BIOCHEMICAL DETECTION OF INSECTICIDE RESISTANCE MECHANISMS IN THE SELECTED SPECIES OF MOSQUITO VECTOR

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to the University of Calicut for
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DOCTOR OF PHILOSOPHY IN ZOOLOGY

Submitted by

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CERTIFICATE

This is to certify that the thesis entitled **"BIOCHEMICAL DETECTION OF INSECTICIDE RESISTANCE MECHANISMS IN THE SELECTED SPECIES OF MOSQUITO VECTOR"** submitted to University of Calicut, in partial fulfillment of the requirements for the award of the degree of **Doctor of Philosophy in Zoology** is a record of original and independent research work carried out **Ms. Anju Viswan, K.,** Department of Zoology, University of Calicut, under my guidance and supervision. The Thesis has not formed the basis for the award of any other Degree/Diploma of this or any other University.

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DECLARATION

I do hereby declare that this thesis entitled **"BIOCHEMICAL DETECTION OF INSECTICIDE RESISTANCE MECHANISMS IN THE SELECTED SPECIES OF MOSQUITO VECTOR"** submitted to the University of Calicut in partial fulfillment for the Doctoral degree in Zoology is a bonafide research work done by me under the supervision and guidance of **Dr. E. Pushpalatha**, Assistant Professor, Department of Zoology, University of Calicut and no part of the thesis has been presented by me for the award of any other degree, diploma or similar title.

Place: **Anju Viswan, K**
Date:

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ABBREVIATIONS

ace	Acetylcholinesterase gene
AChE	Acetylcholinesterase
ACT	Artemesinin combination therapy
Alu 1	Athrobacter luteus restriction enzyme
ASCHI	Acetylcholine iodide
AS-PCR	Allele specific PCR
BHC	Benzene hexachloride
CDNB	Chlorodinitrobenzene
Cyp	Cytochrome p450 gene
DDT	Dichloro diphenyl trichloroethane
DEET	N, N-Diethyl-meta-toluamide
DHF	Dengue haemorrhagic fever
DMSO	Dimethylsulfoxide
DTNB	Dithiobis 2 nitro benzoic acid
EA	Ethyl acetate
EDTA	Ethylene diamine tetra aceticacid
est	Esterase gene
GABA	Gamma amino butyric acid
GCMS	Gas chromatography mass spectrometry
GST	Glutathione S transferase
GV	Granulosis virus
HCH	β- Hexa chloro cyclohexane
IRAC	insecticide resistance action committee
JEV	Japanese Encephalitis Virus
kdr	knock down resistance gene
LC_{50}	Lethal concentration for 50%
LC_{90}	Letahl concentration for 90%
LCMS	Liquid chromatography mass spectrometry

LFL	Lower fiducial limit
LLINs	Long lasting insecticidal nets
LT_{50}	Letahl time for 50%
MFO	Mixed function oxidase
NA	Naphthyl acetate
NFCP	National filarial control programme
NMR	Nuclear magnetic resoance
NPV	Nuclear polyhydrosis virus
NVBDCP	National vector borne disease control programme
OP	Organophosphorous
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PPM	Parts per million
RR	Resistance ratio
SDS	Sodium lauryl sulphate
TMB	$3,3^1,5,5^1$ Tetramethyl benzidine
TMS	Tetra methyl silane
UFL	Upper fiduacial limit
WHO	World health organization
WNV	West Nile Virus

1.1 INTRODUCTION

The global incidence of the outbreak of vector borne diseases is mainly contributed by mosquitoes the most important group of arthropods and can be found across all climate zones and zoogeographical regions. They are very tolerant group of insects and can withstand varied temperature and other climatic factors. From an ecological perspective, mosquitoes are integral to the aquatic and terrestrial food chain as they form the source of food for fishes, frogs and birds and other insectivores. However, for the human species, they are quite a menace even on an auditory angle. The buzzing or whining sound is quite annoying and disturbing for the ear as well as for the mind. The mosquito population thrives on the ability to reproduce and disperse with great efficiency and also on their natural ability to survive even in harsh environmental conditions.

Mosquitoes are widely regarded as a social menace in human life mainly due to their painful and annoying bites which can cause skin allergies leading to itching for several days, loss of sleep and restlessness. The bites can sometimes be fatal too as mosquitoes act as vectors for many serious diseases like malaria, chikungunya, dengue fever, filariasis, Japanese encephalitis, zika etc. There are primarily four genera of mosquitoes, namely *Anopheles*, *Culex*, *Aedes* and *Mansonia,* who contribute most to distribution of diseases among humans. The diseases spread by mosquitoes have an economic impact too due to decline in commercial and labor outputs, especially in tropical and subtropical countries. The rapid increase in mortality rates for vector-borne diseases is quite alarming. *Aedes aegypti* and *Ae. albopictus* has been identified as major vector which carries mostly viruses of serious diseases like dengue, dengue hemorrhagic fever (DHF), chikungunya, zika etc. *Culex,* the vector of filariasis and Japanese encephalitis is quite widely distributed and inflict common chronic symptoms. *Anopheles*

is the major vector of malaria in several countries of the Middle East and Indian subcontinent.

Of all the genera of mosquitoes, the Culicine mosquitoes, particularly the Filariasis vector *Culex quinquefasciatus* Say is predominantly an endophilic and endophagic mosquito. They are the primary transmitters of the two deadly diseases, Filariasis and Japanese encephalitis. *Culex* breed mostly in polluted water sources like drains, ponds, rice fields and any polluted water logged areas. They are active in the night and are identified as night biters. The adults of the species can be controlled by effective indoor spraying of residual insecticide. The effective combating of the spread of this vector species is by decimating its larval population by using a variety of larvicides in the open drains, latrines, ditches etc. and by fumigating the closed drains.

Lymphatic Filariasis, a prominent disease of the Indian subcontinent, is caused by the combination of *Wuchereria bancrofti,* the causative organism and *Cx. quinquefasciatus,* the vector. It is a major public health concern and remains a challenging socio-economic problem in many of the tropical countries and endemic in India (Udonsi, 1986). *W. bancrofti* is the most prevalent filarial nematode and is characterized by progressive debilitating swelling at the extremities, scrotum and breast of an infected individual (Myung *et al*., 1998). It is approximated that more than 100 million individuals are infected by *Cx. quinquefasciatus* annually (Rajasekariah *et al.,* 1991).

Serious efforts are being made globally to control the spread of these vector mosquitoes in order to control mosquito-borne diseases. The use of effective insecticides is a very important aspect of this effort. The four overlapping aims of mosquito control are to prevent mosquito bites, keep mosquito population at acceptable densities, minimize vertebrate contact and reduce the longevity of female mosquitoes. The successful meeting of these

four aims would mean that the harmful effect of the bites is minimized and that the pathogen transmission is interrupted. The most direct and hassle free approach to prevent the bites is personal caution and protection. It is important to avoid being outdoors during peak periods of mosquito activity. The most reliable and traditional tool in mosquito population management is habitat monitoring and modification. Regular cleaning of plant pots, surroundings, removal of tires, waste pots, bottles and kernels will prevent oviposition, hatching and larval development. Smoke repelling is also an easier method but it prevents only the bites and is not very effective in reducing the mosquito population.

Over the years, Scientists have been experimenting with different methods to control or eradicate mosquito-borne diseases extensively. Among the numerous prevention strategies tried, chemical control using insecticides appears to be the norm for both public health and household insect pest control (Yap *et al.,* 2000). The most feasible, effective, and practical method in controlling mosquito vector species is through the use of insecticides (Tikar *et al*., 2009). Insecticide, also sometimes called pesticides, is a substance used to kill insects and includes ovicides, larvicides and adulticides used against insect eggs, larvae and adults respectively. Insecticides are used in agriculture, medicine and industrial implementation. They are stated to be a major factor behind the increase in agricultural productivity of the 20th century. Nearly all insecticides have the potential to alter ecosystems quite significantly with many being toxic to humans and some even propagating along the food chain. Organochlorines, organophosphates, pyrethroids, carbamates, pyrroles and phenyl pyrazoles are the commonly used and widely recommended insecticides against adult mosquitoes (Xu *et al*., 2012). Indoor residual spraying and long lasting nets coated with pyrethroids and DEET is being used widely these days to combat the occurrence of mosquito borne diseases.

Control regimes followed world over to check vector borne diseases have become tougher as the disease causing parasites have almost become immune to drugs and mosquitoes have developed resistance to insecticides (Brown *et al*., 2001). There is a chance for developing detoxification mechanism against insecticides in mosquitoes by using chemical insecticides. The detoxification mechanism of an insect describes the decreased susceptibility of an insect population to an insecticide that was previously effective in controlling that insect. Insect species evolve resistance via natural selection. The most resistant specimens survive and pass on their genetic traits to their offspring. In fact it is said that the genetic modifications in an insect body due to extensive use of synthetic insecticides has resulted in the development of resistance in many insects (IRAC, 2007). The steady development of resistance to chemical insecticides is a matter of serious concern both on an economic as well as sanitary level (Georghiou and Taylor, 1980, Parimi *et al.,* 2003). This type of insecticide resistance results in rebounding vectorial capacity, making it an important area of investigation in environmental health (Hemingway *et al.*, 2004). The repetitive use of synthetic organic insecticides interrupted the natural biological control system thus resulting in resurgence and resistance in target population and destroyed the beneficial fauna which was not targeted. The residues from the pesticides show elevated levels of bio magnifications by infiltrating the eco system and spreading through the food chain. The repercussions of such insecticide usage brought on the need for research and development of environmentally safe and bio-degradable methods for controlling and eliminating mosquito population.

Owing to the repeated use of the common insecticides, mosquitoes developed resistance to it and have caused a number of outbreaks of mosquito borne diseases in the recent years (Hemingway *et al*., 2002, Kelvin, 2011). Mosquitoes have developed resistance over the years, to all kinds of

insecticides including biocides (Brogdon and Allister, 1998). In many countries, the breeding sites of *Cx. quinquefasciatus* have been sprayed with organophosphorus insecticides (Ketterman *et al*., 1993), thereby leading to the development of resistance to them. Temephos, an organophosphate (OP) insecticide, is recommended as a larvicide by World Health Organization to control mosquitoes, midges, blackfly larvae and other insects (WHO, 2009).

In India, Temephos has been recommended for controlling mosquito larvae and its use is patronized by the Government of India under their National Vector Borne Diseases Control Programme (NVBDCP). It is also used in several other countries like Brazil, USA, South Africa and numerous Southeast Asian countries (Laurentino *et al*., 2004). Temephos is widely used for the control of *Aedes*. However, the indiscriminate use of Temephos has led to development of resistance, in Cambodia, federal districts of Brazil, and Southeast Asia (Paeporn *et al*., 2003).

Indiscriminate and continuous use of insecticides to control the insect vectors and pests result in the genetic factors of anthropogenic selections facilitating insecticide resistance on the vectors and pests. The resistance level depends on the volume and frequency of insecticide applied on them and also to the inherent characteristics of the insect species involved. The most significant factor influencing the development of insecticide development is the life cycle of the insect and the number of progenies produced. Interestingly, mosquitoes have all the characteristics suited for rapid resistance development, including short life span with abundant progeny. The identification of resistance mechanism helps forecast the cross resistance spectrum and the choice of alternative insecticides (WHO, 1992). Thus, the identification process can be used to improve vector control programs by the resistance management option (Nazni *et al*., 1997).

The mechanism contributing to this undesirable trait of insecticide resistance is characterized into four factors mainly: increased metabolism for non-toxic compounds, decreased site sensitivity, decreased rates of insecticide penetration and the increased rates of insecticide excretion. Among these factors, the metabolic resistance is the most important one as it enhances the activities of specific enzymes, thus aiding in the detoxification and degradation of insecticides. The major groups of enzymes involved in insecticide metabolism are esterases, monooxygenases and glutathione-S-transferases (Hemingway *et al.*, 2004). The metabolic enzymes provide resistance in two major ways: over production of the enzymes which consequently can lead to increased metabolism or sequestration and also by altering the catalytic center activity of the enzyme, thereby increasing the rate of insecticide metabolism by the enzyme unit. These mechanisms acting individually or in combination confer resistance towards the different classes of insecticides at an extremely high level in most cases. However, many insecticides like DDT and permethrin influence behavioral changes in the insect like reducing the rate of mosquito entry into the house, increasing the rate of early exit and inducing a shift in bite timings (Lines *et al*., 1987, Miller *et al.,* 1991, Mbogo *et al*., 1996, Mathenge *et al.,* 2001). For reducing the rate of insecticide penetration, some mosquitoes have also evolved thicker or altered cuticles (Stone and Brown, 1969, Apperson and Georghiou, 1975).

The target sites differ with each different type of insecticide. For pyrethroids and DDT, the target sites involved are sodium channels, for organophosphates and carbamates, it is acetylcholinesterases while for the HCH gamma cyclodienes, it is the GABA receptor. The resistance mechanism against pyrethroid and DDT is known as knockdown resistance or kdr and the same has been linked to mutations in the para-type sodium channel genes. This type of resistance has been in several insect pests and vectors (Soderlund and Knipple, 2003). Besides knockdown resistance, metabolic resistance

mechanism is also present in pyrethroid / DDT resistant mosquitoes. Elevated levels of monooxygenases, esterases and glutathione -S- transferases may enhance the insecticide tolerance in the different mosquito populations (Brogdon *et al*., 1999, Ranson *et al*., 2000).

The detoxification of several organophosphorus pesticides in the insect body is carried out by Carboxyl esterase. Increased rates of Carboxyl esterase activity is observed in insects resistant to organophosphorus insecticides (Hemingway and Georghiou, 1984, Fournier *et al*., 1987, Mouches *et al*., 1987). All esterases have a similar role in resistance attribution, i.e., rapid insecticide binding and slow release of the metabolites (Karunaratne *et al.,* 1993 and 1995). Resistance to organophosphorus insecticides in the mosquito *Cx. quinquefasciatus,* the major vector of filariasis, is associated with co-amplification of the *est*a21 and *est*b21 esterase genes in more than 90% of its population world-wide (Hemingway and Karunaratne 1998).

Glutathione -S- transferases (GSTs), also called as phase II detoxification enzymes, are a large group of multifunctional enzymes present mostly in aerobic organisms, plants, and animals. The primary role of these enzymes is to detoxify hydrophobic toxicants such as drugs, herbicides and insecticides by catalyzing the nucleophilic attack of the tripeptide glutathione (GSH) on the electrophilic center of substrates (Armstrong, 1991). As a result of the reaction they render the insecticide as a water soluble product and thus more readily excretable from the cell than the non-GSH conjugated substrates (Habig *et al*., 1974). Besides the detoxification aspect, GSTs also play a role in a wide range of biological processes like protection of the cells from the harmful effects of oxidative stress and is also involved in various biosynthetic pathways (Wilce and Parker, 1994). During intracellular storage and transport, some of the GSTs non-catalytically bind to a wide range of endogenous and exogenous ligands (Dulhunty *et al*., 2001, Lo Bello *et al*.,

2001). GSTs have the capacity to bind with hydrophobic compounds which are not actually their substrates and are associated with sequestration, storage and transportation of drugs, hormones and other metabolites such as fatty acids, bilirubin and heme (Hayes and Pulford, 1995). The interest in finding out the role of GSTs in insecticide resistance has led to the discovery of the molecular basis and mechanism of insecticide resistance developed by GSTs (Hemingway *et al*., 2004, Enayati *et al*., 2005).

As on the date, six classes of insect GSTs have been identified, which are delta, epsilon, omega, sigma, theta, and zeta classes (Tang and Tu, 1994, Ortelli *et al*., 2003). The delta and epsilon classes are found exclusively in insects and are the major GST classes in insects. Members of both classes have been implicated in resistance to organophosphates, organochlorines and pyrethroid insecticides (Fournier *et al*., 1992, Grant and Hammock, 1992, Kostaropoulos *et al*., 2001, Ranson *et al*., 2004).

Cytochrome P450-dependent monooxygenases or mixed function oxidases are an important and diverse family of hydrophobic, heme containing enzymes involved in the metabolism of numerous endogenous and exogenous compounds. They mainly act in the detoxification of the substrate, though the activation of OP insecticides from the phosphorothionate to the more toxic oxon form is a notable exception. There are several reports demonstrating the elevated P450 monooxygenase activities in insecticide-resistant mosquitoes, frequently in conjunction with altered activities of other enzymes. Vulule *et al.*, 1999 reported the elevated oxidase and esterase levels in permethrin-resistant *An. gambiae* from Kenya. Brogdon *et al*., 1999 demonstrated an oxidase-based and esterase-based resistance mechanism alone and in combination in permethrin-resistant *An. albimanus* from Guatemala.

Acetylcholinesterase, responsible for neurotransmitter degradation at the cholinergic nerve synapse, is the target of both organophosphate and carbamate insecticides. Selection of modified AChE less sensitive to these insecticides has been shown to be a common resistance mechanism, and has been observed in numerous arthropod pest species. In the mosquito gene, *ace*-1, it encodes the synaptic AChE1 responsible for insensitivity to insecticides (Weill *et al*., 2003).

The scope of the current study aims to assess and analyze the resistance status of field populations of *Cx. quinquefasciatus* against the organophosphorus insecticide temephos of the Calicut, Cochin, Malappuram, Thrissur and Palakkad town areas of Kerala. Temephos is an organophosphate (OP) insecticide, recommended as a larvicide by the World Health Organization (WHO) to control mosquitoes, midges, blackfly, and other infesting insects (WHO, 2009). Studies have shown temephos to be effective in controlling *Ae. aegypti* in several parts of India (Katyal *et al*., 2001, Mukhopadhyay *et al*., 2006). Shetty *et al*., 2013 and Kumar *et al*., 2014 reported different populations of *Ae. aegypti* Bangalore and Mumbai showing a high tolerance to temephos, when compared with the WHO recommended diagnostic dose.

OBJECTIVES OF THE STUDY

1. To find out the resistance/susceptibility status of field populations of *Culex quinquefasciatus* Say larvae collected from Cochin Municipal Corporation, Kozhikode Corporation, Palakkad Municipality, Ponnani Municipality (Malappuram) and Thrissur Municipal Corporaion against the organophosphorous larvicide temephos.

2. To analyze the detoxification enzymes (carboxylesterase, glutathione - S - transferase, mixed function oxidase) and acetylcholinesterase enzyme levels in the selected field and laboratory population of *Cx. quinquefasciatus* in the three consecutive years 2014, 15 and 16 by biochemical assays.

3. To determine the susceptibility/resistance status of field collected *Cx.quinquefasciatus* against malathion (5%), cyfluthrin (0.15%), deltamethrin (0.05%) using WHO susceptibility kit.

4. To identify the presence/absence of site specific mutations in the *ace*1 gene and *kdr* gene of the selected field collected *Cx. quinquefasciatus.*

1.2. REVIEW OF LITERATURE

The development of resistance to different classes of insecticides is a serious threat to vector control programmes in India and in global scenario and is well documented. Reports reveal that in many cases insects have become tolerant to more than one insecticide group that chemical control has becom e more difficult. Resistance is known to affect all major vector species against the four recommended classes of insecticides. Since 2010, a total of 61 countries have reported resistance to at least one class of insecticide, with a 50 of those countries reporting resistance to 2 or more classes. The major problem of development of resistance is due to lack of adequate mechanisms for routine monitoring of insects and vector control regimes followed world over (WHO, 2017).

