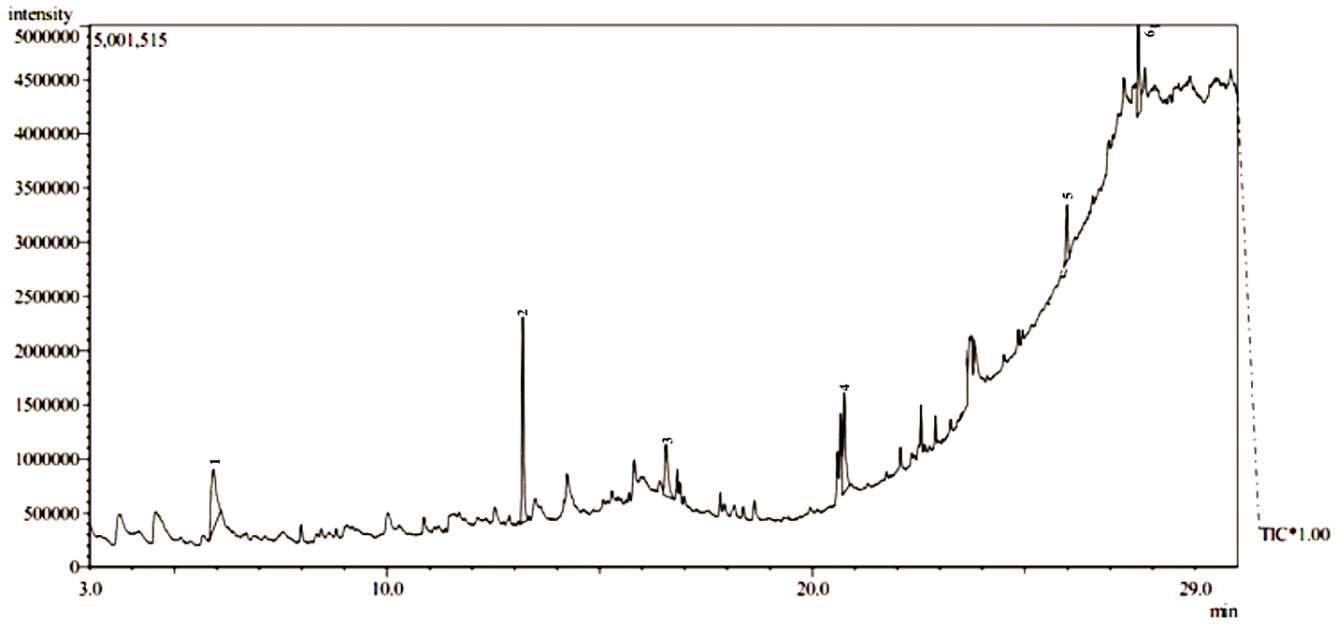
4.5.3 Gas Chromatography-Mass Spectroscopy Analysisbioactive leave fractions of *Datura stramonium* and *Ocimum gratissimum*

The chromatogram of the bioactive leave fractions of *Datura stramonium* and *Ocimum gratissimum* revealed the present of six (6) and seven (7) peaks respectively.

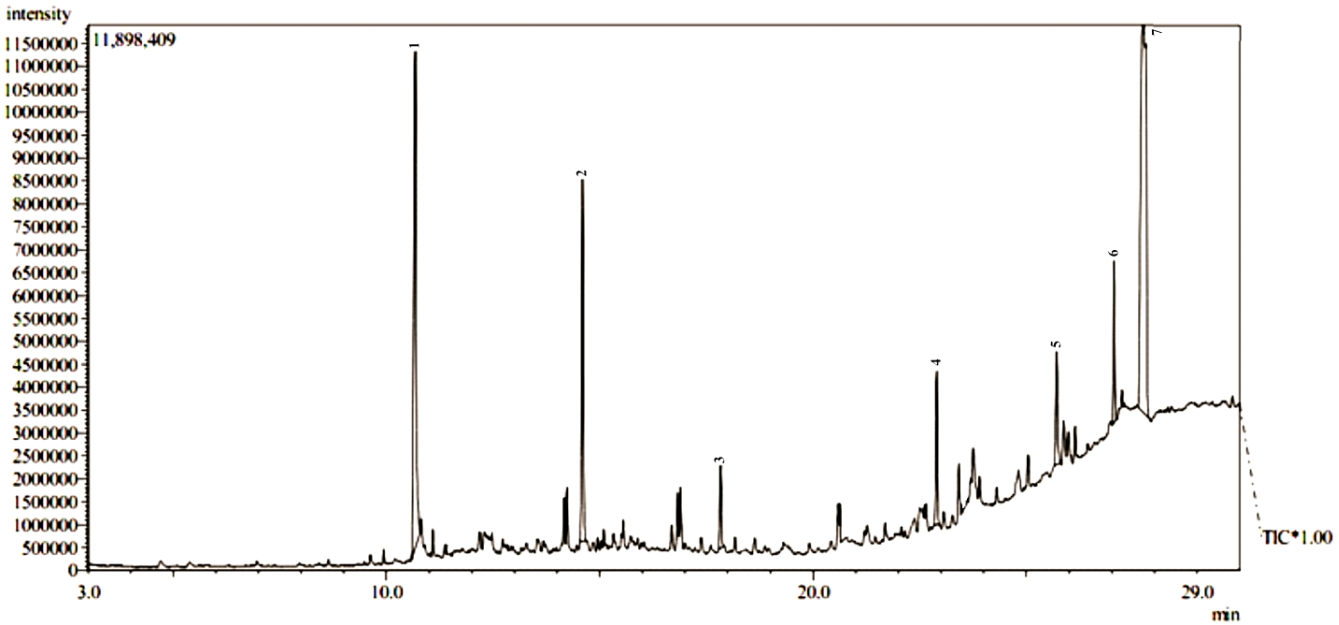
The studies on the bio-active principles in the ethanolic leave fraction 1 of *Datura stramonium* by GC-MS analysis clearly showed the presence of six compounds. The active principles with their retention time (RT), molecular formulae, molecular weight (MW), and concentration (peak area%) are presented in Table 4.5.3.1. The GC-MS chromatogram of the six peak of the compounds detected was shown in Figure 4.3. The compounds identified by the mass spectroscopy were presented with compound name and percentage peak of the individual compounds are showed as followed. The results revealed Glycerin (22.19%), 6-Pentyl-5,6-dihydro-2H-pyran-2-one (26.49%) and 2,2-Dimethylpropanoic acid, tridec-2-ynyl ester (19.16%) was found as the 3 major components in the ethanol fraction.The three minor compounds such as Hexadecanoic acid (12.21%), Heneicosane (7.59%) and Di-n-octyl phthalate (12.36%) also were revealed. In DSEE bioactive fraction 6-Pentyl-5,6-dihydro-2H-pyran-2-one and Glycerin could be the most likely aboundance compound in the bioactive fraction that is suspected to be responsible for the larvicidal activity.