Cx. quinquefasciatus (originally named *Cx. fatigans*), commonly known as the Southern house mosquito, is a medium-sized mosquito belonging to the *Cx. pipiens* species complex. *Cx. quinquefasciatus* was described in 1823 by Thomas Say from a specimen collected in Southern US. Recent studies indicate that this species was originated in Southeast Asia and then spread to the New World through slave ships and colonized Africa (Fonseca *et al.*, 2006). It is the principal vector of Bancroftian filariasis and also acts as the vector of avian malaria and several arboviruses like St. Louis Encephalitis Virus, West Nile virus etc. (Sardelis *et al*., 2001, Turell *et al*., 2005, Arensburger *et al*., 2010, Sarkar *et al*., 2011). It has a high reproductive potential and shows ecological plasticity owing to its adaptability to diverse ecological niches (Bhattacharya *et al*., 2016).

The females of *Culex* contribute a single egg raft averaging 150-200 eggs. Development of immature stages during the life cycle is dependent on temperature, nutrition and population density. The development of the larvae to the adult stage takes around 10 – 14 days and can even be as short as 7 days

under optimal conditions (Reuda *et al.*, 1990). This species breeds in specific environments like mildly to highly polluted, organically rich water in open drains, flooded latrines, pit latrines, septic tanks and polluted water collected in pits or containers (Weinstein *et al.*, 1997, Laird, 1995).

The southern house mosquitoes are highly anthropophagic with 50-76% of them feeding on human beings and impacts hosts both indoors and outdoors (Reuben *et al.*, 1992). The females of the species also feed on amphibians, pigs, horses, cattle, sheep and birds (Bhattacharya *et al.*, 1982, Holder *et al.*, 1999). *Cx. quinquefasciatus* is considered as an important invasive species that has significant impact on native species or ecosystem as well as on human and vertebrate animal health (Juliano and Lounibos, 2005). This invasive species has the plasticity to adapt to diverse habitats. This ecological plasticity might be due to its amplified immunity gene repertoire (Bartholomy *et al.,* 2010).

Lymphatic filariasis (LF) is a major public health problem in India. India contributes to 41% of the global disease burden (Agarwal and Sashindran, 2006). In India, Lymphatic filariasis was recorded as early as 6th century BC by the famous Indian physician Susruta in his book, 'Susruta Samhita' (Bhaskar *et al.*, 2000). The description of the signs and symptoms of the disease has been well documented by Madhavakara (7th century AD) in his treatise 'Madhava Nidhana'. It proves that the disease was well studied by our ancestors (Sabesan *et al.*, 2010). Two types of Lymphatic filariasis are seen in India. Among the two, the infection by *Wuchereria bancrofti* (Bancroftian filariasis) is more common and that caused by *Brugia malayi* (brugian filariasis) is very rare. In the mainland, *W. bancrofti* transmitted by *Cx. quinquefasciatus* contributes to 99.4% of the infection (Agarwal and Sashindran, 2006).

According to the district-level endemicity map created in 2000, by a survey covering 289 districts across India, as many as 257 were found to be endemic for Lymphatic filariasis. Altogether seventeen states and six union territories were found to be endemic with about 553 million people exposed to the risk of infection. Kerala ranks second in endemicity preceded only by Bihar (over 17%) (NVBDCP, 2010). Out of the 14 districts in Kerala, 11 coastal districts (Thiruvananthapuram, Kollam, Kottayam, Alappuzha, Cochin, Trissur, Palakkad, Malappuram, Kozhikode, Kannur and Kasargod) are endemic for this disease (Sabesan *et al.*, 2000).

Vector control plays an important role in the eradication or control of the disease. The National Filaria Control Programme (NFCP), launched in 1955, insisted on providing due importance to vector control by anti-larval measures in urban areas, and indoor residual spraying in rural areas. The assessment of NFCP in 1960 revealed ineffectiveness of indoor spray of insecticides, due to high resistance in the insect vectors (Agarwal and Sashindran, 2006). However, the continuous application of synthetic insecticides renders development of resistance in target insects. Insecticide resistance is defined as "an inherited characterization that allows the development of ability in some individuals of a given organism to tolerate doses of a toxicant which would prove lethal to a majority of individuals in a normal population of the same species" (WHO, 2008).

The development of resistance in vector species, and emergence of new resistant genotypes among the vector population has been a major setback in the global efforts to control vector-borne diseases (Hemingway and Ranson, 2000). According to the World Health Organization, insecticide resistance is "the biggest single obstacle in the struggle against vector borne diseases" (WHO, 1976). Resistance has gradually developed to every chemical class of insecticide, including microbial drugs and insect growth

regulators (Brogdon and McAllister, 1998). In the last several decades, many species of insects have acquired resistance to insecticides. This resistance is inherited and has proved to be the biggest single barrier to successful control of insects using chemicals. Organochlorides, organophosphates, carbamates and pyrethroids are the major groups of insecticides used widely.

There are four possible types of resistance mechanisms to the main insecticide groups in almost all insects which are increased metabolism to non-toxic products, decreased target site sensitivity, decreased rates of insecticide penetration and increased rates of insecticide excretion.

Many studies have shown that multiple, complex resistance mechanisms, particularly increased metabolic detoxification of insecticides and decreased sensitivity of the target proteins or genes are responsible for insecticide resistance. Gene over-expression and amplification, and mutations in protein-coding gene regions, has also frequently been implicated (Pasteur and Raymond, 1996, Liu, 2015). Insecticide resistance mechanisms have a biochemical foundation. The two major biochemical resistance mechanisms are target site resistance, wherein the insecticide no longer binds to its target site, and detoxification enzyme based resistance where enhanced levels or modified activities of esterases, oxidases, or glutathione-S-transferases prevent the insecticide from reaching its site of action. Thermal stress response is another type of resistance mechanism but its importance has not yet been assessed fully (Patil *et al*., 1996).

Resistance based on detoxification enzymes occurs when enhanced levels or modified activities of detoxification enzymes like esterases, oxidases, or glutathione-s-transferases (GST) prevent the insecticide from reaching its site of action and target-site resistance occurs when the insecticide no longer binds to its target (Brogdon and McAllister, 1998). Target-site resistance mechanisms include knockdown resistance (Soderlund

and Knipple, 2003), known as kdr, caused by alterations in the sodium channel, which is the target site of DDT and pyrethroids, and the insensitivity of acetylcholinesterase (Hemingway and Georghiou, 1983) which is the target of organophosphate(OP) and carbamate insecticides.

Alterations of amino acids responsible for insecticide binding at the site of action cause the insecticide to be less effective or even ineffective of its action. The target of organophosphorous (temephos, malathion, fenitrothion) and carbamate (propoxur, sevin) insecticides is acetylcholine esterase in nerve synapses, and target of organochlorines (DDT) and synthetic pyrethroids are the sodium channels of the nerve sheath.

Mosquitoes that have been under continued selection pressure with one or a range of different insecticides may develop cross or multiple resistance. Cross resistance means that the strain is not only resistant to one insecticide of a particular class (of a given mode of action) but also (often to a lesser degree) to other insecticides in the same class (with similar mode of action) even when it has never been exposed to the other insecticides. DDT- pyrethroid cross- resistance by single amino acid changes (one or both of two known sites) in the axonal sodium channel insecticide binding site is reported by Miyazaki *et al*., 1996. This cross-resistance leads to shift in the sodium current activation curve and cause low sensitivity to pyrethroids (Vais *et al*., 1997). Reduced sensitivity to OP and carbamate insecticides is due to point mutations of acetylcholinesterase (AChE) - insecticide binding site. The phenomenon of multiple resistances is considerably more important due to the separate detoxification mechanisms for unrelated insecticides, which results in the evolution of an insect population resistant to different classes of insecticides with dissimilar mode of action. This in turn makes the chemical control of that population extremely difficult.

A carbamate is an organic compound derived from carbamic acid (NH_2COOH). Carbamate insecticides contain the carbamate ester functional group. Examples of carbamates include aldicarb (Temik), carbofuran (Furadan), carbaryl (Sevin), ethienocarb, fenobucarb, oxamyl and methomyl. Carbamates act by reversibly inhibiting the acetylcholinesterase enzyme in the nerve synapses (Fukuto, 1990). Organophosphates are esters of phosphoric acid. They act on the cholinesterase enzyme through irreversible covalent inhibition (Peter *et al*., 2014).

Organophosphorous and carbamate insecticides target the acetylcholinesterase in nerve synapses. Mosquitoes show widespread and strong resistance to organophosphates (Liu, 2015). Coto *et al*., (2000) demonstrated that the use of OPs for the control of *Ae. aegypti* results in the development of high level resistance in *Culex* populations,while the target species remained susceptible to the insecticide. The esterase (cholinesterase and carboxylesterase) constitute a large group of enzymes in insects and they are involved in the resistance mechanism towards organophosphates and carbamates insecticides. The enzyme can be studied by their action on substrate (α- naphthyl acetate) which is split in to an ester (α-naphthol) and an acid. The ester may be coupled with a variety of diazonium salts to form a highly stable diazoate blue complex. The activity of esterases can be measured by the insoluble dyes spectrophotometrically.

There are two genes that encode different isoforms of acetylcholine esterase in mosquito's namely *ace*-1 and *ace*-2. At least 5 point mutations in the acetylcholine esterase insecticide binding site have been identified, which singly or in combination, cause varied degrees of reduced sensitivity to OPs and carbamate insecticides (Mutero *et al.*, 1994). Three different amino acid substitutions have been identified so far in the *ace*-1 gene, resulting in reduced sensitivity to OP and carbamate insecticides owing to a single

mutation in the gene: G119S in *An. gambiae, Cx. quinquefasciatus, Cx. vishnui* and *Cx. pipiens*, F290V in *Cx. pipiens* and F331W in *Cx. tritaeniorhynchus* (Nabeshima *et al.,* 2004, Weill *et al*., 2004, Alout and Berthomieu, 2007, Alout and Labb´e, 2009). Of these the most important and most studied is the G119S mutation (resulting from a single point mutation GGC to AGC in the gene *ace*-1) in *Cx. pipiens* and *An. gambiae* (Weill *et al*., 2003).

Over-production of non-specific carboxylesterases as an evolutionary response to organophosphorus and carbamate insecticide selection pressure has been documented in numerous arthropod species including mosquitoes. The most widely studied mosquito species demonstrating this resistance mechanism are members of the *Culex* genus, including *Cx. pipiens, Cx. quinquefasciatus and Cx. tritaeniorhynchus* (Hemingway and Karunaratne, 1998).

Two gene loci, *est*-2 and *est*-3, have genes that produce resistance by the over production of esterases owing to the amplification or the transcriptional up-regulation of the genes (Raymond *et al*., 1998). Esterase gene amplification is well documented in resistant strains of the mosquitoes *Cx. quinquefasciatus, Cx. pipiens, Cx. tarsalis* and *Cx. tritaeniorhynchus* (Mouches *et al.,* 1986, Karunaratne *et al.,* 1998, Field and Devonshire*,* 1998). Only minor role is played by monooxygenases in organophosphate resistance, and little, if any, in carbamate resistance. Monooxygenase-based cross-resistance to carbamates is unusual in mosquitoes and has only been reported for Propoxur (Brooke *et al*., 2001).

Many cases of organophosphate resistance have been reported in species of *Culex* and *Aedes* (Georghiou *et al.,* 1966). The development of resistance to malathion and diazinon in *Cx. fatigans* in the Cameroons (Hamon and Sales, 1963) and Sierra Leone (Thomas, 1962), to malathion in

Cx. tarsalis in California (Lewallen, 1961) and to diazinon, malathion and trichlorfon in *Ae. aegypti* in Puerto Rico (Fox and Garcia-Moll, 1961, Kerr *et al*., 1964) are evidences for the wide-scale resistance to OPs and Carbamates. Phenomenal organophosphorus resistance has been described in larvae of *Ae. nigromaculis* from California, which achieved a 4000- fold resistance to parathion, 20-fold to methyl parathion and fenthion, and 10-fold to malathion (Brown *et al.,* 1963).

Synthetic pyrethroids are organic compounds similar to the natural pyrethrins produced by the flowers of pyrethrum (*Chrysanthemum cinerariafolium* and *C. coccineum*). DDT belongs to the class of organochlorine pesticides. It is among the first pesticides used in mosquito control programmes and generally used more as insecticide, but is banned in the United States since 1972 and later on in many other countries due to its bioaccumulation properties that pose a threat to human beings and other non-target species. Both these classes of insecticides act as axonic excitotoxins that prevent the closure of the voltage-gated sodium channels of the axonal membranes causing paralysis and thereby death of the insect (Soderlund and Bloomquist, 1989).

Pyrethroids account for approximately 25% of the world's insecticide market. They are used in coils, mats and aerosols. Pyrethroids are popular due to their very low toxicity in humans and rapid killing effect on the insect (Hemingway *et al.,* 2004). Pyrethroids are the only class of insecticides approved for treating mosquito nets as they are safe for humans (Zaim *et al*., 2000). In India and foreign countries Malaria control programmes are highly dependent on pyrethroids by way of using it in indoor residual spraying (IRS) and also through the treatment of pyrethroid impregnated bed nets. They are being increasingly deployed in IRS programmes in Africa and other malaria endemic areas (World Malaria Report, WHO, 2008). Pyrethroids are also

widely used in the control of various agricultural pests worldwide and ultimately reach the breeding places of mosquitoes which finally affect the mosquitoes by developing the resistance specifically against them.

Mass distribution of insecticide-treated bed nets and artemisinin-combination therapies (ACTs) have been used for malaria control in many African countries. Studies in Kenya, Senegal and Gambia during the period 2007–2010 have shown that these policies substantially reduced malaria morbidity, mortality and prevalence (Ceesay *et al*., 2008, O'Meara *et al*., 2008, WHO, 2010). However, Trape *et al*., 2011 reported an increase in malaria morbidity in Senegal from the end of 2010, ie; after 27 – 30 months of the introduction of LLINs (Long Lasting Insecticidal Nets). This instance of malaria morbidity in older children and adults was even higher than that during the period proceeding to the introduction of LLINs.

An uncontrolled use of pyrethroids in the field has led to ineffective vector control activities (Othman *et al*., 2013). This may be due to the possibility that mosquitoes may have developed resistance to pyrethroids due to selection pressure (Somboon *et al.,* 2003). Moreover, those already resistant to DDT may rapidly become resistant to pyrethroids due to the possibility of cross-resistance (Miller, 1988). There are two major mechanisms of pyrethroid resistance in insects: increases in the rate of metabolic detoxification of the insecticide, or changes in target site sensitivity which cause the knockdown resistance by *kdr* mutation (Brengues *et al*., 2003). Metabolic detoxification is often associated with changes in monooxygenase activity, producing pyrethroid-specific resistance (Berge *et al*., 1998).

Many recent works done in resistance status of mosquito vectors have reported the emergence of resistance towards pyrethroids (Wan-Norafikah *et al*., 2013, Ponce, *et al*., 2015, Wanjala *et al*., 2015, Yadouléton *et al*., 2015,

Scott *et al*., 2012, Smith *et al*., 2016) which may be due the extensive and over use of pyrethroids for the control of mosquitoes in the field which in turn have an influence of the development of resistance against this class of insecticides in common. Use of pyrethroids for the control of agricultural pests also have led to the development of resistance in mosquito vectors as they are exposed in less than lethal doses to the insecticide for a longer period (UN, 2006).

Many studies on permethrin resistance have been conducted all over the world. For instance, development of permethrin resistance was found in the *Cx. quinquefasciatus* collected from 19 counties throughout the USA (Liu *et al.,* 2006). Meanwhile, an incipient resistance to permethrin was detected among *Cx. quinquefasciatus* collected from two filarial endemic districts of Northern India (Kumar *et al.,* 2011). Later in 2012, Fofana and team reported that *Cx. quinquefasciatus* collected from four districts in the township of Yopougon, Abidjanwere resistant to 1% permethrin. In Malaysia, permethrin was found to be least effective against *Cx. quinquefasciatus* (Vythilingam *et al*., 1992).

Furthermore, Hamdan *et al.,* 2005 reported that permethrin resistance was developing at a higher rate compared to malathion and temephos in *Cx. quinquefasciatus*. This trend is similar to a study conducted by Nazni *et al.,* (1998) where the field collected *Cx. quinquefasciatus* larvae which were already resistant to malathion and permethrin, showed a resistance ratio of 96.2-folds and 9.4-folds, respectively in comparison to a susceptible laboratory strain, which developed higher resistance to permethrin compared to malathion after been subjected to selection pressure with malathion (eight generations) and permethrin (nine generations). Nazni *et al.,* (2005) also observed that *Cx. quinquefasciatus* has developed a high level of resistance to

permethrin in the Ampang Hill and Pantai Dalam with a resistance ratio value of 12.20 and 10.95 respectively.

Resistance to pyrethroid insecticides and DDT in mosquitoes is mainly conferred by two mechanisms: (1) *kdr* mutation and (2) elevated levels of microsomal monooxygenases [cytochrome P-450] (Liu *et al.*, 2006). Several *cyp* genes have been associated with pyrethroid resistance in mosquitoes, particularly in the case of permethrin (Hemingway *et al.*, 2004). DDT resistance can also be conferred by a class of glutathione-S-transferase (Brown and Perry, 1956, Ranson *et al*., 2001). Esterases do not play a major role in pyrethroid resistance (Nauen, 2007).

The term *kdr* is used to describe resistance cases in insects towards DDT and pyrethroids due to reduced sensitivity of the voltage-gated sodium channel, the target-site of these insecticides. A point mutation (TTA→ TTT) in the S6 hydrophobic trans-membrane segment of domain-II (IIS6 domain) of voltage-gated sodium channel that substituted phenylalanine for leucine in the 1014th position (L1014F) led to reduced affinity of the target site for the insecticides (O'Reilly *et al*., 2006). This was first described in *Musca domestica* in early 1950s (Milany, 1954). *Kdr* is a common mechanism of resistance among pest insects, providing a wide spectrum of cross-resistance to DDT and pyrethroids (Williamson *et al*., 1996, Dong, 1997, Torres *et al*., 1998, Soderlund and Knipple, 2003). In *Culex*, only few instances of this mutation have so far been reported from different parts of the world (Chandre *et al*., 1998, Torres *et al*., 1999, McAbee *et al*., 2004, Xu *et al*., 2005, Wondji *et al.*, 2008, Zhou *et al*., 2009).

A second variant of mutation at position 1014 was identified in pyrethroid resistant *H. virescens* population, where leucine was replaced with histidine (L1014H) (Park and Taylor, 1997), as well as two mutations at locations not previously associated with pyrethroid resistance: V410M (Park

et al., 1997), D1549V and E1553G (Head *et al*., 1998). Studies with knockdown-resistant populations of *Cx. pipiens* and *An. gambiae* identified a third variant at position 1014, which is a replacement of leucine with serine (L1014S) (Torres *et al*., 1999, Ranson *et al*., 2000).

In addition to *kdr,* more than 20 mutations in insect sodium channels have been identified that are involved in reducing channel sensitivity to insecticides or neurotoxins (Park and Taylor, 1997, Liu and Pridgeon, 2002, Pridgeon *et al*., 2002). *Kdr* mutation in mosquitoes have attracted worldwide attention due to the importance of pyrethroids in mosquito control programmes, especially in malaria control (Zaim, *et al.*, 2000), and due to cases of outbreaks of mosquito-borne diseases that have begun to arise due to the increased pyrethroid resistance in mosquitoes (Sina and Aultman, 2001).

The presence of *kdr* mutation (L1014F) can be found out by molecular diagnostic tests like Allele-Specific PCR with resistant and susceptible-specific primers, where amplification depends upon the exact match of a primer to the DNA sequence of only one of two or more alleles of interest, thus resulting in amplification of products that are specific to particular alleles. (Torres *et al.,* 1998, Sarkar *et al.*, 2009).

P-450 Monooxygenases (or mixed function oxidases) also play a major role in conferring pyrethroid resistance to mosquitoes. Cytochrome P450-dependent monooxygenases are an important and diverse family of hydrophobic, heme-containing enzymes involved in the detoxification and/or activation of xenobiotics such as drugs, pesticides, plant toxins, chemical carcinogens and mutagens, as well as in the metabolism of endogenous compounds such as hormones, fatty acids, and steroids (Hemingway *et al*., 2004, Liu *et al*., 2015). There is a wide array of different P450 monooxygenases in insects. Analysis of *An. gambiae* genome revealed a total of 111 P450 monooxygenases arranged mostly in clusters within the genome

(Ranson *et al.*, 2002). Multiple P450 genes have been found to be co-overexpressed in individual insect species via several constitutive over expression and induction mechanisms, which in turn are co-responsible for high levels of insecticide resistance (Liu *et al*., 2015).

Elevated transcript levels (due to transcriptional up-regulation) of an adult-specific *Cyp*6 P450 gene, *Cyp*6z1, have been found in a pyrethroid-resistant strain of *An. gambiae* from East Africa (Nikou *et al*., 2003). Another *Cyp*6 gene implicated in insecticide resistance is *Cyp*6f1 from the mosquito *Cx. quinquefasciatus*. Slightly elevated levels (~2.5-fold) of *Cyp*6f1 transcript has been reported in a strain with permethrin resistance (Kasai *et al*., 2000).

A study conducted to characterize the expression profiles of 204 P450 genes in the mosquito *Cx. quinquefasciatus* at a whole genome level using real-time quantitative PCR analysis, identified multiple P450 genes as up-regulated in individual resistant mosquito strains leading to the conclusion that multiple P450 genes are co-over expressed. This in turn is responsible for the detoxification of insecticides and the development of insecticide resistance in these mosquito strains (Yang and Liu, 2011).

The first case of pyrethroid resistance in *Cx. quinquefasciatus* was reported in California by laboratory selection of a strain with permethrin and was mainly due to a knockdown resistance (*kdr*) gene (Priester and Georghiou, 1978). DDT resistance in *Cx. quinquefasciatus* was first recorded in 1958 in Cote d'Ivoire and Burkina Faso (Adam *et al*., 1958, Hamon *et al*., 1958) and later on in Mali (Hamon *et al*., 1961). In 1968, twelve populations of *Cx. quinquefasciatus* from seven West African countries were all found to be resistant to dieldrin and most of them also to DDT (Mouchet *et al*., 1968).

A study conducted in Mexico to find out the frequency of occurrence of *kdr* mutation in 16 field populations from Western Mexico found all of the

populations analyzed to be having the *kdr* genotype with frequencies ranging from 3.33% - 63.63% (Ponce *et al*., 2016). Insecticide exposure, synergist and biochemical assays conducted on pyrethroid resistant *A. funestus* of South Africa and Southern Mozambique suggested that elevated levels of mixed function oxidases were responsible for the detoxification of pyrethroids in resistant mosquitoes in these areas (Brooke *et al*., 2001). High resistance towards the pyrethroid, permethrin in four field strains of *Cx. quinquefasciatus* in Malaysia was attributed to increased activity of mixed function oxidases (confirmed by synergist PBO) and the presence of kdr mutation (Wan-Norafikah *et al*., 2013). Studies done on *Cx. quinquefasciatus* by Sarkar *et al.,* (2009) confirm the occurrence of *kdr* allele in this vector in north-eastern India. This has serious consequences for the control of filariasis in India as *Cx. quinquefasciatus* is the principal vector of Bancroftian filariasis in India.The use of insecticide mixtures or rotational use of insecticides to delay the development and/or spread of resistance is one strategy that is worth consideration (Curtis *et al*., 1998) and is now under investigation on a large scale in Mexico (Penilla *et al*., 1998).

The initial step to identify a potential problem is to detect changes in the susceptibility of vector populations through bioassay, biochemical assay and molecular assay. WHO has developed susceptibility bioassay kits for mosquitoes. Biochemical and molecular methods can only detect resistance mechanism in an individual insect and therefore can confirm resistance with the use of only a small number of insects. The purpose of the biochemical methodology is not to elucidate *invivo* enzymology of the resistance enzymes of the mosquito species studied, but recognize resistant phenotypes and mechanism likely to produce resistance. Brogdon and Dickson, 1983 first developed the test using microplate system to measure acetylcholinesterase (AChE) and nonspecific esterases activities and protein levels in mosquitoes. Identification of resistance mechanism helps to determine the cross-

resistance spectrum and facilitate the choice of alternate insecticides and helps in the mapping of areas with insecticide resistance population (WHO, 1992). Microplate assay similar to other resistance detection assay should be simple and detect detoxification enzyme levels in single mosquitoes. The results of the tests are more accurate and obtained within minutes. Through molecular diagnostic procedures, the point mutations that cause target site resistance or changes in detoxification enzyme specificity can be analyzed easily.

1.3. MATERIALS AND METHODS

1.3.1. Test Organism: *Culex quinquefasciatus* Say

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Diptera
Family	:	Culicidae
Genus	:	*Culex*
Species	:	*quinquefasciatus*

Culex quinquefasciatus Say, the common southern house mosquito, is a medium sized, mosquito found in tropical and subtropical and lower latitudes of temperate regions of the world. The body of adult varies from 3.96 to 4.25 mm in length and is brown in colour and the proboscis, thorax, wings, and tarsi are darker than the rest of the body (Lima *et al*., 2003). It undergoes complete metamorphosis with the life cycle consisting of egg, larval, pupal and adult stages (Plate 1). The entire lifecycle may be completed in 10–14 days, but it is prolonged by cold weather. Males of *Cx. quinquefasciatus* live only for 1-2 weeks but the females may live upto two months. Both males and females feed on plant sap to obtain energy for flight. Females require a blood meal to obtain necessary proteins for the development of eggs. It is during blood feeding that the transmission of pathogens occurs through the saliva of the mosquito. Eggs are laid one at a time, but are stuck together to a form of a raft of about 200-300 eggs. A raft of eggs looks like speck of soot floating on the water and is about 1/4 inch long and 1/8 inch wide. Incubation period is ranged from 3-5 days. The larval and adult stages are mainly used for identification. The presence of long

respiratory siphon in the larval stage is the main feature that helps in identifying Culicines.

1.3.1.1. Identification of test organism:

Mosquito larvae were collected from the field and brought to the laboratory and reared in 0.08% saline solution. The larvae were fed *ad libitum* with fine powder of dog biscuit and yeast in the ratio 3:1. The pupae from the rearing trays were collected and kept in a standard cage for emergence. After emergence the adults were subjected to identification (Barraud, 1934).

Adult: Proboscis without pale ring (entirely dark); Post spiracular area without pale scale patch; Abdomen with basal bands.

Larva: Siphon with several pairs of sub-posterior hair tufts, usually paired; Thoracic hair always single; Lateral hair on saddle 1- branched.

1.3.1.2. Culture maintenance of test organism in the laboratory:

The adult mosquitoes were maintained at 29±2°C and 75±5% relative humidity at 12 hour day and night cycle mimicking the natural conditions in a 15 inch square cage covered with wire mesh. The adults were fed on 10% sucrose solution soaked in cotton and placed in a glass jar. The adult females were fed with blood from an immobilized quail in the twilight hours. Plastic egg cups with water were placed in the cage for oviposition. The egg rafts were collected after oviposition and transferred to the rearing trays. After hatching, the first instar larvae were transferred to a plastic tray containing water and were provided with powdered mixture of dog biscuit and yeast in the ratio 3:1 as larval food. The feeding was continued until the larvae reached the fourth instar. 30 Fourth instar larvae were taken from each sample for biochemical assays. The rest of the larvae were allowed to reach the adult stage and were then used for the susceptibility tests.

1.3.2. Areas of sample collection:

The larvae of *Cx. quinquefasciatus* were collected from five different areas of Kerala state, India viz, Cochin Municipal Corporation, Kozhikode Corporation, Palakkad Municipality, Ponnani Municiplaity (Malappuram) and Thrissur Municipal Corporaion. From each of the area, depending upon the mosquito control regimes in existence, collections were made from two different locations ie, i) the regularly insecticide spraying locations (T) and ii) scarcely spraying locations (U). In all these areas temephos was used as a larvicide for the control of *Culex* mosquitoes by the Municpality/Corporation. Recently, they have started using *Bti* as larvicide in all the five areas and alternatively, Temephos and *Bti* were predominantly used by Ponnani Municipality (as per the reports of Municipality Health Dept.). The District vector control unit also sprays temephos as larvicide in all these areas as a control measure for *Culex* mosquitoes.

Susceptible Laboratory Populations were collected from CRME (Centre for Research in Medical Entomology) ICMR, Madurai.

The areas of collection are denoted as

LAB - Laboratory

EKM - Cochin Municipal Corporation

CLT - Kozhikode Corporation

PKD - Palakkad Municipality

MPM - Ponnani Municipality

TCR - Thrissur Corporation

1.3.3. Bioassay:

Bioassays were conducted as per the protocol of WHO 1981. 0.01ppm concentration of Temephos (analytical grade: Temephos PESTANAL

SIGMA-ALDRICH) with chemical formula $C_{16}H_{20}O_6P_2S_3$ was used for the bioassay. Temephos (trade name: Abate) is an organophosphate larvicide used to treat water infested with disease carrying insects like mosquitoes, midges and black fly larvae. When the animal is exposed to Temephos, it accelerates the chemical block to the production and action of cholinesterase, an essential nervous system enzyme.

The bioassay was carried out in glass tumblers containing 250ml tap water each of desired concentration and 10 larvae were placed in each glass. Water without insecticide was also kept as control. Adequate replicates were also maintained. Mortality was recorded at 15 minute intervals until all the larvae dies or up to 3hours after the commencement of the experiment. The time taken to kill 50% of the larvae was noted and the LT_{50} in minutes was calculated using the probit analysis developed by Finney (1971). Resistance ratio in response to LT_{50} of *Cx. quinquefasciatus* collected from various sampling sites was calculated using the formula of Dhang *et al*., 2008.

Resistance ratio (RR) = LT_{50} of field strain / LT_{50} of laboratory strain.

RR values > 1 indicated resistance, while values ≤ 1 were considered susceptible.

1.3.4. Biochemical assays:

Biochemical assays were used to quantify levels of monooxygenases and non-specific esterases, as well as to detect the presence of elevated acetylcholine esterase in 4^{th} instar larvae of the F1 generation. 30 fourth instar larvae were taken from each of the samples for the assays. The assays were done according to the protocol provided in techniques to detect insecticide resistance mechanism; Field and Laboratory Manual, WHO (1998) [Techniques to detect insecticide resistance mechanisms, WHO/ CDS/ CPC/ MAI: / 98; 6]

The larvae were homogenized in 200 µl distilled water using a homogenizer. The homogenate was immediately placed on ice and later stored at -20°C. After the acetylcholinesterase assay, the remainder of the insect homogenate is spun in a microfuge at 14K for 30 seconds. The centrifuged sample was used for all the rest of the assays. The assays were completed using the homogenate as rapidly as possible to ensure accuracy of results.

1.3.4.1. Total protein assay (Bradford, 1976)

Reagents required:

1. Protein standard: 5 mg bovine serum albumin in 100ml distilled water (0.05mg/ml)
2. Bradford's reagent: Dissolve 100 mg Coomassie Blue G-250 in 50 ml 95% ethanol, add 100 ml 85% (w/v) phosphoric acid to this solution and dilute the mixture to 1 litre with water and stored at 4°C.

Methodology:

The Bradford reagent is designed to be diluted 5-fold with protein. So, the standards and samples are diluted to 160µl with distilled water to which 40µl of the reagent is added. Pipette 0, 15, 30, 45, 70, 100, 130 and 160µl of Bovine Serum Albumin (BSA) (0.05 mg/ml) into assigned wells of a 96-well plate, and made upto 160 µl with distilled water. Pipette up to 16 µl of unknown samples into individual wells of a 96-well plate and made upto 160 µl with distilled water. Added 40 µl of Bradford Reagent into all wells containing standard or sample and incubated at room temperature for 30 minutes and red the absorbance at 595 nm in a micro plate reader.

1.3.4.2. Carboxylesterase assay

Reagents required:

1. 30mM 1- naphthyl acetate (NA) (0.2793g 1-NA in 50ml acetone).
2. 30mM 2- naphthyl acetate (NA) (0.2793g 2-NA in 50ml acetone). Solutions a and b can be made up and stored separately in tightly stoppered bottle at 4ºC for several months.
3. Freshly prepared working naphthyl acetate solutions: 1ml of 30mM stock in 99 ml of phosphate buffer 0.02M pH 7.2.
4. Freshly prepared Stain (150 mg Fast blue B dissolved in 15ml distilled water, and then add 35 ml of 5%Sodium lauryl sulphate (SDS).

Methodology:

Take 2 X 10 µl replicates of homogenate was taken and placed in separate wells of microtitre plate. 100 µl of 1-NA working solution was added to one replicate and 100 µl of 2-NA working solution was added to the second replicate and the plate was left at room temperature for 15 minutes. 25 µl of fast blue stain solution was added to each replicate. One or more plate blank was included per set. This contained 10 µl distilled water, 100 µl of 1-NA or 2-NA solution and 25 µl of stain. The plate was read at 570 nm as an end point.

1.3.4.3. Glutathione-S-transferase assay

Reagents required:

1. 10 mM GSH i.e. reduced glutathione (0.0081g GSH in 2.5 ml 0.1 M phosphate buffer, pH 6.5).
2. 63 mM chlorodinitrobenzene (0.1278 g CDNB in l0ml methanol).

3. Working solution: add 125 µl of CDNB solution to 2.5 ml GSH solution.

All solutions were prepared freshly and used within 1-2 hrs.

Methodology:

2 X 10µl replicates of the homogenate were taken in separate wells of the mirotitre plate. 200 µl of GSH / CDNB working solution was added to the wells. 2 plate blanks were kept per microplate having 10µl distilled water + 200µl of GSH + CDNB working solution. The plate was red at 340 nm continuously for 5 minutes within 20 minutes of adding the reagents.

1.3.4.4. Monooxygenase titration assay

Reagents required:

1. 3% hydrogen peroxide.
2. 0.625 M potassium phosphate buffer, pH 7.2.
3. 0.01g $3,3^{1},5,5^{1}$- tetramethyl benzidine in 5ml methanol.
4. 0.25 M sodium acetate buffer pH 5.0.

Methodology:

2 X 10 µl replicates of homogenate were taken in separate wells of a microtitre plate. 40 µl of 0.625 M potassium phosphate buffer (pH 7.2) is added to each replicate. 5 ml methanol solution of tetramethylbenzidine (TMB) was mixed with 15 ml of 0.25 M sodium acetate buffer (pH 5.0) and 100 µl of the above mixture is added to each replicate. 12.5 µl of 3% hydrogen peroxide is added to each replicate and the mixture was left for 2 hours at room temperature and absorbance was red at 650nm. Controls were run with 10 µl of buffer in place of the insect homogenate.

1.3.4.5. Acetylcholinesterase assay

Reagents required:

1. 10ml 0.01M dithiobis 2-nitrobenzoic acid (0.0396g DTNB + 10ml 0.1M phosphatebuffer pH 7.0).
2. 20ml 0.01M acetylthiocholine iodide (0.0578g ASCHI + 20ml distilled water).
3. 10ml 0.1M propoxur (0.292g propoxur in l0ml acetone).
4. Split the ASCHI solution into two 10ml aliquots, to one aliquot adds 20µl of 0.1M propoxur.
5. 1 % Triton X-100 in 0.lM phosphate buffer pH 7.8.

All solutions were freshly prepared except 0.1M propoxur .

Methodology:

2 X 25µl of the crude insect homogenate was taken in separate wells of microtitreplate and 145µl of Triton phosphate buffer (1% Triton X-100 in 0.1 M phosphate buffer pH 7.8) was added to each replicate. 10µl 0.01M DTNB (Dithiobis2-nitrobenzoic acid) was added to the mixer of each replicate.

25µl 0.01M ASCHI (Acetylthiocholine iodide) was added to one replicate while 25µl of 0.01M ASCHI + 0.1M Propoxur was added to the second replicate. Two or more blanks were kept per plate which contain 25µl distilled water and 10µl DTNB + 25µl ASCHI + 145µl triton phosphate buffer without insect homogenate.

The wells were incubated at room temperature for one hour and readings were taken at 405 nm in a microplate reader as endpoint.

1.3.5. Insecticide susceptibility/Resistance tests:

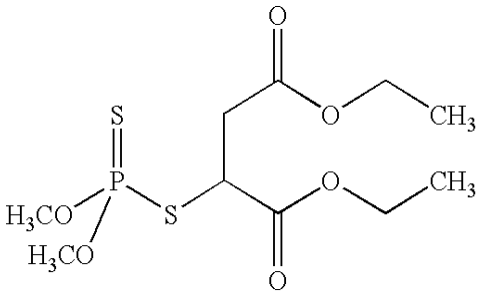
The susceptibility tests were carried out according to the standard WHO specifications using WHO insecticide susceptibility kit. Test papers impregnated with diagnostic doses of test insecticides (Organophosphate: Malathion -5%, Pyrethroids: Cyfluthrin - 0.15% and Deltamethrin - 0.05%), holding and exposure tubes and other necessary apparatus needed for the assay were procured from the WHO collaborating centre, Penang, Malaysia via NVBDCP, New Delhi. Bioassays were performed according to standard methodology prescribed by WHO (1998).

1.3.5.1. Insecticides impregnated papers used for the test:

The following insecticide impregnated papers were used to conduct the bioassay. The control papers for each of the insecticide impregnated papers were also supplied with the WHO kit was used to compare the results.

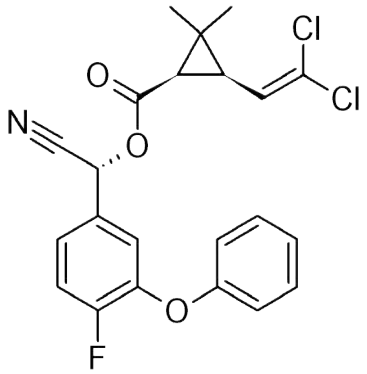
1.3.5.1.1. Malathion 5% (Organophosphate (OP) insecticide. MF: $C_{10}H_{19}O_6PS_2$)

It is used to control aphids, leafhoppers, and Japanese beetles in agriculture fields. This is also used in public health pest control programs for controlling mosquito-borne illnesses. It is an adulticide used for ground application (fogging equipment mounted on trucks). In situations of heavy mosquito presence across large geographic areas, it is aerially applied.



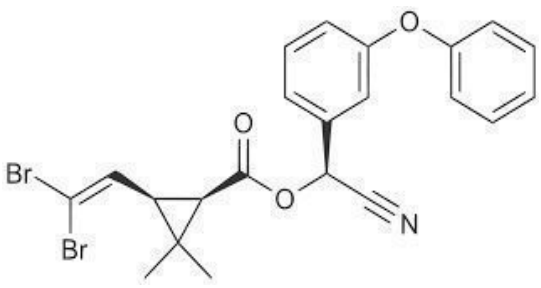
1.3.5.1.2. Cyfluthrin 0.15% (Synthetic pyrethroid insecticide, MF: $C_{22}H_{18}Cl_2FNO_3$)

It acts as both contact and stomach poison. It is a non-systemic chemical used to control cutworms, ants, silverfish, cockroaches, termites, grain beetles, weevils, mosquitoes, fleas, and many others. Cyfluthrin is used in insecticidal sprays such as Temprid (Bayer) which uses a combination of (beta-) cyfluthrin and imidacloprid**.** Also used for household uses in mosquito repellent coils and mats.