While Table 4.5.3.2 rveal result of GCMS specra for OGnHE fraction number three (3), it revealed the present of seven peaks with compound name and percentage peak of the individual compounds are showed as followed. These include Thymol (24.34%), Caryophyllene oxide (10.91%) and Di-n-octyl phthalate (41.36%) was found as the 3 major components in the n-Hexane fraction of *O. gratissimum* leave. On the other hand four minor compounds such as Hexahydrofarnesyl acetone (7.67%), Stearic acid, methyl ester (4.88%), DELTA.8-Tetrahydrocannabinol (6.56%) and dl-2-Ethylhexyl chloroformate (4.28%).

In the OGnHE bioactive fraction Thymol, Caryophyllene oxide and Di-n-octyl phthalate are the most likely aboundance compound in the bioactive fraction that icould be suspected to be responsible for the larvicidal activity.



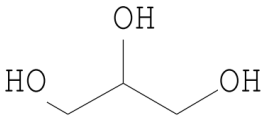
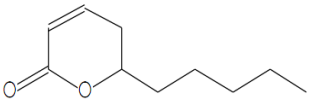
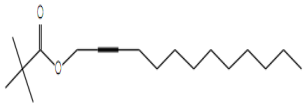
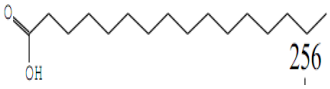
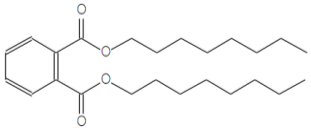
(a) Spectrum of *D. stramonium* Ethanol extract (DSEE) fraction-1



(b) Spectrum of *O. grattisimum*n-Hexane extract (OGnHE) fraction-3

Figure 4.3: GC-MS spectrum for fraction DSEE and OGnHE fraction

Table 4.5.3.1: Major Compounds present in the leave bioactive fraction of *Datura stramonium*ethanol leave extractby GCMS analysis

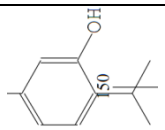
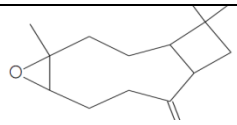
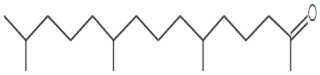
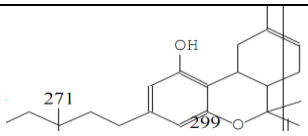
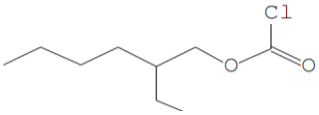
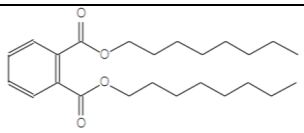
PN	RT	Compound Name	Structure	(Peak Area/SI)%	MW
1	5.917	Glycerin		22.19/96	92
2	13.192	6-Pentyl-5,6-dihydro-2H-pyran-2-one		26.49/93	168
3	16.561	2,2-Dimethylpropanoic acid, tridec-2-ynyl ester		12.21/79	280
4	20.754	Hexadecanoic acid		19.16/92	256
5	25.990	Heneicosane		7.59/89	296
6	27.667	Di-n-octyl phthalate		12.36/83	390

PN = peak number

SI = similarity index

MW = Molecular weight

Table 4.5.3.2: Major Compounds present in the leave bioactive fraction of *O. grattissimum* ethanol extract by GCMS

PN	RT	Compound Name	Structure	(Peak Area/SI)%	MW
1	10.676	Thymol		24.34/92	150
2	14.598	Caryophyllene oxide		10.91/91	220
3	17.832	Hexahydrofarnesyl acetone		7.67/95	268
4	22.899	Stearic acid, methyl ester		4.88/93	298
5	25.711	DELTA.8-Tetrahydrocannabinol		6.56/60	314
6	27.058	dl-2-Ethylhexyl chloroformate		4.28/84	192
7	27.749	Di-n-octyl phthalate		41.36/92	390

PN = peak number

SI = similarity index

MW = Molecular weight

CHAPTER FIVE

5.0 DISCUSSION

Plants produce a wide spectrum of allele-chemicals; however, many of such chemicals have not been explored for their physiological properties (Norduland and Sauls, 1981). Phytochemicals are known tospecifically inhibit growth, morphogenesis, metamorphosis andreproduction disease causing organisms (Ahmad, 2007). Alkaloids, Tanins, Flavonoids, Terpenoids and sterols were present in both plants.