1.3.5.1.3. Deltamethrin 0.05% (Synthetic Pyrethroid, MF: $C_{22}H_{19}Br_2NO_3$)

It is the most powerful synthetic pyrethroid that kills insect on contact and through digestion. Rapidly paralyzes the insect nervous system giving a quick knockdown effect. It has good residual activity for outdoor uses (field crops, cattle dip, and tsetse) and for indoor uses (mosquitoes, stable flies, horseflies, fleas, cockroaches, stored product insects) and it provides broad spectra of control.



1.3.5.2. Methodology:

Adult bioassays were conducted using 1–3 day old F1 generation females exposed for one hour to insecticide-impregnated and control papers. Adult response was assessed from 3 replicates, 15 – 25 females/replicate, with mortality measured 24hrs after the 1hour exposure. Control assays included exposures of samples of mosquitoes to papers treated only with the solvent of the insecticide in question.

All exposures lasted for 1 hour. Knockdown was recorded every 10 min and final mortality was recorded after a 24hrs holding period during which time a 10% sucrose solution was made available to survivors.

1.3.6. Detection of presence/absence of site specific mutations:

Survivors and non-survivors of the test with pyrethroid and malathion exposure were stored separately at -20°C for further molecular characterization. DNA were isolated by Phenol – Chloroform - Isoamyl alcohol extraction and was used for AS-PCR assay for determining the presence of *kdr* genotype and acetylcholinesterase gene amplification and restriction using Alu1enzyme for detection of mutation in *ace*1 gene.

1.3.6.1. DNA Isolation

Reagents required:

1. PBS (Phosphate buffered saline)

1. Na_2HPO_4 - 1.09g
2. NaH_2PO_4 - 0.32g
3. NaCl - 9g
4. Double distilled Water - 100ml

Mix, dissolve and adjust the pH to 7.2 and store at room temperature.

2. Lysis buffer

1. 0.5M EDTA (18.6g EDTA in 80ml dd.H_2O. adjust pH to 8 using NaOH pellets)
2. 5M NaCl (29.22 g NaCl in 100 ml dd. H_2O)
3. 1M TrisHCl (12.11g Tris base in 80ml dd. H_2O, adjust pH with HCl & make up to 100ml with dd.H_2O)
4. 10% SDS

Mix 0.5M EDTA, 5M NaCl, 1M Tris HCl, 10% SDS in the ratio 10: 2: 1: 10.

3. 3M Sodium acetate

1. $NaOAc \cdot 3H_2O$ - 40.8g
2. Double distilled Water - 100ml

Dissolve NaOAc in 80ml dd.H_2O, adjust pH to 5.2 with glacial acetic acid and made up to 100ml with dd. H_2O

4. Proteinase K - Sigma Aldrich

5. Phenol Chloroform Isoamyl alcohol- Sigma Aldrich

Methodology:

Grind the sample with PBS (phosphate buffered saline) and dip in liquid nitrogen for 5 minutes. Add 1ml Lysis buffer + proteinase K. Incubate for 20 - 30 minutes at 37°C and centrifuged at 12,000 rpm for 20 minutes. An equal volume of phenol-chloroform-isoamylalcohol (25:24:1) is added to the supernatant and centrifuged at 12,000 rpm for 10 minutes. Then, the clear aqueous solution is taken and added equal volume of 100% ethanol and 300 - 500µl of 3M Sodium acetate; incubated at -20°C for a minimum of one hour and a maximum of 1-2 days. After incubation, it was centrifuged at 10,000 rpm for 10 minutes and discarded the supernatant. To the pellet, added 1 ml 70% ethanol and centrifuged at 5000 rpm for 3-5 minutes. Repeated the

procedure twice and air dried the pellet, added 50 - 100µl 1 x TE buffer or nuclease free water and store at -20°C.

1.3.6.2. Allele - Specific PCR (AS-PCR) assay

Primer selection:

Four primers (Primer 1, 2, 3 and 4) were selected from the region II of para-type voltage gated sodium channel (vgsc) gene of *Cx. pipiens* (Martine-Torres *et al.*, 1999; M. Sarkar *et al.*, 2009).

Two primers [Primer 1 (forward) 5′-GTGGAACTTCACCGACTTC - 3′and Primer 2 (reverse) 5′-GCAAGGCTAAGAAAAGGTTAAG- 3′] were used to amplify the fragment of sodium channel gene containing the *kdr* mutation site. The other two primers [Primer 3 (forward) 5′-CCACCGTAGTGATAGGAAATTTA- 3′ and Primer 4 (forward) 5′-CCACC GTAGTGATAGGAAATTTT- 3′] were allele-specific primers used in genotyping of knockdown susceptible (Primer 3) and knockdown resistant (Primer 4) alleles by allele-specific PCR assay. The allele-specific primers were identical except at the 3′-OH end where 'A' in Primer 3 was replaced by 'T' in Primer 4. Both primers 3 and 4 could amplify a 380bp corresponding region.

Methodology:

20 pyrethroid survivors (10 deltamethrin survivors and 10 cyfluthrin survivors) and 20 non-survivor mosquitoes were selected for AS-PCR assay. The PCR was performed according to Martin-Torres *et al.,* (1999) with modifications to detect *kdr* mutation in the mosquito population. Two PCR reactions were run in parallel. One reaction contained the primers 1, 2 and 3 (10 pmol each). In the other reaction primer 3 was replaced by primer 4. 10ng of mosquito DNA was added as template in each reaction.

The reaction mixture contained:

DNA sample	-	1 µl (10 ng)
Primer 1	-	1 µl (10 pmol)
Primer 2	-	1 µl (10 pmol)
Primer 3 / Primer 4	-	1 µl (10 pmol)
1 x PCR smart mix	-	10 µl
Nuclease free water	-	6 µl
Total volume	-	20 µl

The PCR conditions were 5 min at 94 °C for the first cycle, followed by 1 min at 94 °C, 2 min at 49 °C and 2 min for 72 °C for 29 cycles, and 10 min at 72 °C for the final extension. The DNA fragments were separated by electrophoresis on 1.5% agarose gel and were visualized by ethidium bromide staining under UV light. The presence of 380bp band corresponding to resistant and susceptible specific primers revealed the genotype of the mosquitoes.

1.3.6.3. Detection of mutation in *ace*1 gene by restriction enzyme digestion:

Primer selection:

Two primers [Primer 1 (forward) 5′- CGACTCGGACCCACTCGT - 3′and Primer 2 (reverse) 5′- GACTTGCGACACGGTACTGCA - 3′] were used to amplify the partial sequence of *ace*1 (374bp) gene (Osta *et al*., 2012).

Methodology:

DNA was isolated from the malathion survivors and non survivors (15 each) and used for the detection of mutation in *ace*1 gene.

DNA sample	-	1 µl (10 ng)
Primer 1	-	1 µl (10 pmol)
Primer 2	-	1 µl (10 pmol)
1X PCR smart mix	-	10 µl
Nuclease free water	-	7 µl
Total reaction volume	-	20 µl

The PCR conditions were 5 min at 94 °C for the first cycle, followed by 1 min at 94 °C, 1 min at 60 °C and 1min for 72 °C for 30 cycles, and 10 min at 72 °C for the final extension. The DNA fragments were separated by electrophoresis on 1.5% agarose gel and were visualized by ethidium bromide staining under UV light. The presence of 374bp band is corresponding to *ace*1 partial gene.

Restriction digestion using Alu1 enzyme:

Nuclease free water	-	9µl
Buffer Alu1 (10X)	-	2µl
*ace*1	-	8µl
Alu1 enzyme	-	1µl
Total reaction volume	-	20 µl

The PCR conditions were 5 min at 94 °C for the first cycle, followed by 1 min at 94 °C, 1 min at 60 °C and 1min for 72 °C for 30 cycles, and 10 min at 72 °C for the final extension. The DNA fragments were separated by electrophoresis on 1.5% agarose gel and were visualized by ethidium bromide staining under UV light. The presence of 374bp band is corresponding to *ace*1 partial gene, it is the susceptible one and it is homozygous (SS) susceptible. Homozygous resistant is represented by RR with 2 bands; one at 272bp and

other at 102bp. Heterozygous resistant is represented by 3 bands (SR); bands at 374bp, 272bp, and 102bp.

1.3.7. Statistical Analysis

Statistical analysis was performed using Statistical package SPSS 20.0.

1.4. RESULTS

1.4.1. Bioassay:

Cx. quinquefasciatus larvae collected from different sampling sites of the selected districts in Kerala and the laboratory populations were subjected for bioassay. The bioassay was conducted over a period of three consecutive years' using 0.01ppm temephos and the mortality was recorded. Table 1 shows the data on time taken (in minutes) for 50% mortality of larvae using 0.01ppm temephos tested against the laboratory and field populations of *Cx. quinquefasciatus* in the three consecutive years 2014, 2015 and 2016. The LT_{50} values of the laboratory population observed were 37.02, 36.98 and 37.08 min in the years 2014, 2015 and 2016 respectively. *Cx. quinquefasciatus* collected from insecticide regularly treating and scarcely treating area at MPM shows the LT_{50} values 51.47, 52.31, 53.56 minutes and 40.71, 41.14 and 41.99 minutes respectively. The larvae collected from the scarcely treated areas of PKD, TCR, CLT and EKM showed the LT_{50} values of 42.31, 43.44, 44.54, 46.64 in the year 2014, 43.47, 44.88, 45.05, 48.82 in the year 2015 and 45.64, 45.65, 48.86, 54.46 in 2016 respectively and those from the regularly treated locations of the same area showed the values 55.63, 63.51, 69.24, and 72.95 in the year 2014, 65.32, 74.65, 79.32, 85.69 in the year 2015 and 70.24, 76.64, 84.32, 90.04 in 2016 respectively.

Table 1: Data on LT_{50} (minutes) and associated statistics of *Cx. quinquefasciatus* Say collected from different sampling sites tested against 0.01ppm Temephos.

Sl. No:	Area of Collection	LT_{50} (Minutes) (LFL-UFL) 95% CL		
		2014	2015	2016
1	LAB	37.02 (35.69-38.34)	36.98 (35.02-37.94)	37.08 (35.04- 39.06)
2	MPM U	40.71 (32.84- 54.82)	41.14 (39.88-45.22)	41.99 (38.88- 44.86)
3	MPM T	51.47 (49.88-53.29)	52.31 (50.30-54.45)	53.56 (49.58- 55.44)
4	PKD U	42.31 (42.30-43.25	43.47 (42.88-45.29)	45.64 (42.54- 47.75)
5	PKD T	55.63 (53.10-47.18)	65.32 (53.32-57.67)	70.24 (68.82- 73.42)
6	TCR U	43.44 (42.36- 45.54)	44.88 (42.24- 46.76)	45.65 (42.25- 47.76)
7	TCR T	63.51 (60.10-66.13)	74.65 (73.11-76.18)	76.64 (71.56- 80.84)
8	CLT U	44.54 (42.22- 48.62)	45.05 (42.25- 47.85)	48.86 (44.46- 52.64)
9	CLT T	69.24 (63.22-67.68)	79.32 (53.32-57.67)	84.32 (80.88- 88.42)
10	EKM U	46.64 (43.34- 49.98)	48.82 (43.85- 51.54)	54.46 (49.94- 58.28)
11	EKM T	72.95 (69.69-75.27)	85.69 (83.45-87.70)	90.04 (85.56- 95.94)

LAB= Laboratory population,
U= Samples collected from insecticide scarcely treating area,
T= Samples collected from insecticide regularly treating area,
MPM =Ponnani, TCR= Thrissur, PKD= Palakkad, CLT= Kozhikode, EKM= Cochin

1.4.1.1. Resistance Ratio:

Resistance ratio in response to LT_{50} of *Cx. quinquefasciatus* collected from various sampling sites was calculated using the formula of Dhang *et al*., 2008.

Resistance ratio (RR) = LT_{50} of field strain / LT_{50} of laboratory strain.

RR values > 1 indicated resistance, while values ≤ 1 were considered susceptible.

The data on resistance ratio of all the *Cx. quinquefasciatus* tested using LT_{50} values are provided in table 2. In all the field populations the resistance ratio is greater than 1. According to Dhang *et al.,* 2008 if the ratio is higher than 1 which indicates the populations may have developed resistance towards the insecticides. The RR values of MPM T with laboratory populations were 1.39, 1.41 and 1.44 for the years 2014, 2015 and 2016. RR values in response to LT_{50} of PKD T and TCR T with that of LAB *Culex* were 1.50, 1.76, 1.89 and 1.76, 2.02, 2.06 respectively. The CLT and EKM population also had a high level of RR ratio with values 1.87, 2.14, 2.27 and 1.97, 2.32, 2.48 in the year 2014, 15 and 16 respectively.

Table 2: Data on Resistance ratio of field populations of *Cx. quinquefasciatus* collected from different sampling sites with laboratory population using LT_{50} values

Sl. No	Area of Collection	Year		
		2014	2015	2016
1	MPM U	1.099	1.112	1.13
2	MPM T	1.39 (1.26)	1.41 (1.27)	1.44 (1.27)
3	PKD U	1.14	1.17	1.23
4	PKD T	1.50 (1.31)	1.76 (1.50)	1.89 (1.53)
5	TCR U	1.17	1.21	1.23
6	TCR T	1.76 (1.46)	2.01 (1.66)	2.06(1.67)
7	CLT U	1.20	1.21	1.31
8	CLT T	1.87 (1.55)	2.14 (1.76)	2.27 (1.72)
9	EKM U	1.25	1.32	1.46
10	EKM T	1.97 (1.56)	2.31 (1.75)	2.42 (1.65)
Values in parenthesis show the resistance ratio of regularly insecticide treated area with that of scarcely treated area.				

1.4.2. Biochemical assay results:

The results of biochemical assays are described below.

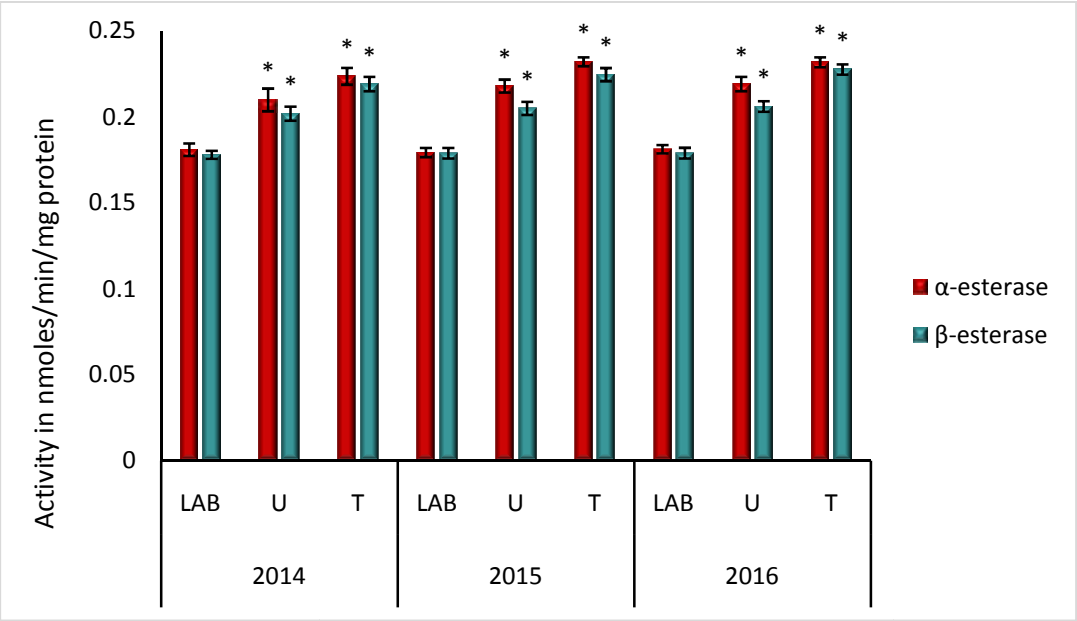
1.4.2.1. Carboxylesterase activity:

The carboxylesterases levels indicated the degree of detoxification to insecticides. High levels of these enzymes are seen in several organophosphate resistant strains of a number of insect species as compared to susceptible strains of the same insect. In the present study, alpha and beta esterase (carboxylesterases) assays were conducted to find out the elevated levels of the enzymes and represented in the following graphs (Fig.1-5).

The carboxylesterase activity of laboratory reared and field collected samples of *Cx. quinquefasciatus* from MPM during 2014 to 2016 were represented in figure 1. The LAB had values of 0.181±0.0036, 0.179±0.0026, 0.181±0.0023 nanomoles/min/mg of protein α- esterase during 2014 to 2016. The MPM U and MPM T populations have the values of 0.21±0.0065, 0.218±0.0037, 0.219±0.0041 and 0.224±0.0048, 0.232±0.0025, 0.232± 0.0028 respectively.

The beta esterase levels of LAB, MPM U and MPM T were 0.178±0.002, 0.179± 0.003, 0.179± 0.003; 0.202± 0.004, 0.205±0.004, 0.206±0.003; 0.219±0.004, 0.224±0.004, 0.228±0.003 in the three consecutive years.

Figure 1: Carboxylesterase activity of *Cx. quinquefasciatus* from MP M



Level of significance p<0.05; * = p<0.05

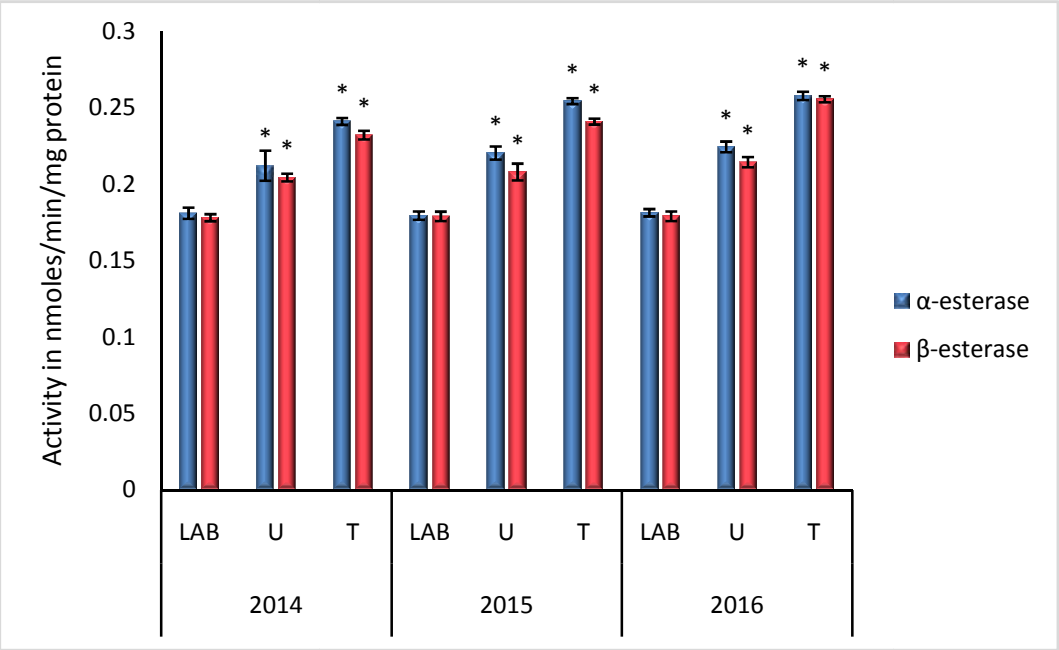
Table 3 provided the data on statistical analysis of α and β esterase activity of *Cx. quinquefasciatus* collected from MPM. In all the cases the p value was less than 0.00001 hence the results were highly significant and there was observable change in the values of laboratory and field population.