Currently there is resurgence of interest in plant derived compounds for developing them commercially as ecofriendly insecticides. The results of phytochemical analysis of the leaves of *D. Stramonium* and *O. gratissimum*are presented in Table 4.1.1 using different solvent and it was observed that the extract contained phytochemicals like alkaloids, tanins, flavonoids, terpenoids, anthraquinone and steroid (Afolabi, *et al*., 2007; Olofintoye, *et al*., 2011). These compounds contained therein are substances that control cell growth and division, reduce inflammation, stimulate blood cells formation and fight infections previously reported by Ejele, *et al.*, (2012). This phytochemicals have been shown to have insecticidal property against several egg, larvae, pupae, nymph and adult of insect. The presence of alkaloids in the leaf extract (LE) *D. stramonium*is an indication that they may be useful in alleviating some of the symptoms associated with pains (Egunyomi *et al.*, 2009). Flavonoids act on the gastro-intestinal tract to increase the peristalsis action. Tannin constituent has an astringent property on mucus membrane. The presence of cardiac glycosides indicates that they may be potent in curing cardiac insufficiency, coughs and circulatory problems. In *O. Gratissimum* LE of N-Hexane reveal high percentage of sterol and Terpenoids which could responsible for the oily active ethnobotanicals for topical application as mosquito larvicide and repellents property (Egunyomi, et al 2010).

Redfern *et. al.,* 1982 and Pathak and Tiwari (2010) reported that some phytochemicals act as toxicants which generally kill different life stages of the insect while various other interfere with growth and metamorphosis.

Evaluation of the mosquito larvicidal potential of the crude Aqueous, ethanol, ethylacetate and n-hexane LE of *D. stramonium* and *O. gratissimum,* against the fourth-instar larvae mosquito (*Culex quinquefasciatus*) vector of avian malaria, lymphatic filariasis. Results from Table 4.2.1 and 4.2.2 showed that the larval mortality of the ethanol LE *D stramonium* registered significant mortality of 99% at the concentration of 50ppm after 24hr while the other solvent extract did not exceed 60% indicating no significant toxicity, This toxicity could be attributed to the presence of many chemical ingredients such as triterpenoids, alkaloid and sterol in ethanol extract these observation thus, correlates those made by Kilonzo (1991), Jackai and Oyediran (1991), and Mbailao *et al*., (2006) who found neem kernel powder to have a high toxic effect on feeding and survival of different pest species and insects under Laboratory condition. On the other hand n-Hexane LE of *O. gratissimum* caused 100% larval mortality of *Culex quinquefasciatus* at a concentration of 1000ppm after 24hr with aqueous LE with the least percentage mortality below 50% even at 1000ppm after 48hr. Similarly the development of the different intermediate stages (Larval-Pupal, Pupal-Adult) and the formation of abnormal pupae and adult are also an indication of the insect growth regulation properties of the plant extracts which were indicative of malfunctioning of endocrine system. Formation of the malformed larval-pupal intermediate is also reported to be physiological effect of Neem (Rahaman, *et. al*., 2005).

This present study showed high activity of *O*. *gratissimum,* n-hexane extract with lower LC_{50} value against late 3^{rd} early 4^{th} instar larvae of *Culex quinquefasciatus.* It showed an excellent toxicity with LC_{50} values of 2.52ppm with corresponding LC_{90} values of 30.511ppm. Acetone

extract of Ocimum sanctum were reported to have a toxic effect against the larvae of Spodoptera litura, Aedes aegypti and *Culex quinquefasciatus* (Bagavan *et. al*., 2008; Kamaraj *et. al*., 2008).

The larvicidal properties of the various fractions obtained from the column chromatographic purification of DSEE and OGnHE, it was observed that DSEE-F1and OGnHE-F3 exhibited the best larvicidal activity among the various fractions with Percentage mortality of 99% at 100ppm and 100% at 50ppm all after 24hr respectively. Hence this high Larvicidal activity of the active fractions were attributed to the presence of Glycerin, 6-Pentyl-5,6-dihydro-2H-pyran-2-one, 2,2-Dimethylpropanoic acid, tridec-2-ynyl ester, Hexadecanoic acid, Di-n-octyl phthalate and Heneicosane while in OGnHE fraction are Thymol, Caryophyllene oxide and Di-n-octyl phthalate could be the compounds responsible for the larvicidal activity. Previous studies of assessment of the larvicidal potentials of thymol derivatives on anopheles mosquitos by Jack, *et al*., (2006) and Sen-Sung, *et al*., (2008) appreciable mortality were found both in the present study mortality in the larvae was found to be in a dose dependant pattern.

The high mortalities and percent mortalities for the Glycerin, 6-Pentyl-5,6-dihydro-2H-pyran-2-one, Thymol and Di-n-octyl phthalate are indicators that they are potential larvicides.

This could be attributed partly to decreased solubility of these derivatives as the molecular mass increases. Spreading thymol derivatives over water surface obstruct the breathing of the larvae thereby suffocating them or acting as poison. Glycerin and 6-Pentyl-5, 6-dihydro-2H-pyran-2-one groups are better electron withdrawing groups which are electron releasing groups previousely reported by Jack, *et al*., (2006).

However, on further purification, the efficacy of the larvicidal active fractions was slightly enhance and increased as compared to the crude extract in a dose-dependant manner. During this study it was demonstrated that the *D. stramonium* Ethanol and *O. grattissimum* n-Hexane all of leaf extract contain Heneicosane and Caryophyllene oxide as a potent and effective larvicide

whose LD50 (2.21 and 1.49)ppm and LC90 (7.53 and 4.283)ppm where establish as a source of mosquito larvicidal compounds. The present work and detection of compounds is in consonance with the work reported by other scientists in different plant species viz., *Psoraleacory lifolia* Linn (Virendra, *et al.,* 2013, Virendra, et al., 2013, Prakash et al., 2008).