Table 3: Data on Statistical analysis of α and β esterase of *Cx. quinquefasciatus* collected from MPM

Year	Area of sample collection	Mean enzyme activity ± SD (nmol α/β naphthol/min/ mg protein		One – way ANOVA	
		α-esterase	β- esterase	α-esterase	β-esterase
2014	LAB	0.181±0.004	0.178±0.002	F = 551.21 p< 0.00001 The result is significant at p< .05	F = 995.80 p< 0.00001 The result is significant at p< .05
	U	0.21±0.007	0.20±0.004		
	T	0.22±0.005	0.22±0.004		
2015	LAB	0.179±0.0027	0.179±0.003	F = 2463.11 p< 0.00001 The result is significant at p< .05	F = 1252.17 p< 0.00001 The result is significant at p< .05
	U	0.22±0.004	0.21±0.004		
	T	0.23±0.003	0.22±0.004		
2016	LAB	0.181±0.002	0.179±0.003	F = 2080.29 p< 0.00001 The result is significant at p< .05	F = 1983.23 p< 0.00001 The result is significant at p< .05
	U	0.22±0.004	0.21±0.003		
	T	0.23±0.002	0.23±0.003		

Figure 2 illustrate the carboxylesterase activity levels of *Cx. quinquefasciatus* collected from PKD during 2014 to 2016. According to the results, samples from the insecticide regularly applying areas have the highest level of enzyme activity. Mosquitoes cultured in the laboratory condition shows the lowest α & β esterase activity and samples from the insecticide scarcely treating area showed intermediate mode of insecticide detoxification. Year wise enzyme activity showed an upward trend with time in the field populations and the values of α-esterase range from 0.212 to 0.224 and 0.241 to 0.258 and that of β-esterase ranges from 0.204 to 0.214 and 0.232 to 0.255 in PKD U and PKD T respectively.

Figure 2: Carboxylesterase activity of *Cx. quinquefasciatus* from PKD



Level of significance p<0.05; * = p<0.05

Table 4 provides α& β esterase activity of laboratory reared and field collected strains of *Cx. quinquefasciatus* from PKD during 2014 to 2016. Year wise enzyme activity showed an upward trend with time, through that exhibited significant (p<0.05) increase in resistance.

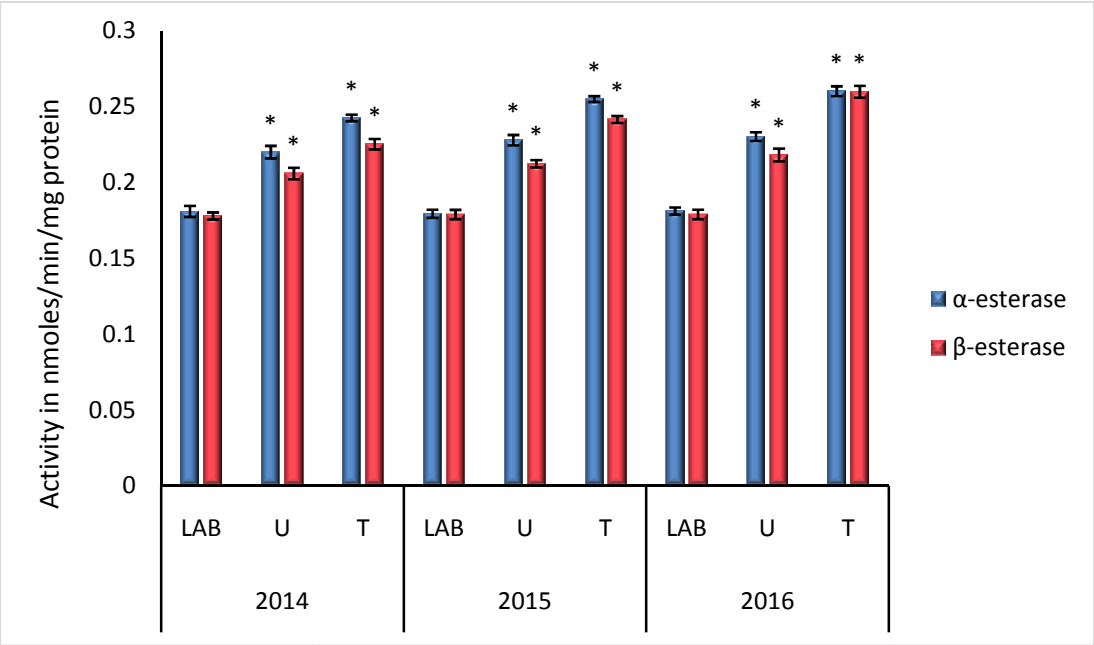
Table 4: Data on Statistical analysis of α and β esterase of *Cx. quinquefasciatus* collected from PKD

Year	Area of sample collection	Mean enzyme activity ± SD (nmol α/β naphthol/min/ mg protein		One – way ANOVA	
		α-esterase	β- esterase	α-esterase	β-esterase
2014	LAB	0.18±0.003	0.17±0.002	F = 723.13 p< 0.00001 The result is significant at p< .05	F = 3482.11 p< 0.00001 The result is significant at p< .05
	U	0.21±0.009	0.20±0.003		
	T	0.24±0.002	0.23±0.003		
2015	LAB	0.17±0.002	0.17±0.003	F = 4513.74 p< 0.00001 The result is significant at p< .05	F = 2049.49 p< 0.00001 The result is significant at p< .05
	U	0.22±0.004	0.21±0.006		
	T	0.25±0.002	0.24±0.002		
2016	LAB	0.18±0.002	0.17±0.003	F = 5395.10 p< 0.00001 The result is significant at p< .05	F = 5373.82 p< 0.00001 The result is significant at p< .05
	U	0.22±0.003	0.21±0.003		
	T	0.26±0.003	0.26±0.002		

Figure 3 showed the carboxylesterase activity of laboratory reared and field collected *Cx. quinquefasciatus* from TCR during 2014 to 2016. The α-esterase values of laboratory population observed were 0.18±0.003, 0.17±0.002 and 0.18±0.002 nanomoles/min/mg of protein during 2014 to 2016 and the TCR U and TCR T populations have the values 0.22±0.004, 0.23±0.003, 0.23±0.003 and 0.24±0.002, 0.26±0.002, 0.26±0.003respectively.

The β- esterase values of laboratory population observed were 0.178±0.002, 0.179±0.003 and 0.179±0.003 nanomoles/min/mg of protein in 2014, 2015 and 2016 and the TCR U and TCR T populations have the values 0.206±0.004, 0.212±0.002, 0.218±0.004 and 0.225±0.003, 0.242±0.002, 0.26±0.004 respectively.

Figure 3: Carboxylesterase activity of *Cx. quinquefasciatus* from TCR



Level of significance p<0.05; * = p<0.05

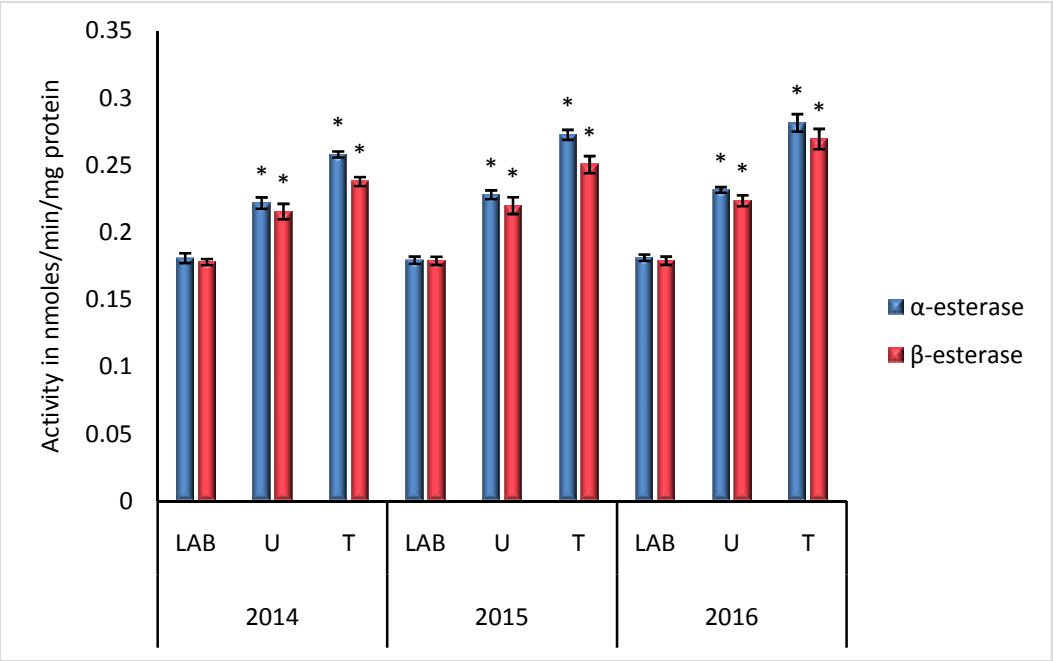
Table 5 provides the data on statistical analysis of α and β esterase of *Cx. quinquefasciatus* collected from TCR. The α esterase F values for 2014, 2015 and 2016 year were 2583.02, 5967.55, 6034.16 and β esterase F values were 1662.18, 4442.25, and 3593.62 respectively. In all the cases the p value was less than 0.00001, hence the results were highly significant and there was change in the values of laboratory population and field population.

Table 5: Data on Statistical analysis of α and β esterase of *Cx quinquefasciatus* collected from TCR

Year	Area of sample collection	Mean enzyme activity ± SD (nmol α/β naphthol/min/ mg protein		One – way ANOVA	
		α-esterase	β- esterase	α-esterase	β-esterase
2014	LAB	0.181±0.0036	0.178±0.002	F = 2583.02 p< 0.00001 The result is significant at p< .05	F = 1662.18 p< 0.00001 The result is significant at p< .05
	U	0.22±0.004	0.21±0.004		
	T	0.24±0.002	0.23±0.003		
2015	LAB	0.179±0.0027	0.179±0.003	F = 5967.55 p< 0.00001 The result is significant at p< .05	F = 4442.25 p< 0.00001 The result is significant at p< .05
	U	0.23±0.003	0.21±0.002		
	T	0.26±0.002	0.24±0.002		
2016	LAB	0.181±0.002	0.179±0.003	F = 6034.16 p< 0.00001 The result is significant at p< .05	F = 3593.62 p< 0.00001 The result is significant at p< .05
	U	0.23±0.003	0.22±0.004		
	T	0.26±0.003	0.26±0.004		

Figure 4 express the carboxylesterase activity levels of *Cx. quinquefasciatus* collected from CLT during 2014 to 2016. According to the results, samples from the insecticide regularly applying areas have the highest level of enzyme activity. Strains cultured in the laboratory condition shows the lowest α& β esterase activity.

Figure 4: Carboxylesterase activity of *Cx. quinquefasciatus* from CLT



Level of significance $p<0.05$; * = $p<0.05$

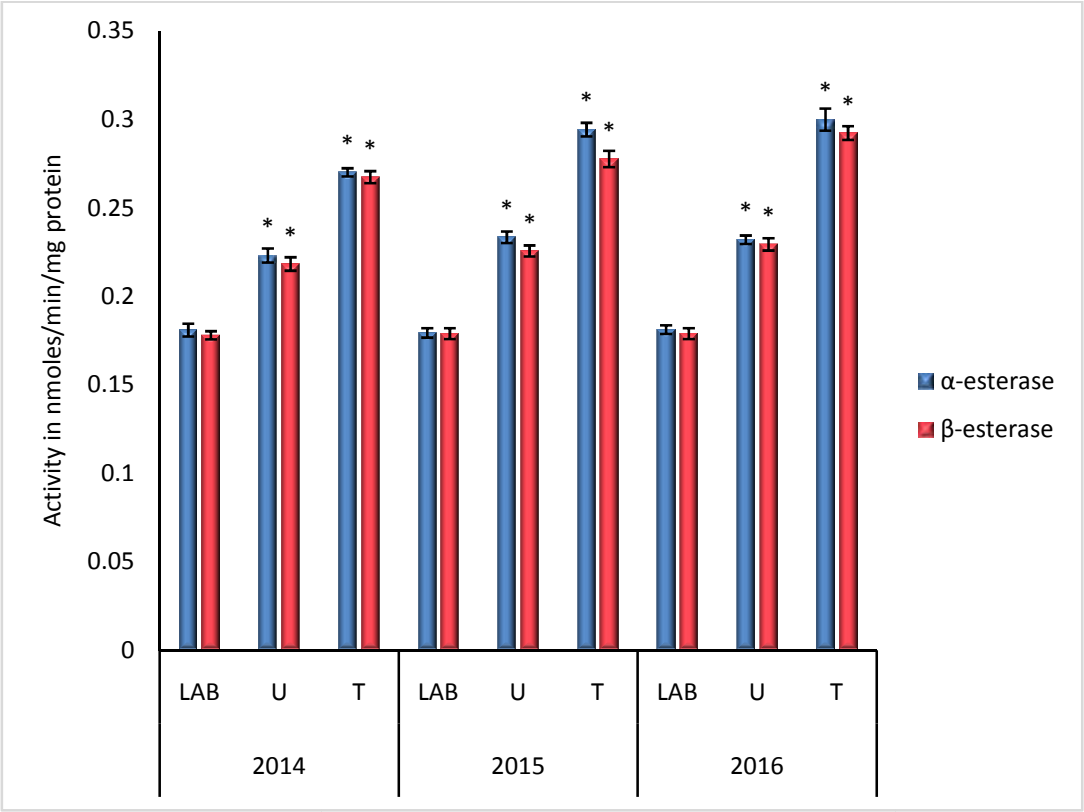
Table 6 provides α& β esterase activity of laboratory reared and field collected *Cx.quinquefasciatus* from CLT during 2014 to 2016. Year wise enzyme activity data of insecticide regularly treating area showed an upward trend with time, through that exhibited significant ($p<0.05$) increase in resistance.

Table 6: Data on Statistical analysis of α and β esterase of *Cx. quinquefasciatus* collected from CLT

Year	Area of sample collection	Mean enzyme activity ± SD (nmol α/β naphthol/min/ mg protein		One – way ANOVA	
		α-esterase	β- esterase	α-esterase	β-esterase
2014	LAB	0.181±0.0036	0.178±0.002	F = 3795.75 p< 0.00001 The result is significant at p< .05	F = 1709.35 p< 0.00001 The result is significant at p< .05
	U	0.22±0.004	0.22±0.006		
	T	0.26±0.002	0.24±0.003		
2015	LAB	0.179±0.0027	0.179±0.003	F = 6444.03 p< 0.00001 The result is significant at p< .05	F = 1347.76 p< 0.00001 The result is significant at p< .05
	U	0.23±0.003	0.22±0.006		
	T	0.27±0.004	0.25±0.006		
2016	LAB	0.181±0.002	0.179±0.003	F = 4518.29 p< 0.00001 The result is significant at p< .05	F = 2263.36 p< 0.00001 The result is significant at p< .05
	U	0.23±0.002	0.22±0.004		
	T	0.28±0.006	0.27±0.008		

Figure 5 show the carboxylesterase activity of laboratory reared and field collected *Cx. quinquefasciatus* from EKM during 2014 to 2016. The α-esterase and β- esterase values of laboratory population are 0.181±0.0036, 0.179±0.0027, 0.181±0.002 and 0.178±0.002, 0.179±0.003 and 0.179±0.003 nanomoles/min/mg of protein during 2014, 2015 and 2016. The EKM U and EKM T populations have the α- esterase values 0.22±0.004, 0.23±0.003, 0.23±0.002 and 0.27±0.002, 0.29±0.004, 0.3±0.006 and β- esterase values of 0.218±0.004, 0.226±0.003, 0.229±0.002 and 0.267±0.003, 0.278±0.005, 0.292±0.004 nanomoles/min/mg of protein in the three consecutive years respectively.

Figure 5: Carboxylesterase activity of *Cx. quinquefasciatus* from EKM



Level of significance p<0.05; * = p<0.05

Table 7 provided the data on statistical analysis of α and β esterase activity of *Cx. quinquefasciatus* collected from EKM. In all the cases the p value was less than 0.00001 hence the results were highly significant and there were changes in the values of laboratory and field population.

Table 7: Data on Statistical analysis of α and β esterase of *Cx. quinquefasciatus* collected from EKM

Year	Area of sample collection	Mean enzyme activity ± SD (nmol α/β naphthol/min/ mg protein		One – way ANOVA	
		α-esterase	β- esterase	α-esterase	β-esterase
2014	LAB	0.181±0.0036	0.178±0.002	F = 5401.63 p< 0.00001 The result is significant at p< .05	F = 5867.15 p< 0.00001 The result is significant at p< .05
	U	0.22±0.004	0.22±0.004		
	T	0.27±0.002	0.27±0.003		
2015	LAB	0.179±0.0027	0.179±0.003	F = 9526.01 p< 0.00001 The result is significant at p< .05	F = 5583.41 p< 0.00001 The result is significant at p< .05
	U	0.23±0.003	0.23±0.003		
	T	0.29±0.004	0.28±0.005		
2016	LAB	0.181±0.002	0.179±0.003	F = 6425.96 p< 0.00001 The result is significant at p< .05	F = 8065.99 p< 0.00001 The result is significant at p< .05
	U	0.23±0.002	0.23±0.004		
	T	0.3±0.006	0.29±0.004		

The increased enzyme activity is represented in the following tables. It is calculated using the formula: (The activity in the present year - The activity in the previous year)/ The activity in the previous year X 100.

Table 8: Increased activity of α esterase level in the field and laboratory populations of *Cx.quinquefasciatus*

Area of sample collection	Increased activity % in 2015 from 2014	Increased activity % in 2016 from 2015	Increased activity % in 2016 from 2014
LAB	-1.105	1.117	0
MPM U	3.810	0.459	4.286
MPM T	3.571	0.000	3.571
PKD U	3.774	1.818	5.660
PKD T	5.394	1.575	7.054
TCR U	3.636	0.877	4.545
TCR T	4.938	1.961	6.996
CLT U	2.703	1.754	4.505
CLT T	5.814	3.297	9.302
EKM U	4.484	-0.429	4.036
EKM T	8.889	2.041	11.111

α esterase activity increase in EKM population was 11.11% in the year 2016 as compared with the year 2014. The level of increase from the 2015 to 2016 was less as compared with level of increase in 2014 to 2016. More than 5% activity increase was obtained in PKD, TCR, CLT and EKM. But only at PKD the scarcely treating area also shows a value of increased activity more than 5.

Table 9: Increased activity of β esterase level in the field and laboratory populations of *Cx. quinquefasciatus*

Area of sample collection	Increased activity % in 2015 from 2014	Increased activity % in 2016 from 2015	Increased activity % in 2016 from 2014
LAB	0.562	0.00	0.562
MPM U	1.485	0.488	1.980
MPM T	2.283	1.786	4.110
PKD U	1.961	2.885	4.902
PKD T	3.879	5.809	9.914
TCR U	2.913	2.830	5.825
TCR T	7.556	7.438	15.556
CLT U	1.852	1.818	3.704
CLT T	5.882	7.143	13.445
EKM U	3.670	1.327	5.046
EKM T	4.120	5.036	9.363

Increased activity of β esterase was shown by TCR and CLT as compared with other areas. The activity increase percentage of TCR *Culex* population in 2015/2014, 2016/2015, 2016/2014 was 7.556, 7.438 and 15.556 respectively.

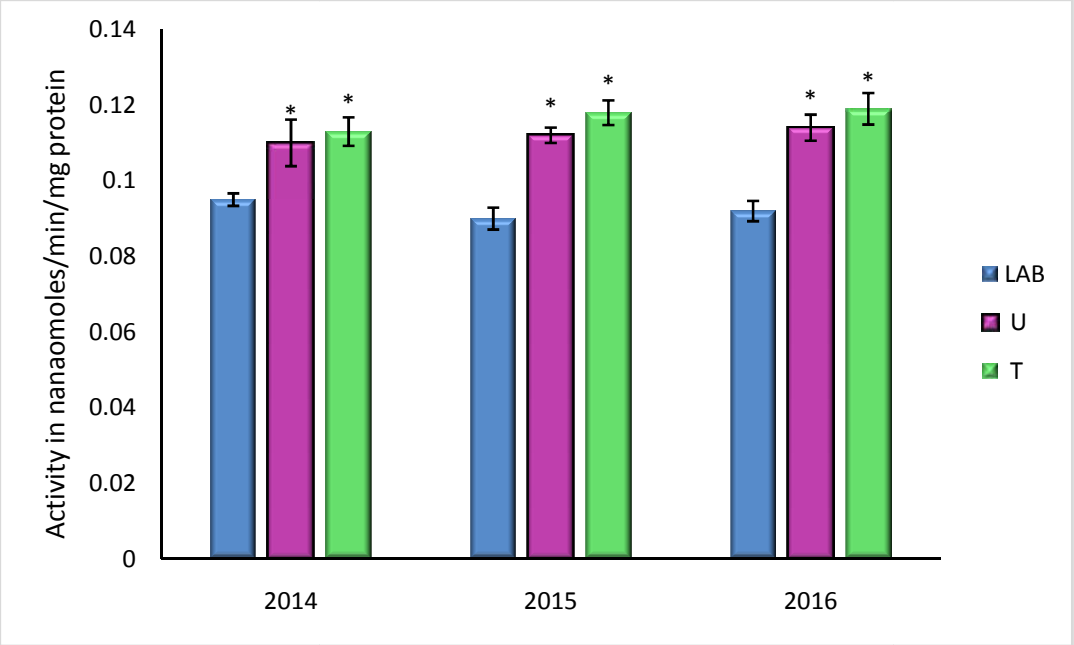
1.4.2.2. Glutathione-S-transferase activity:

The glutathione-S-transferases (GSTs) are members of a large family of multifunctional intracellular enzymes involved in the detoxification of endogenous and xenobiotic compounds via glutathione conjugation, dehydrochlorination, glutathione peroxidase activity or passive/sacrificial

binding (Hayes and Wolf, 1988; Mannervik *et al*., 1988; Pickett and Lu, 1989; Yang *et al.,* 2001). Elevated levels of GST activity have been found to be associated to insecticide resistance in many insects. One or more GSTs have often been implicated in the resistance to organophosphates (OPs), organochlorine (OC) and pyrethroids. In the present study the GST levels were elevated in all cases and the results are as follows.

Figure 6 exhibit the data on GST activity of *Cx. quinquefasciatus* collected from MPM. The laboratory population has the value of 0.0945±0.001, 0.0928±0.002, 0.0921±0.113 nanomoles/min/mg of protein GST activity in the years 2014, 15 and 16. The MPM U and MPM T GST values of the three consecutive years were 0.11±0.006, 0.111±0.002, 0.113±0.003 and 0.113±.003, 0.117± 0.003, 0.118±0.004 respectively.

Figure 6: GST activity of *Cx. quinquefasciatus* from MPM



Level of significance p<0.05; * = p<0.05

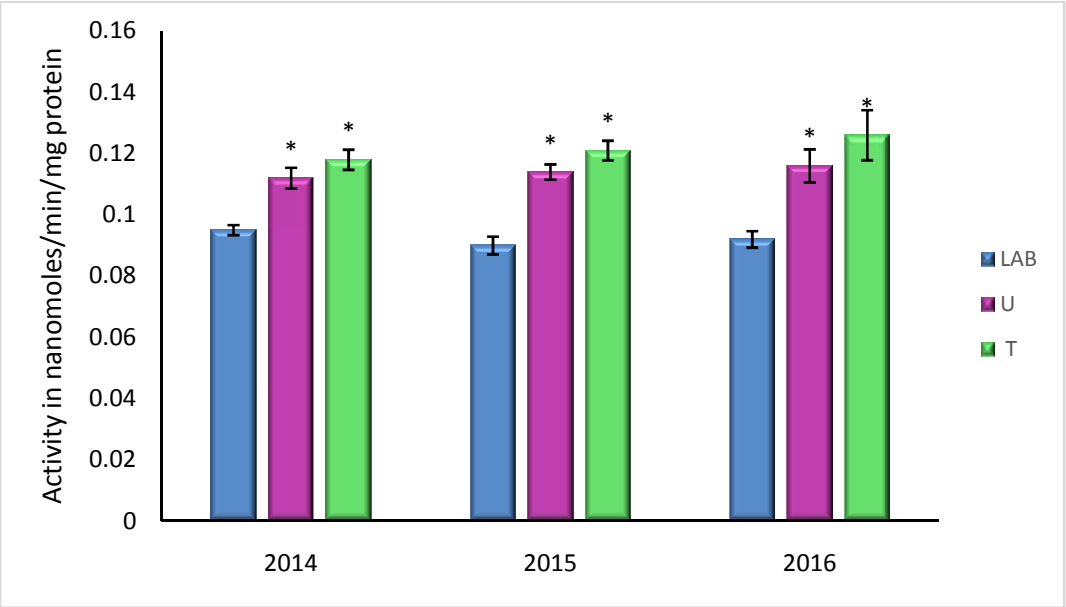
Table 8 provided the data on statistical analysis of GSTactivity of *Cx. quinquefasciatus* collected from MPM. In all the cases the p value was less than 0.00001 hence the results were highly significant for both laboratory and field populations.

Table 10: Data on Statistical analysis of GST activity of *Cx. quinquefasciatus* collected from MPM

Year	Area of sample collection	Mean enzyme activity ± SD (nmol /min/ mg protein)	One – way ANOVA
2014	LAB	0.09±0.002	F = 162.28 p< 0.00001 The result is significant at p< .05
	U	0.11±0.006	
	T	0.11±0.003	
2015	LAB	0.09±0.003	F = 677.62 p< 0.00001 The result is significant atp< .05
	U	0.11±0.002	
	T	0.12±0.003	
2016	LAB	0.09±0.003	F = 507.93 p< 0.00001 The result is significant at p< .05
	U	0.11±0.003	
	T	0.12±0.004	

Figure 7 shows the data on GST activity of *Cx. quinquefasciatus* collected from PKD. 0.0945±0.001, 0.0928±0.002, 0.0921±0.113 nanomoles/min/mg of protein GST activity was observed in laboratory population where the PKD U and PKD T GST values were 0.112±0.003, 0.114±0.002, 0.116±0.005 and 0.118±.003, 0.121± 0.003, 0.126±0.008 respectively in 2014, 2015 and 2016.

Figure 7: GST activity of *Cx. quinquefasciatus* from PKD



Level of significance p<0.05; * = p<0.05

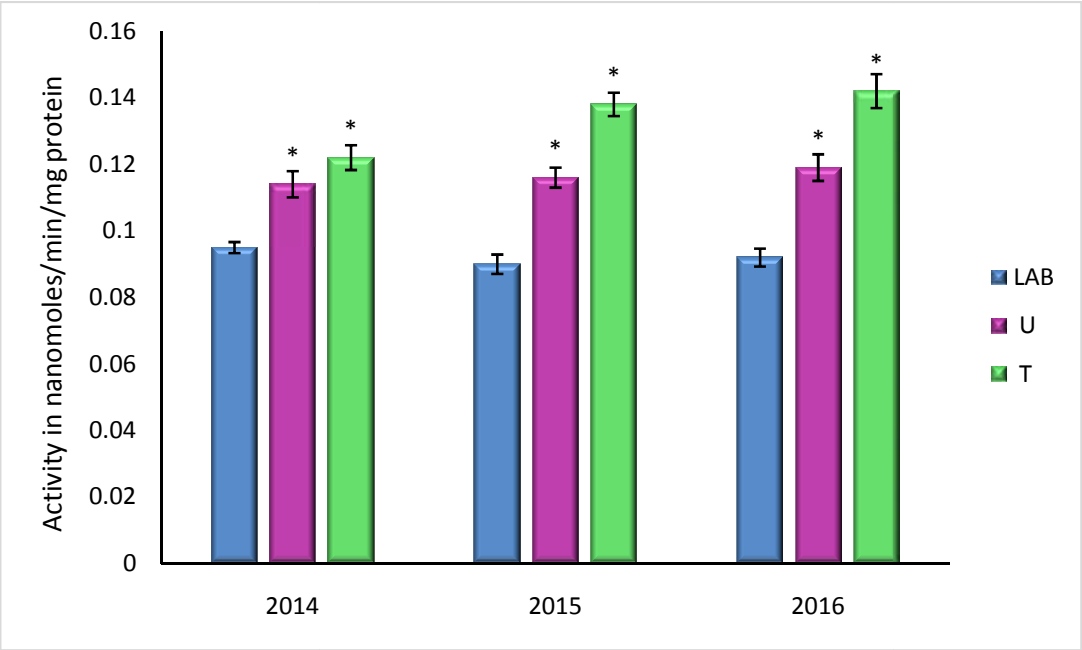
Table 11 exhibits the data on statistical analysis of GST of *Cx. quinquefasciatus* collected from PKD. The GST F values for 2014, 2015 and 2016 years were 541.03, 811.21 and 272.99 respectively. In all the cases the p value was less than 0.00001, hence the results were highly significant and there was change in the values of laboratory population and field populations.

Table 11: Data on Statistical analysis GST activity of *Cx. quinquefasciatus* collected from PKD

Year	Area of sample collection	Mean enzyme activity ± SD (nmol /min/ mg protein)	One – way ANOVA
2014	LAB	0.09±0.002	F = 541.03 p< 0.00001 The result is significant at p< .05
	U	0.11±0.003	
	T	0.12±0.003	
2015	LAB	0.09±0.003	F = 811.21 p< 0.00001 The result is significant at p< .05
	U	0.11±0.002	
	T	0.12±0.003	
2016	LAB	0.09±0.003	F = 272.99 p< 0.00001 The result is significant at p< .05
	U	0.12±0.005	
	T	0.13±0.008	

GST activity of *Cx. quinquefasciatus* collected from TCR was represented in Figure 8. The laboratory population has the value of 0.0945±0.001, 0.0928±0.002, 0.0921±0.113 nanomoles/min/mg of protein GST activity during 2014, 2015 and 2016. The TCR U and TCR T GST values of the three consecutive years were 0.114±0.003, 0.116±0.003, 0.119±0.003 and0.122±0.003, 0.138±0.003, 0.142±0.005 respectively.

Figure 8: GST activity of *Cx. quinquefasciatus* from TCR



Level of significance p<0.05; * = p<0.05

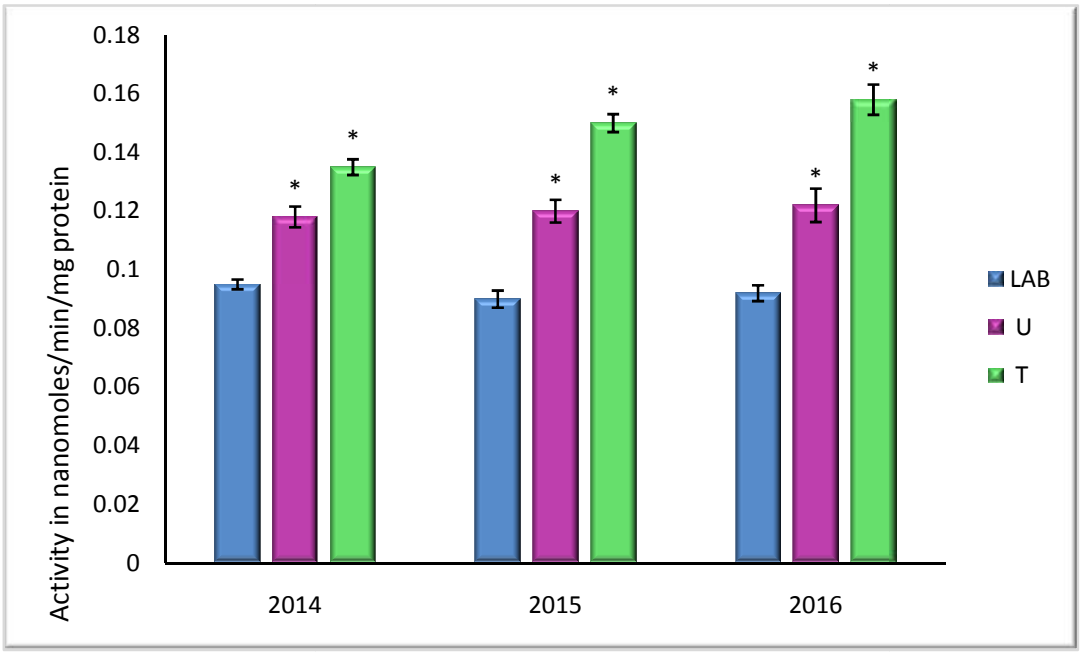
Table 12 provides the GSTactivity of laboratory reared and field collected strains of *Cx.quinquefasciatus* from TCR during 2014 to 2016. Year wise enzyme activity showed an upward trend with time, through that exhibited significant (p<0.05) increase in resistance.

Table 12: Data on Statistical analysis GST activity of *Cx. quinquefasciatus* collected from TCR

Year	Sample	Mean enzyme activity ± SD (nmol /min/ mg protein)	One – way ANOVA
2014	LAB	0.09±0.002	F = 570.49 p< 0.00001 The result is significant at p< .05
	U	0.11±0.004	
	T	0.12±0.004	
2015	LAB	0.09±0.003	F = 1559.22 p< 0.00001 The result is significant at p< .05
	U	0.12±0.003	
	T	0.14±0.004	
2016	LAB	0.09±0.003	F = 1183.61 p< 0.00001 The result is significant at p< .05
	U	0.12±0.004	
	T	0.14±0.005	

GST activity of *Cx. quinqueafsciatus* collected from CLT was represented in Figure 9. The laboratory population, CLT U and CLT T have the GST values of 0.0945±0.001, 0.0928±0.002, 0.0921±0.113; 0.118±0.003, 0.12±0.003, 0.122±0.005 and 0.135±0.002, 0.15± 0.003, 0.158 ±0.005 nanomoles/min/mg of protein activity in the years 2014,15 and 16.

Figure 9: GST activity of *Cx. quinquefasciatus* from CLT



Level of significance $p<0.05$; * = $p<0.05$

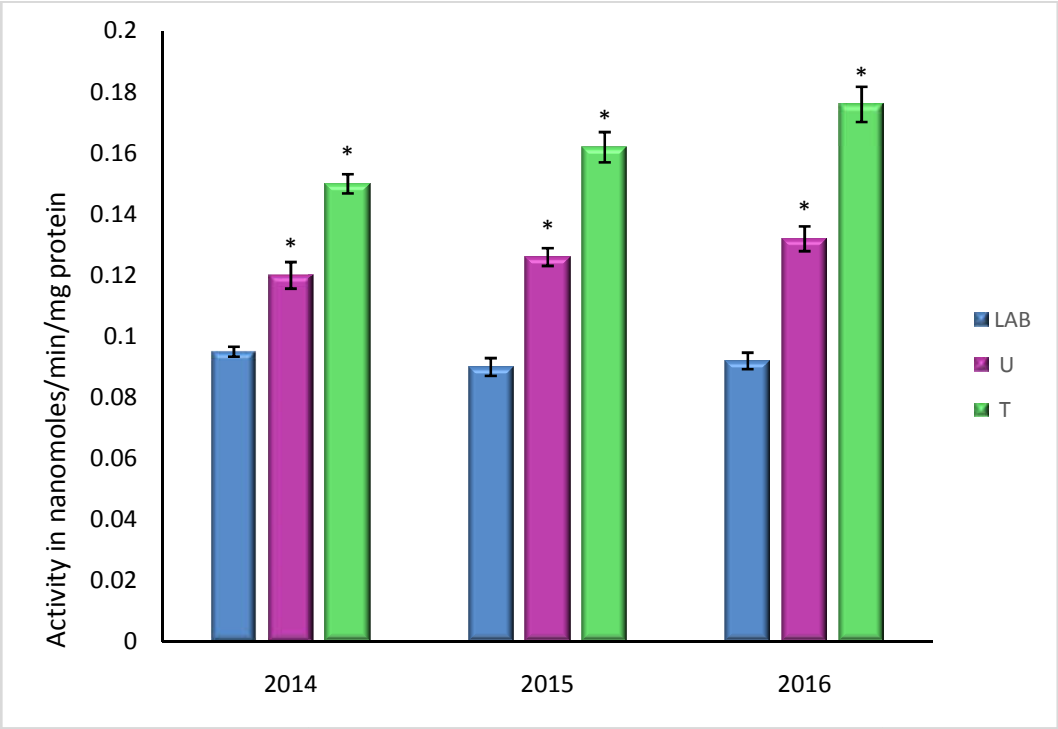
Table 13 provides the GST activity of laboratory reared and field collected strains of *Cx. quinquefasciatus* from CLT during 2014 to 2016. Year wise enzyme activity showed an upward trend with time and there is significant change between the laboratory and field populations.

Table 13: Data on Statistical analysis GST activity of *Cx. quinquefasciatus* collected from CLT

Year	Sample	Mean enzyme activity ± SD (nmol /min/ mg protein)	One – way ANOVA
2014	LAB	0.095±0.002	F = 570.49 p< 0.00001 The result is significant at p< .05
	U	0.118±0.004	
	T	0.135±0.003	
2015	LAB	0.093±0.003	F = 1559.22 p< 0.00001 The result is significant at p< .05
	U	0.12±0.004	
	T	0.15±0.003	
2016	LAB	0.092±0.003	F = 1183.61 p< 0.00001 The result is significant at p< .05
		0.122±0.006	
		0.158±0.005	

Figure 10 represents the data on GST activity of *Cx. quinquefasciatus* collected from EKM. The laboratory population has the value of 0.094±0.001, 0.092±0.002, 0.092±0.113 nanomoles/min/mg of protein GST activity in the years 2014, 15 and 16. The EKM U and EKM T GST values of the three consecutive years were 0.12±0.004, 0.12±0.002, 0.13±0.004 and 0.15±.003, 0.16± 0.004, 0.17±0.005 respectively.

Figure 10: GST activity of *Cx. quinquefasciatus* from EKM



Level of significance p<0.05; * = p<0.05

Table 14 provides the GST activity of laboratory reared and field collected strains of *Cx. quinquefasciatus* from EKM during 2014 to 2016. Year wise enzyme activity data of insecticide regularly treating area showed an upward trend with time, through that exhibited significant (p<0.05) increase in resistance.