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The mosquito larvae of *Culex quinquifaciatus* was grown in laboratory, maintained at 29^0C, 60mmHg relative humidity and 12hours photoperiod until third And forth instar was obtained. The crude extracts from *Datura stramonium* and *Ocimum gratissimum* leaf using different solvent extract were tested for mosquito larvicidal activity against *Culex quinquifaciatus*. Both crude extracts and isolated fraction showed larvicidal activity. Further purification of fraction DSEE-F1 and OGnHE-F3 by prep-TLC a single Band (band A and band B) respectively. The Larvicidal activity of band A and band B were found to be 100% at 12.5ppm and 25ppm after 24hours against the third and forth instar larvae of *Culex quinquifaciatus*. The larvicidal activity of the isolated band A and B were higher than the isolated fraction; these may be probably due to removal of impurities and antagonist in the fraction. Generally the isolated compound showed best larvicidal activity with low LC50 compared with those reported from literature. The two purified bands were subjected to GC-MS analysis for structure elucidation. Single compound each was revealed in band A and band B namely Heneicosane and Caryophyllene oxide respectively. Heneicosane, (an ecospecific-toxic insect growth regulator) and Caryophyllene oxide, (a sisquiterpene with unstable structure and reactive electrophilic intermidiate) in Band A and Band B respectively could be the potent botanical larvicide for mosquito control program.

5.2 Recommendations

Results of this study suggest that this compounds; Heneicosane and Caryophyllene oxide are potential natural mosquito larvicides. Moreover, these findings could be useful in the search for more selective, biodegradable and natural larvicidal compounds or can be used as leads for the development of environmentally friendly larvicides. It should be stated that the known toxicity of Caryophyllene oxide needs to be considered for this application. The *D. stramonium* and *O. gratissimum* should be scaled up during extraction to increase the yield in order to fully exploit the phytochemicals. This will enable the structural elucidation of the active compounds whose structures were not elucidated due to low yield.

Suggestion for further research; despite heavy limitations of necessary research equipment. It is the recommendation of this work that these compounds can be investigated further for product development. Repeated bioassay coupled with analytical fractionation is needed to confirm the actual active compounds responsible for larvicidal activity of *D. stramonium* and *O. gratissimum*. More advanced purification techniques such as HPLC and ^{13}C NMR should be applied to maximize the yield and the structural confirmation. The mode of action of the active compound as well as it possible effects on non target organism should be studied before it can be practically used as natural mosquito control agent. The products of this research should be synthesized to confirm their structure and be recommended as a candidate for formulations as larvicide

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APPENDIX 1.0

Percentage yield of extracts of *D. stramonium*and *O. grattissimum*leaf following extraction with aqueous, ethanol, ethylacetate and n-hexane.

APPENDIX 2.0

CALCULATION FORMULA FOR THE CORRECTED MOTALITY

$$\text{Mortality (\%)} = \frac{\text{Number of dead larvae}}{\text{Number of larvae introduced}} * 100$$

$$\text{Corrected \% mortality} = \left(1 - \frac{\text{n in Co before treatment X n in T after treatment}}{\text{n in Co after treatment X n in T before treatment}}\right) \times 100$$

Where: n = Insect population, T = treated, Co = control

APPENDIX 4.0

APPENDIX 5.0

Combined yield of fractions with the same Rf value and combined yield as % of total extract fractionated of *D. stramonium*.

S/N	Fractions pooled	Rf values	Combined yield of fractions with same Rf values (g)	Combined fraction yield as % of total fractions collected	Combined fraction yield as % of total crude fractionated
1	7	0.69	0.15	12.93	7.98
2	8, 9, 10, 11	0.77	0.06	5.17	3.19
3	12, 13, 14	0.83	0.05	4.31	2.66
4	15, 16	0.38	0.15	12.93	7.98
5	17, 18	0.4	0.13	11.21	6.91
6	19, 20, 21, 22, 23, 24	0.44	0.23	19.83	12.23
7	25, 26, 27, 28, 29, 30, 31, 32-35	0.54	0.39	33.62	20.74
Total		-	1.16 g	100%	61.69%