Table 14: Data on Statistical analysis GST activity of *Cx. quinquefasciatus* collected from EKM

Year	Area of sample collection	Mean enzyme activity ± SD (nmol /min/ mg protein)	One – way ANOVA
2014	LAB	0.09±0.002	F = 2286.24
	U	0.12±0.004	p< 0.00001
	T	0.15±0.003	The result is significant at p< .05
2015	LAB	0.09±0.003	F = 2666.42
	U	0.13±0.003	p< 0.00001
	T	0.16±0.005	The result is significant at p< .05
2016	LAB	0.09±0.003	F = 2851.25
	U	0.13±0.004	p< 0.00001
	T	0.18±0.006	The result is significant at p< .05

Table 15 exhibits data on the increased levels of GST enzyme activity in the field and laboratory populations from the previous years, the comparison of data reveals the hike of enzyme activity. EKM, CLT and TCR population have a higher value of increase in the enzyme activity whereas the MPM population has the least value.

Table 15: Increased activity of GST level in the field and laboratory populations of *Cx. quinquefasciatus*

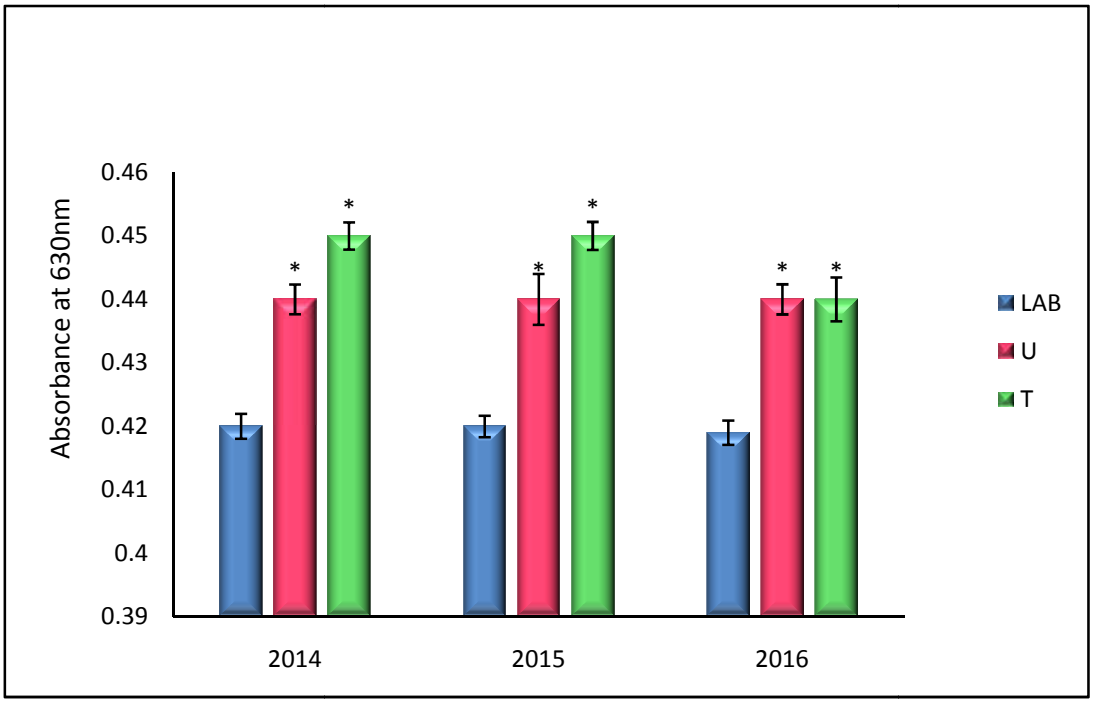
Area of sample collection	Increased activity % in 2015 from 2014	Increased activity % in 2016 from 2015	Increased activity % in 2016 from 2014
LAB	-2.105	-1.075	-3.158
MPM U	1.818	1.786	3.636
MPM T	4.425	0.847	5.310
PKD U	1.786	1.754	3.571
PKD T	2.542	4.132	6.780
TCR U	1.754	2.586	4.386
TCR T	13.115	2.899	16.393
CLT U	1.695	1.667	3.390
CLT T	11.111	5.333	17.037
EKM U	5.000	4.762	10.000
EKM T	8.000	8.642	17.333

1.4.2.3 Monooxygenase activity:

P450 mediated monooxygenase (MFOs) are known to be involved in the detoxification of organophosphate, pyrethroid and carbamate insecticides (Hemingway, 2000). Among these mixed function oxidase-mediated resistance to pyrethroids was more predominant.

The monooxygenase activity of laboratory reared and field collected *Cx. quinquefasciatus* from MPM during 2014 to 2016 were represented in figure 11. The laboratory population had values of 0.42±0.002, 0.42±0.001, 0.42±0.002 Optical density (OD) during 2014 to 2016. The MPM U and MPM T populations have the values of 0.44±0.002, 0.44±0.004, 0.44±0.004 and 0.45±0.002, 0.45±0.002, 0.44±0.003 respectively.

Figure 11: Monooxygenase activity in *Cx. quinquefasciatus* from MPM



Level of significance $p<0.05$; * = $p<0.05$

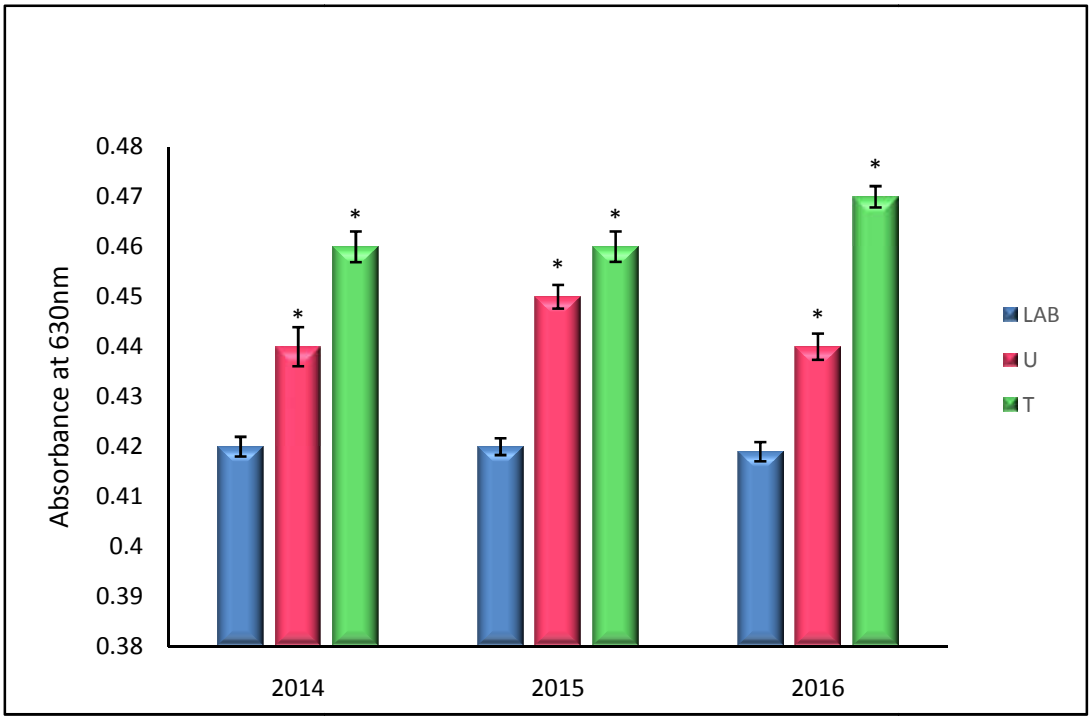
Table 16 provided the data on mean enzyme activity and its statistical analysis on *Cx. quinquefasciatus* collected from MPM. In all the cases the p value was less than 0.00001 and it proves there is significant change between the laboratory and filed collected mosquitoes.

Table 16: Data on monooxygenase activity (absorbance± SD) and statistical analysis of *Cx. quinquefasciatus* collected from MPM

Year	Area os sample collection	Mean enzyme activity ± SD (Absorbance at 630 nm)	One – way ANOVA
2014	LAB	0.42±0.002	F = 1563.03 p< 0.00001 The result is significant at p<0.05
	U	0.44±0.002	
	T	0.45±0.002	
2015	LAB	0.42±0.0016	F = 867.07 p< 0.00001 The result is significant at p<0.05
	U	0.44±0.004	
	T	0.45±0.002	
2016	LAB	0.42±0.002	F = 588.92 p< 0.00001 The result is significant at p<0.05
	U	0.44±0.004	
	T	0.44±0.003	

Figure 12 provides the data on monooxygenase activity of *Cx. quinquefasciatus* collected from PKD. 0.42±0.002, 0.42±0.0016, 0.42±0.02 absorbance at 630nm was observed in laboratory population where the PKD U and PKD T the OD values were 0.44±0.004, 0.45±0.002, 0.44±0.003 and 0.46±.003, 0.46± 0.003, 0.47±0.002 respectively in 2014, 2015 and 2016.

Figure 12: Monooxygenase acivity in *Cx. quinquefasciatus* from PKD



Level of significance p<0.05; * = p<0.05

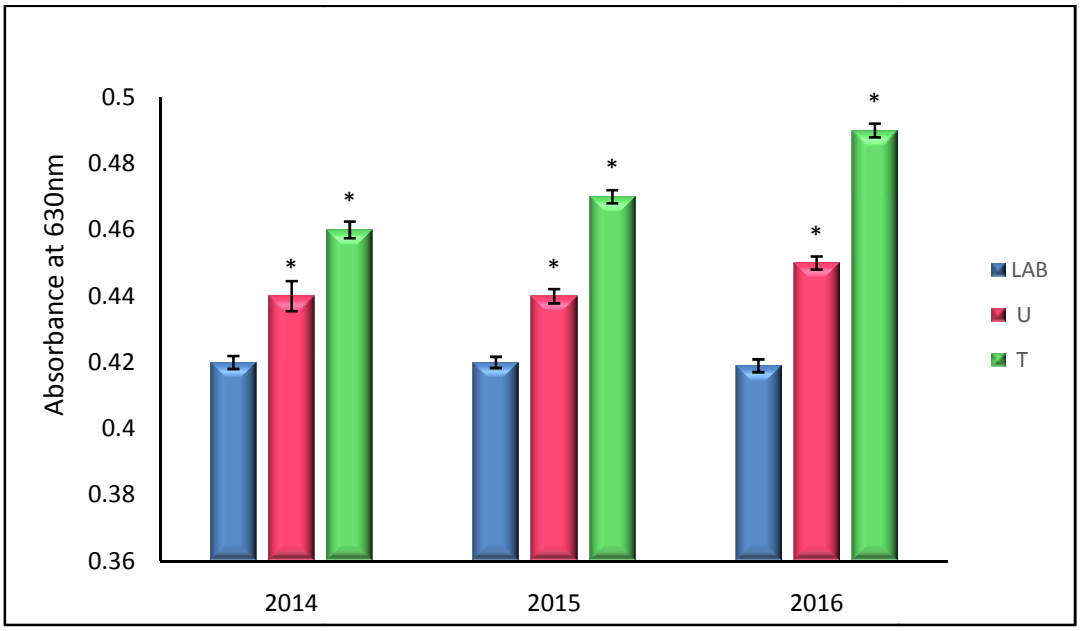
Table 17 show the data on statistical analysis of monoxygenase of *Cx. quinquefasciatus* collected from PKD. The enzyme F values for 2014, 2015 and 2016 years were 1245.04, 2159.49 and 3831.44 respectively. In all the cases the p value was less than 0.00001, hence the results were highly significant.

Table 17: Data on mean enzyme activity± SD and statistical analysis of *Cx quinquefasciatus* collected from PKD

Year	Area of sample collection	Mean enzyme activity ± SD (Absorbance at 630 nm)	One – way ANOVA
2014	LAB	0.42±0.002	F = 1245.04 p< 0.00001 The result is significant at p< .05
	U	0.44±0.004	
	T	0.46±0.003	
2015	LAB	0.42±0.0016	F = 2159.49 p< 0.00001 The result is significant at p< .05
	U	0.45±0.002	
	T	0.46±0.003	
2016	LAB	0.42±0.002	F = 3831.44 p< 0.00001 The result is significant at p< .05
	U	0.44±0.003	
	T	0.47±0.002	

Figure 13 illustrates the monooxygenase activity levels of *Cx. quinquefasciatus* collected from TCR during 2014 to 2016. According to the results, samples from the insecticide regularly applying areas have the highest level of enzyme activity. *Culex* cultured in the laboratory condition shows the lowest activity.

Figure 13: Monooxygenase activity in *Cx. quinquefasciatus* from TCR



Level of significance p<0.05; * = p<0.05

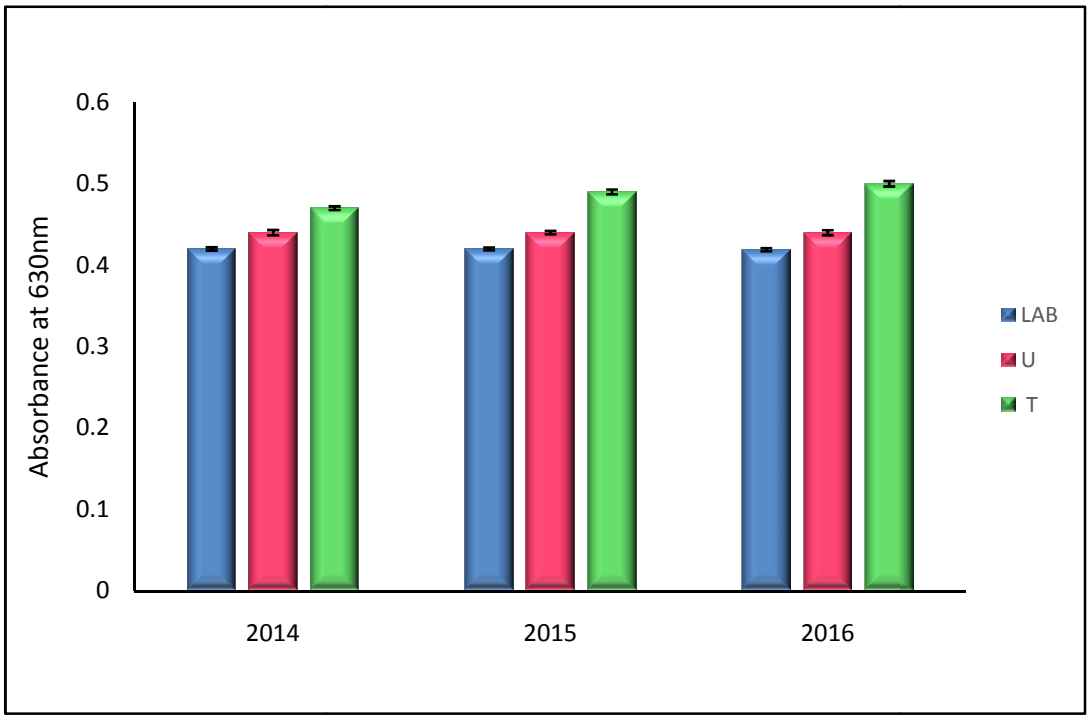
Table 18 exhibits the monooxygenaseactivity of laboratory reared and field collected strains of *Cx.quinquefasciatus* from TCR during 2014 to 2016. Area wise statistical analysis on enzyme activity showed an upward trend with time, through that exhibited significant ($p<0.05$) increase in detoxification.

Table 18: Data on mean enzyme activity± SD and statistical analysis of *Cx. quinquefasciatus* collected from TCR

Year	Area of sample collection	Mean enzyme activity ± SD (Absorbance at 630 nm)	One – way ANOVA
2014	LAB	0.42±0.002	F = 1191.76 p< 0.00001 The result is significant at p< .05
	U	0.44±0.005	
	T	0.46±0.003	
2015	LAB	0.42±0.0016	F = 4945.95 p< 0.00001 The result is significant at p< .05
	U	0.44±0.002	
	T	0.47±0.002	
2016	LAB	0.42±0.002	F = 9739.09 p< 0.00001 The result is significant at p< .05
	U	0.45±0.002	
	T	0.49±0.002	

Monooxygenase activity of *Cx. quinquefasciatus* collected from CLT was represented in Figure 14. The laboratory population, CLT U and CLT T have the OD values of 042±0.002, 0.42±0.0016, 0.42±0.002; 0.44±0.003, 0.44±0.002, 0.44±0.003 and 0.47±0.002, 0.49±0.003, 0.50±0.003 at 630nm in the years 2014,15 and 16.

Figure 14: Monooxygenase activity in *Cx. quinquefasciatus* from CLT



Level of significance p<0.05; * = p<0.05

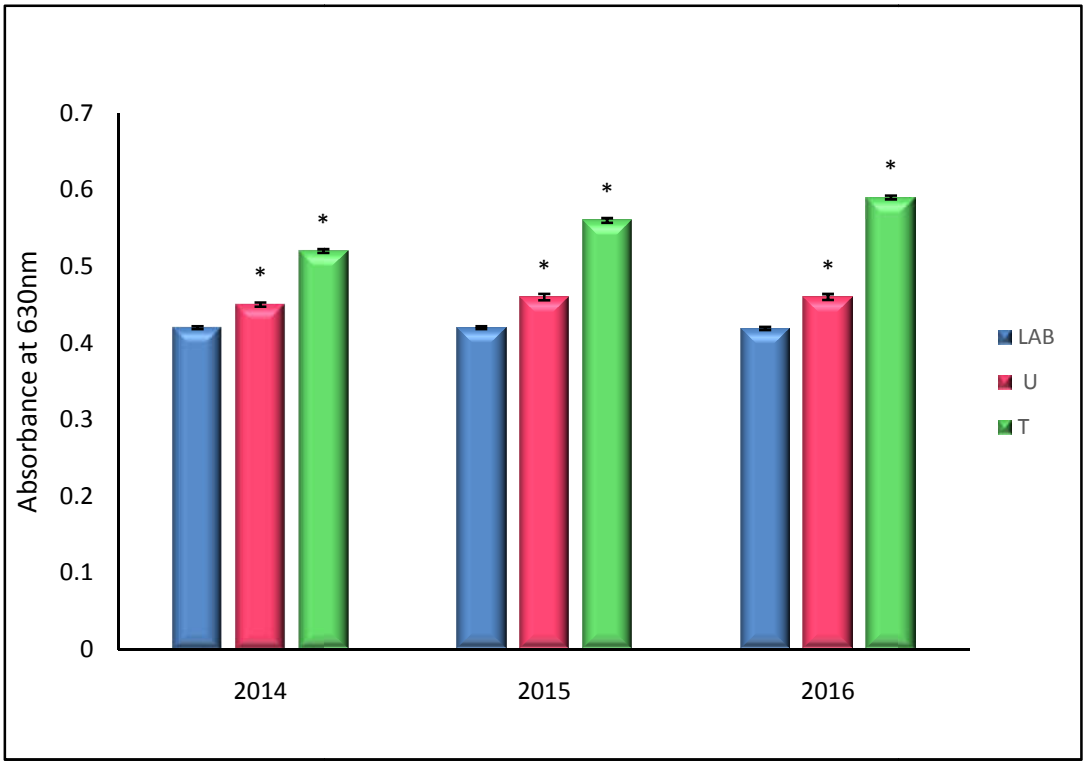
Table 19 provides the monoxygenase activity of laboratory reared and field collected strains of *Cx. quinquefasciatus* from CLT during 2014- 2016. Year wise enzyme activity data of insecticide regularly treating area showed an upward trend with time, through that exhibited significant (p<0.05) increase in resistance.there is significant change between the laboratory and filed populations.