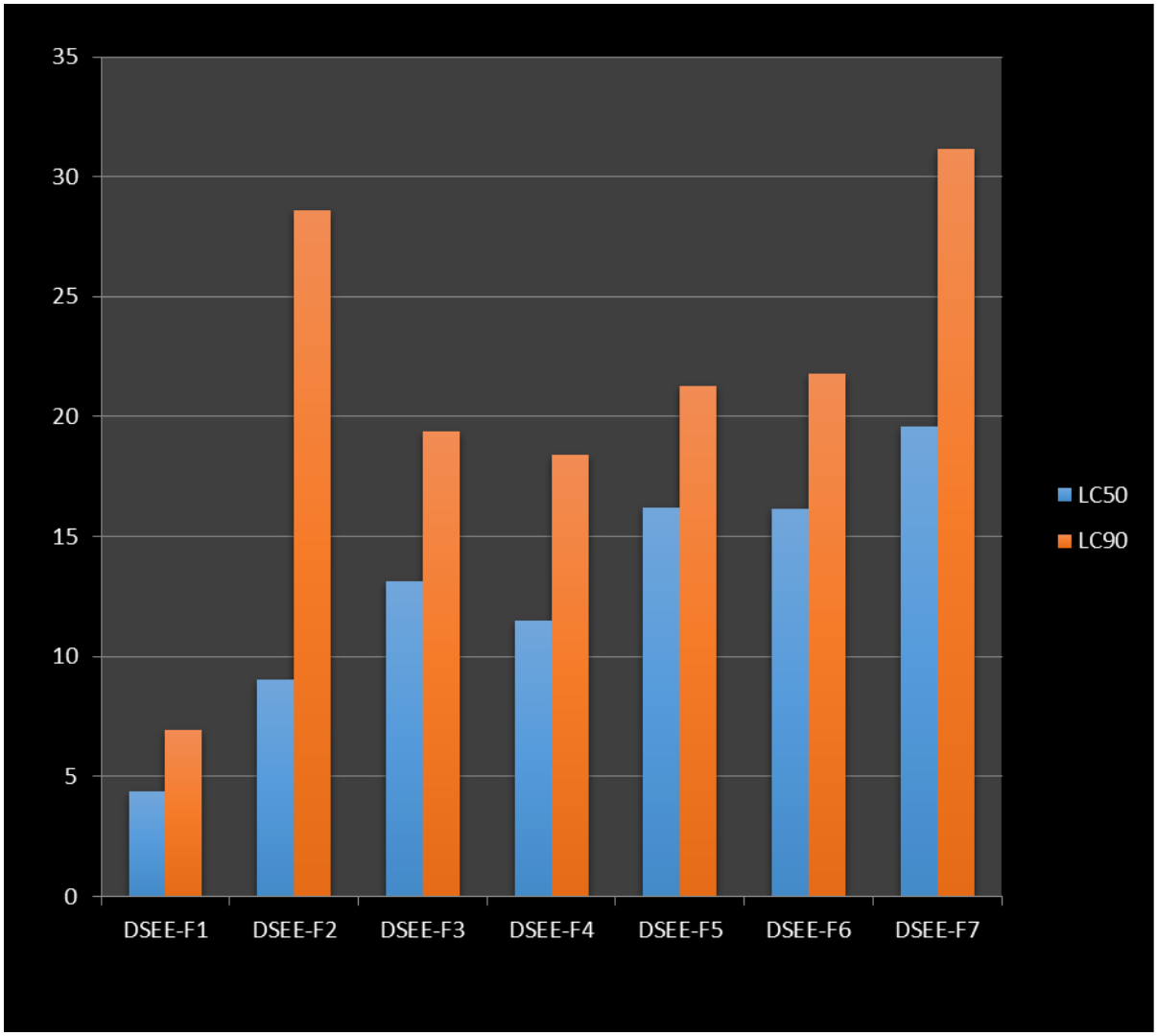
APPENDIX 6.0

Combined yield of fractions with the same Rf value and combined yield as % of total extract fractionated of *Ocimum grattissimum*.

S/N	Fractions pooled	Rf values	Combined yield of fractions with same Rf values (g)	Combined fraction yield as % of total fractions collected	Combined fraction yield as % of total crude fractionated
1	9	0.69	0.15	12.93	7.98
2	10	0.77	0.06	5.17	3.19
3	11	0.83	0.05	4.31	2.66
4	12, 13, 14, 15, 16, 17	0.38	0.15	12.93	7.98
5	18, 19, 20, 21, 22, 23	0.4	0.13	11.21	6.91
6	24, 25, 26, 27	0.44	0.23	19.83	12.23
Total			0.86g	100%	61.69%

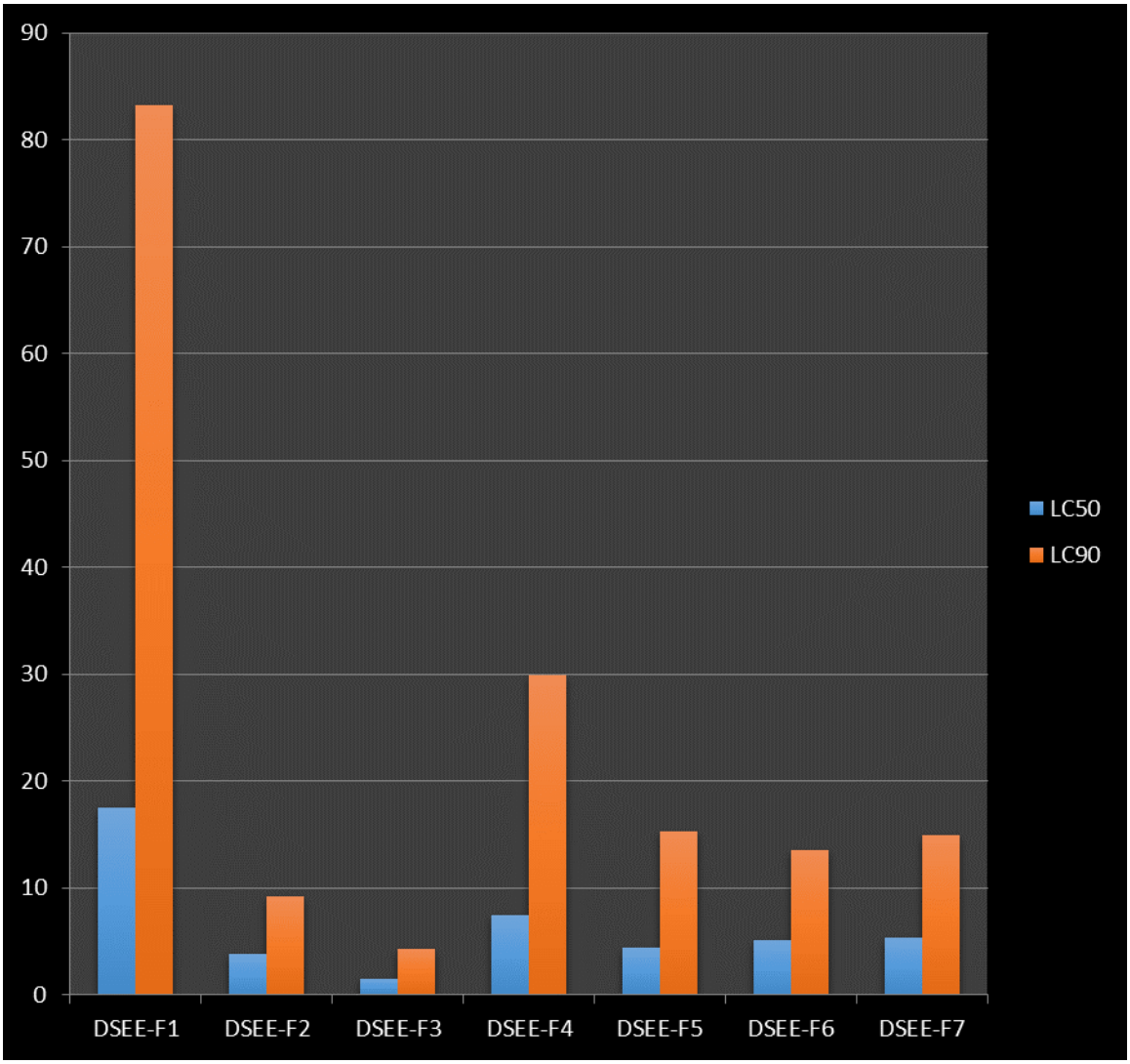
APPENDIX 7.0

A CHART SHOWING THE LOG CONC (PPM) AGAINST FRACTIONS OF D. *stramonium*WAS USED TO ESTIMATE THE LC_{50} AND LC_{90} AGAINST MOSQUITO LARVAE



APPENDIX 8.0

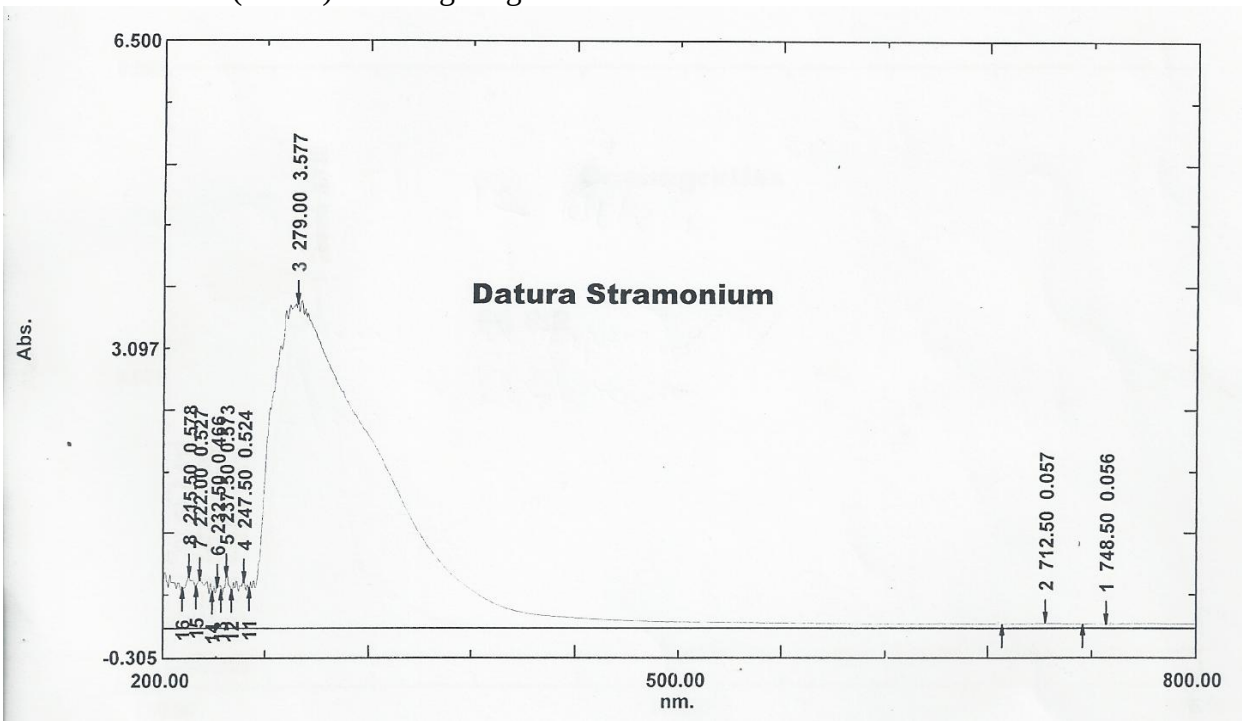
A PLOT SHOWING THE LOG CONC (PPM) AGAINST PROBIT OF THE *Ocimum grattissimum* FRACTION WAS USED TO ESTIMATE THE LC_{50} AND LC_{90} AGAINST MOSQUITO LARVAE



APPENDIX 9.0

UV-VISIBLE SPECTRUM

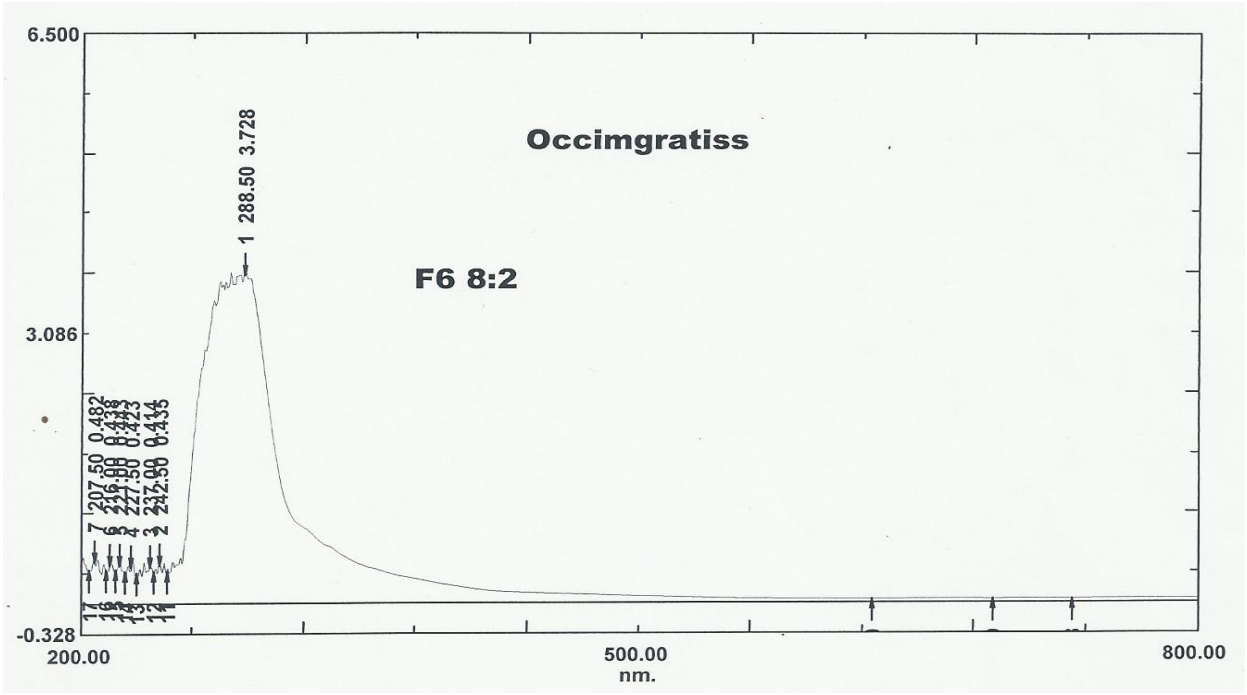
- **UV-Visible spectrum** of leaf Bioactive Fraction of *Datura stramonium* Ethanol extract (DSEE) Showing Fragments Peaks



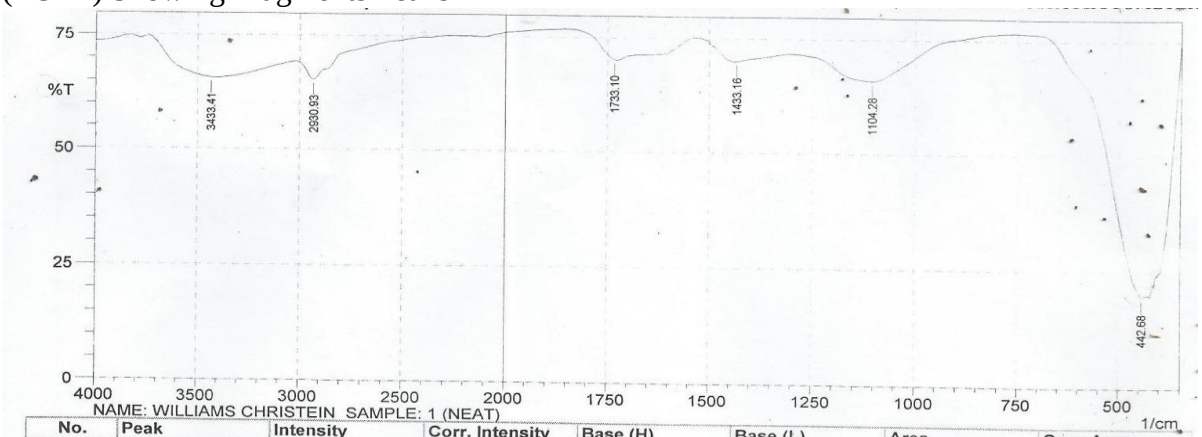
- **UV-Visible spectrum** of leaf Bioactive Fraction of *Ocimum grattisimum* n-Hexane extract (OGnHE) Showing Fragments Peaks

APPENDIX 10.0

FOURIER TRANSFORM INFRARED (FTIR) SPECTRA

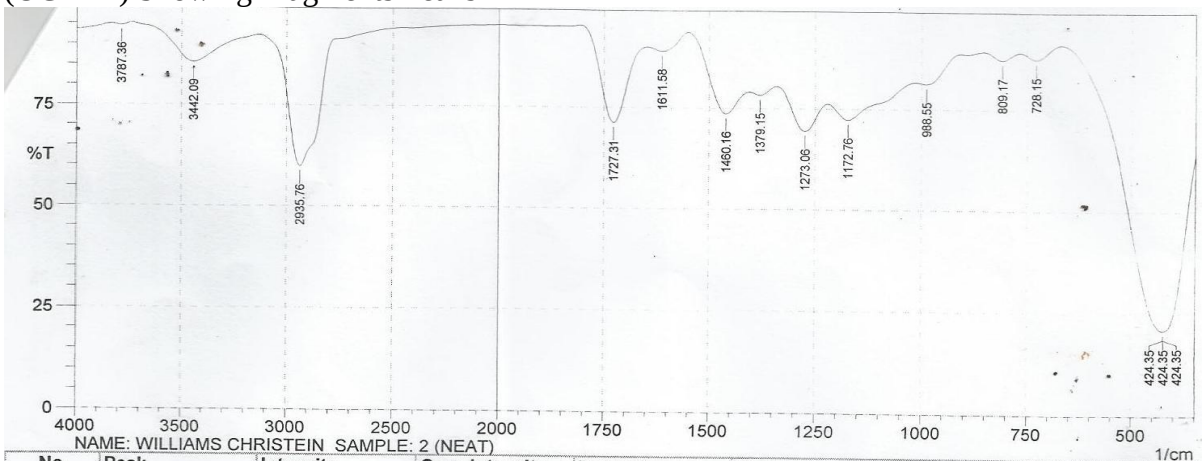


- **FTIR spectrum** of leaf Bioactive Fraction of *Datura stramonium* Ethanol extract (DSEE) Showing Fragments Peaks



No.	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	442.68	19.703	0.253	712.72	441.71	75.684	-35.441
2	1104.28	65.993	7.501	1287.53	768.66	75.933	8.638
3	1433.16	70.048	0.045	1435.09	1389.76	6.907	0
4	1733.1	70.32	0.07	1819.9	1732.13	11.581	-0.214
5	2930.93	65.314	4.727	3017.73	2411.1	89.722	2.849
6	3433.41	65.623	0.009	3516.35	3432.44	15.169	0.031

- **FTIR spectrum** of leaf Bioactive Fraction of *Ocimum grattisimum* n-Hexane extract (OGnHE) Showing Fragments Peaks

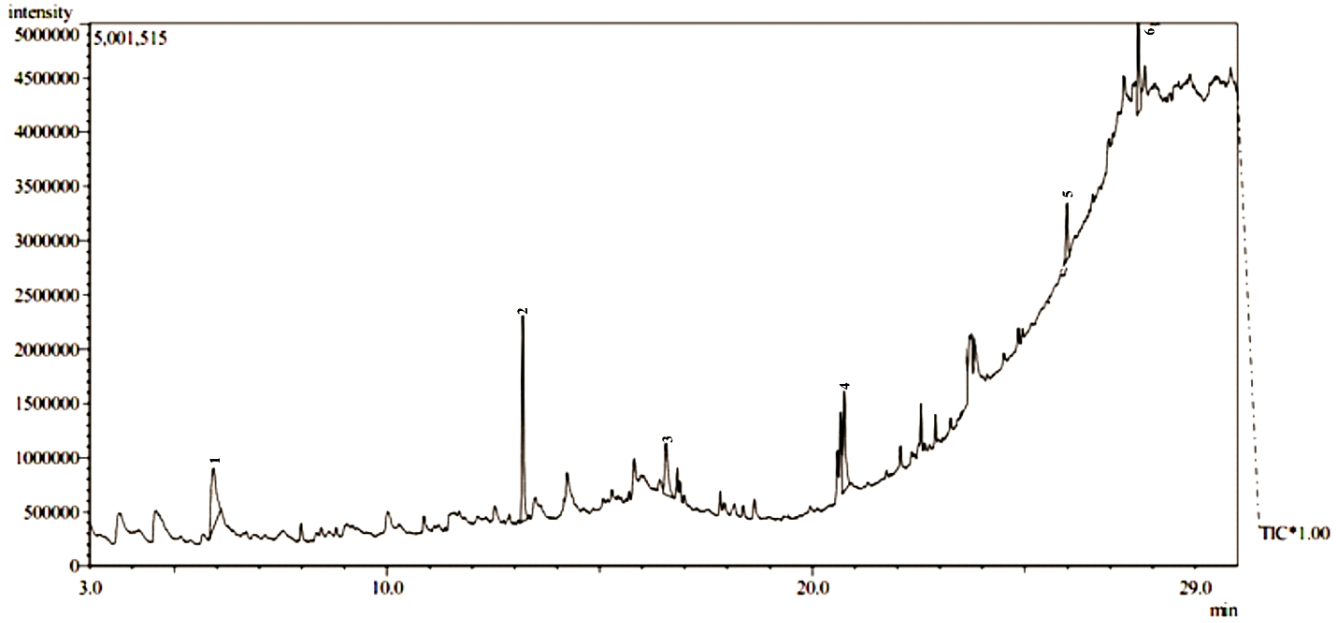


No.	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	424.35	20.811	49.201	668.36	349.13	97.7	60.05
2	424.35	20.811	49.181	669.32	349.13	97.662	59.685
3	424.35	20.811	49.181	669.32	349.13	97.662	59.685
4	728.15	87.656	2.431	766.73	669.32	4.888	0.564
5	809.17	87.369	2.018	853.53	766.73	4.638	0.41
6	3433.41	81.314	2.154	1014.59	907.54	8.005	0.576
7	1172.76	72.276	5.202	1220.98	1014.59	24.172	2.936
8	1273.06	69.337	8.715	1339.61	1220.98	15.51	2.896
9	1379.15	78.248	1.34	1405.19	1339.61	6.686	0.235
10	1460.16	73.577	11.029	1549.86	1405.19	13.26	3.824
11	1611.58	89.078	2.292	1647.26	1549.86	4.23	0.631
12	1727.31	71.24	21.456	1806.4	1647.26	12.655	7.373
13	2935.76	59.832	33.024	3130.57	2448.71	50.948	30.33
14	3442.09	85.547	8.235	3734.31	3130.57	26.443	9.45
15	3787.36	94.548	0.495	3836.54	3734.31	2.367	0.112

APPENDIX 11.0

THE GAS CHROMATOGRAPHY MASS SPECTROSCOPY SPECTRUM

- The GC-MS Spectrum of leaf Bioactive Fraction of *Datura stramonium* Ethanol extract (DSEE) Showing Fragments Peaks



- The GC-MS Spectrum of leaf Bioactive Fraction of *Ocimum grattisimum* n-Hexane extract (OGnHE)Showing Fragments Peaks