Table 19: Data on mean enzyme activity± SD and statistical analysis of *Cx. quinquefasciatus* collected from CLT

Year	Area of sample collection	Mean enzyme activity ± SD (Absorbance at 630 nm)	One – way ANOVA
2014	LAB	0.42±0.002	F = 3008.82 p< 0.00001 The result is significant at p< .05
	U	0.44±0.003	
	T	0.47±0.002	
2015	LAB	0.42±0.0016	F = 7370.85 p< 0.00001 The result is significant at p< .05
	U	0.44±0.002	
	T	0.49±0.003	
2016	LAB	0.42±0.002	F = 6883.15 p< 0.00001 The result is significant at p< .05
	U	0.44±0.003	
	T	0.50±0.003	

Figure 15 represents the monooxygenase activity of laboratory reared and field collected *Cx. quinquefasciatus* from EKM during 2014 to 2016. The OD values of LAB were 0.42±0.002, 0.42±0.0016 and 0.42±0.002 at 630nm during 2014 to 2016. The EKM U and EKM T populations have the absorbance values 0.45±0.003, 0.46±0.004, 0.46±0.004 and 0.52±0.002, 0.56±0.003, 0.59±0.002 respectively.

Figure 15: Monooxygenase activity in *Cx. quinquefasciatus* from EKM



Level of significance p<0.05; * = p<0.05

Table 20 presents the data on statistical analysis of monooxygenase activity of *Cx. quinquefasciatus* collected from EKM. In all the cases the p value was less than 0.00001 hence the results were highly significant between the laboratory and field population.

Table20: Data on mean enzyme activity ± SD and statistical analysis of *Cx. quinquefasciatus* collected from EKM

Year	Area of sample collection	Mean enzyme activity ± SD (Absorbance at 630 nm)	One – way ANOVA
2014	LAB	0.42±0.002	F = 14200.69 p< 0.00001 The result is significant at p< .05
	U	0.45±0.003	
	T	0.52±0.002	
2015	LAB	0.42±0.001	F = 15888.38 p< 0.00001 The result is significant at p< .05
	U	0.46±0.004	
	T	0.56±0.003	
2016	LAB	0.42±0.002	F = 29431.69 p< 0.00001 The result is significant at p< .05
	U	0.46±0.004	
	T	0.59±0.002	

The increased MFO enzyme activity levels of the field and laboratory population from the previous years were depicted in the table 21, the comparison revealed the uphill of enzyme activity during the study period. EKM, *Culex* populations have a higher value (13.46%) of increase in the enzyme activity whereas the MPM, PKD populations have no change in the enzyme activity and the TCR and CLT populations have 6.52 and 6.38% increase from 2014 to 2016.

Table 21: Increased activity of MFO levels in the field and laboratory populations of *Cx. quinquefasciatus*

Area of sample collection	Increased activity % in 2015 from 2014	Increased activity % in 2016 from 2015	Increased activity % in 2016 from 2014
LAB	0.00	0.00	0.00
MPM U	0.00	0.00	0.00
MPM T	0.00	-2.22	-2.22
PKD U	2.27	-2.22	0.00
PKD T	0.00	2.17	2.17
TCR U	0.00	2.27	2.27
TCR T	2.17	4.26	6.52
CLT U	0.00	0.00	0.00
CLT T	4.26	2.04	6.38
EKM U	2.22	0.00	2.22
EKM T	7.69	5.36	13.46

Analysis and Interpretation of Data:

Table 22, 23, 24, 25 provides data on the statistical analysis (ANOVA) of area wise enzymatic activity data in each year. The results proved that there is highly significant difference (p=0.00) in the α, β, GST and MFO activity between the LAB and field collected mosquito samples. The f-value and level of significance (p) is specified in each table and the sample size maintained was 31 in each case.

Table 22a: Level of significance while comparing (ANOVA) the α esterase of the laboratory and filed populations of *Cx.quinquefasciatus* in the year 2014

Area of collection	LAB	MPM U	MPM T	PKD U	PKD T	TCR U	TCR T	CLT U	CLT T	EKM U	EKM T
LAB	a	a	a	a	a	a	a	a	a	a	a
MPMU	a	a	a	b	a	a	a	a	a	a	a
MPM T	a	a	a	a	a	b	a	b	a	b	a
PKD U	a	b	a	a	a	a	a	a	a	a	a
PKD T	a	a	a	a	a	a	b	a	a	a	a
TCR U	a	a	b	a	a	a	a	b	a	b	a
TCR T	a	a	a	a	a	a	a	a	a	a	a
CLT U	a	a	b	a	a	a	a	a	a	a	a
CLT T	a	a	a	a	a	a	a	a	a	a	a
EKM U	a	a	b	a	a	a	a	b	a	a	a
EKM T	a	a	a	a	a	a	a	a	a	a	a
F= 820.717, a= p<0.05, significant b= p>0.05, not significant											

Table 22b: Level of significance while comparing (ANOVA) the α esterase of the laboratory and filed populations of *Cx.quinquefasciatus* in the year 2015

Area of collection	LAB	MPM U	MPM T	PKD U	PKD T	TCR U	TCR T	CLT U	CLT T	EKM U	EKM T
LAB	a	a	a	a	a	a	a	a	a	a	a
MPMU	a	a	a	a	a	a	a	a	a	a	a
MPM T	a	a	a	b	a	a	a	a	a	b	a
PKD U	a	b	a	a	a	a	a	a	a	a	a
PKD T	a	a	a	a	a	a	b	a	a	a	a
TCR U	a	a	a	a	a	a	a	b	a	a	a
TCR T	a	a	a	a	b	a	a	a	a	a	a
CLT U	a	a	a	a	a	b	a	a	a	a	a
CLT T	a	a	a	a	a	a	a	a	a	a	a
EKM U	a	a	b	a	a	a	a	a	a	a	a
EKM T	a	a	a	a	a	a	a	a	a	a	a
F= 2766.37, a= p<0.05, significant b= p>0.05, not significant											

Table 22c: Level of significance while comparing (ANOVA) the α esterase of the laboratory and filed populations of *Cx.quinquefasciatus* in the year 2016

Area of collection	LAB	MPM U	MPM T	PKD U	PKD T	TCR U	TCR T	CLT U	CLT T	EKM U	EKM T
LAB	a	a	a	a	a	a	a	a	a	a	a
MPMU	a	a	a	a	a	a	a	a	a	a	a
MPM T	a	a	a	a	a	a	b	b	a	b	a
PKD U	a	a	a	a	a	a	a	a	a	a	a
PKD T	a	a	a	a	a	a	b	a	a	a	a
TCR U	a	a	a	a	a	a	a	b	a	b	a
TCR T	a	a	b	a	b	a	a	a	a	a	a
CLT U	a	a	b	a	a	b	a	a	a	a	a
CLT T	a	a	a	a	a	a	a	a	a	a	a
EKM U	a	a	b	a	a	b	a	a	a	a	a
EKM T	a	a	a	a	a	a	a	a	a	a	a
F= 2215.86, a= p<0.05, significant b= p>0.05, not significant											

Table 23a: Level of significance while comparing (ANOVA) the β esterase of the laboratory and filed populations of *Cx.quinquefasciatus* in the year 2014

Area of collection	LAB	MPMU	MPM T	PKD U	PKD T	TCR U	TCR T	CLT U	CLT T	EKM U	EKM T
LAB	a	a	a	a	a	a	a	a	a	a	a
MPMU	a	a	a	b	a	a	a	a	a	b	a
MPM T	a	a	a	a	a	a	a	a	a	a	a
PKD U	a	b	a	a	a	b	a	a	a	a	a
PKD T	a	a	a	a	a	a	a	a	a	a	a
TCR U	a	a	a	b	a	a	a	a	a	a	a
TCR T	a	a	a	a	a	a	a	a	a	a	a
CLT U	a	a	a	a	a	a	a	a	a	b	a
CLT T	a	a	a	a	a	a	a	a	a	a	a
EKM U	a	a	b	a	a	a	a	b	a	a	a
EKM T	a	a	a	a	a	a	a	a	a	a	a
F= 1190.727, a= p<0.05, significant b= p>0.05, not significant											

Table 23b: Level of significance while comparing (ANOVA) the β esterase of the laboratory and filed populations of *Cx.quinquefasciatus* in the year 2015

Area of collection	LAB	MPM U	MPM T	PKD U	PKD T	TCR U	TCR T	CLT U	CLT T	EKM U	EKM T
LAB	a	a	a	a	a	a	a	a	a	a	a
MPMU	a	a	a	b	a	a	a	a	a	a	a
MPM T	a	a	a	a	a	a	a	a	a	b	a
PKD U	a	b	a	a	a	a	a	a	a	a	a
PKD T	a	a	a	a	a	a	b	a	a	a	a
TCR U	a	a	a	a	a	a	a	a	a	a	a
TCR T	a	a	a	a	b	a	a	a	a	a	a
CLT U	a	a	a	a	a	a	a	a	a	a	a
CLT T	a	a	a	a	a	a	a	a	a	a	a
EKM U	a	a	b	a	a	a	a	a	a	a	a
EKM T	a	a	a	a	a	a	a	a	a	a	a
F= 440.967, a= p<0.05, significant b= p>0.05, not significant											

Table 23c: Level of significance while comparing (ANOVA) the β esterase of the laboratory and filed populations of *Cx.quinquefasciatus* in the year 2016

Area of collection	LAB	MPM U	MPM T	PKD U	PKD T	TCR U	TCR T	CLT U	CLT T	EKM U	EKM T
LAB	a	a	a	a	a	a	a	a	a	a	a
MPMU	a	a	a	a	a	a	a	a	a	a	a
MPM T	a	a	a	a	a	b	a	b	a	b	a
PKD U	a	a	a	a	a	a	a	a	a	a	a
PKD T	a	a	a	a	a	a	b	a	a	a	a
TCR U	a	a	b	a	a	a	a	b	a	b	a
TCR T	a	a	a	a	b	a	a	a	a	a	a
CLT U	a	a	b	a	a	a	b	a	a	a	a
CLT T	a	a	a	a	a	a	a	a	a	a	a
EKM U	a	a	b	a	a	b	a	a	a	a	a
EKM T	a	a	a	a	a	a	a	a	a	a	a
F= 1987.71; a= p<0.05, significant b= p>0.05, not significant											

Table 24a: Level of significance while comparing (ANOVA) the GST of the laboratory and filed populations of *Cx.quinquefasciatus* in the year 2014

Area of collection	LAB	MPMU	MPM T	PKD U	PKD T	TCR U	TCR T	CLT U	CLT T	EKM U	EKM T
LAB	a	a	a	a	a	a	a	a	a	a	a
MPMU	a	a	b	b	a	a	a	a	a	a	a
MPM T	a	b	a	b	a	b	a	a	a	a	a
PKD U	a	b	b	a	a	b	a	a	a	a	a
PKD T	a	a	a	a	a	a	a	b	a	b	a
TCR U	a	a	b	b	a	a	a	a	a	a	a
TCR T	a	a	a	a	a	a	a	a	a	b	a
CLT U	a	a	a	a	b	a	a	a	a	b	a
CLT T	a	a	a	a	a	a	a	a	a	a	a
EKM U	a	a	a	a	b	a	b	b	a	a	a
EKM T	a	a	a	a	a	a	a	a	a	a	a
F= 440.96, a= p<0.05, significant b= p>0.05, not significant											

Table 24b: Level of significance while comparing (ANOVA) the GST of the laboratory and filed populations of *Cx. quinquefasciatus* in the year 2015

Area of collection	LAB	MPM U	MPM T	PKD U	PKD T	TCR U	TCR T	CLT U	CLT T	EKM U	EKM T
LAB	a	a	a	a	a	a	a	a	a	a	a
MPMU	a	a	a	b	a	a	a	a	a	a	a
MPM T	a	a	a	a	a	b	a	b	a	a	a
PKD U	a	b	a	a	a	b	a	a	a	a	a
PKD T	a	a	a	a	a	a	a	b	a	a	a
TCR U	a	a	b	b	a	a	a	a	a	a	a
TCR T	a	a	a	a	a	a	a	a	a	a	a
CLT U	a	a	b	a	b	a	a	a	a	a	a
CLT T	a	a	a	a	a	a	a	a	a	a	a
EKM U	a	a	a	a	a	a	a	a	a	a	a
EKM T	a	a	a	a	a	a	a	a	a	a	a
F= 1045.935, a= p<0.05, significant b= p>0.05, not significant											

Table 24c: Level of significance while comparing (ANOVA) the GST of the laboratory and filed populations of *Cx.quinquefasciatus* in the year 2016

Area of collection	LAB	MPM U	MPM T	PKD U	PKD T	TCR U	TCR T	CLT U	CLT T	EKM U	EKM T
LAB	a	a	a	a	a	a	a	a	a	a	a
MPMU	a	a	a	b	a	a	a	a	a	a	a
MPM T	a	a	a	b	a	a	a	a	a	a	a
PKD U	a	b	b	a	a	b	a	b	a	a	a
PKD T	a	a	a	a	a	a	a	a	a	a	a
TCR U	a	a	b	b	a	a	a	b	a	a	a
TCR T	a	a	a	a	a	a	a	a	a	a	a
CLT U	a	a	b	a	a	b	a	a	a	a	a
CLT T	a	a	a	a	a	a	a	a	a	a	a
EKM U	a	a	a	a	a	a	a	a	a	a	a
EKM T	a	a	a	a	a	a	a	a	a	a	a
F= 627.96; a= p<0.05, significant b= p>0.05, not significant											

Table 25a: Level of significance while comparing (ANOVA) the MFO activity of the laboratory and filed populations of *Cx.quinquefasciatus* in the year 2014

Area of collection	LAB	MPM U	MPM T	PKD U	PKD T	TCR U	TCR T	CLT U	CLT T	EKM U	EKM T
LAB	a	a	a	a	a	a	a	a	a	a	a
MPMU	a	a	a	b	a	b	a	b	a	a	a
MPM T	a	a	a	a	a	a	a	a	a	b	a
PKD U	a	b	a	a	a	b	a	b	a	a	a
PKD T	a	a	a	a	a	a	b	a	a	a	a
TCR U	a	b	a	b	a	a	a	a	a	a	a
TCR T	a	a	a	a	b	a	a	a	a	a	a
CLT U	a	b	a	b	a	a	a	a	a	a	a
CLT T	a	a	a	a	a	a	a	a	a	a	a
EKM U	a	a	b	a	a	a	a	a	a	a	a
EKM T	a	a	a	a	a	a	a	a	a	a	a
a= p<0.05, significant b= p>0.05, not significant											

Table 25b: Level of significance while comparing (ANOVA) the MFO activity of the laboratory and filed populations of *Cx.quinquefasciatus* in the year 2015

Area of collection	LAB	MPM U	MPM T	PKD U	PKD T	TCR U	TCR T	CLT U	CLT T	EKM U	EKM T
LAB	a	a	a	a	a	a	a	a	a	a	a
MPMU	a	a	a	a	a	b	a	b	a	a	a
MPM T	a	a	a	b	a	a	a	a	a	a	a
PKD U	a	a	b	a	a	a	a	a	a	a	a
PKD T	a	a	a	a	a	a	a	a	a	b	a
TCR U	a	b	a	a	a	a	a	b	a	a	a
TCR T	a	a	a	a	a	a	a	a	a	a	a
CLT U	a	b	a	a	a	b	a	a	a	a	a
CLT T	a	a	a	a	a	a	a	a	a	a	a
EKM U	a	a	a	a	a	b	a	a	a	a	a
EKM T	a	a	a	a	a	a	a	a	a	a	a
F= 5391.59, a= p<0.05, significant b= p>0.05, not significant											

Table 25c: Level of significance while comparing (ANOVA) the MFO activity of the laboratory and filed populations of *Cx.quinquefasciatus* in the year 2016

Area of collection	LAB	MPM U	MPM T	PKD U	PKD T	TCR U	TCR T	CLT U	CLT T	EKM U	EKM T
LAB	a	a	a	a	a	a	a	a	a	a	a
MPMU	a	a	b	b	a	a	a	b	a	a	a
MPM T	a	b	a	b	a	a	a	b	a	a	a
PKD U	a	b	b	a	a	a	a	a	a	a	a
PKD T	a	a	a	a	a	a	a	a	a	a	a
TCR U	a	a	a	a	a	a	a	a	a	a	a
TCR T	a	a	a	a	a	a	a	a	a	a	a
CLT U	a	b	b	a	a	a	a	a	a	a	a
CLT T	a	a	a	a	a	a	a	a	a	a	a
EKM U	a	a	a	a	a	a	a	a	a	a	a
EKM T	a	a	a	a	a	a	a	a	a	a	a
F= 9184.962, a= p<0.05, significant b= p>0.05, not significant											

Table 26a provides the year wise descriptive profile of detoxification enzymes in the laboratory and field population of *Cx. quinquefasciatus.* The f value is 3.698 and p-value is 0.025 which is less than 0.05, so the result is significant ie., the null hypothesis is rejected at 0.05 level of significance. It means that there is significant change in the detoxification enzyme levels in year wise manner.

Table:26a Year wise descriptive profile of detoxification enzymes in laboratory and field collected *Cx. quinquefasciatus*

Year	N	Mean	Std. Deviation	Std. Error	ANOVA	
					F- value	Significane
2014	1364	0.2548	0.1246	0.0034	3.698	0.025
2015	1364	0.2625	0.1264	0.0034		
2016	1364	0.2678	0.1280	0.0035		
Total	4092	0.2617	0.1264	0.0019		

Table 26b provides data on the multiple comparisons of year wise data. There is significant change in the enzyme levels in 2016 with 2014, where p value is 0.019 which is less than 0.05. And in other cases, 2014 to 2015 and 2015 to 2016 the p value is greater than 0.05, hence there is no significant changes.

Table: 26b Multiple Comparisons of year wise data

(I) year	(J) year	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
2014	2015	-.0077323	.0048375	0.246	-.019074	.003610
	2016	-.0130843*	.0048375	0.019	-.024426	-.001742
2015	2016	-.0053521	.0048375	0.510	-.016694	.005990
*. The mean difference is significant at the 0.05 level.						

Table 27a and b provides data on the analysis of enzyme wise changes in the *Cx. quinquefasciatus.* The p- value is 0.00 in the profile; hence the changes in detoxification enzyme levels are highly significant.

Table: 27a Enzyme wise descriptive profile of laboratory and field collected *Cx. quinquefasciatus*

Enzymes	N	Mean	Std. Deviation	Std. Error	ANOVA	
					F- value	Significance
α -esterase	1023	.235490	.0288197	.0009011	25312.863	0.000
β -esterase	1023	.226333	.0272457	.0008518		
GST	1023	.123959	.0190321	.0005950		
MFO	1023	.461032	.0365063	.0011414		
Total	4092	.261704	.1264160	.0019762		

Table: 27b Multiple Comparisons of enzyme wise data

Enzymes	Enzymes	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
α - esterase	β - esterase	.0091564*	.0012638	0.000	.005908	.012404
	GST	.1115310*	.0012638	0.000	.108283	.114779
	MFO	-.2255425*	.0012638	0.000	-.228791	-.222294
			.0012638	0.000	.099127	.105623
β - esterase	GST	.1023746*	.0012638	0.000	-.237947	-.231451
	MFO	-.2346989*				
GST	MFO	-.3370735*	.0012638	0.000	-.340322	-.333825

Table 28a and b proves that there is significant change in the detoxification enzyme levels according to the area wise difference. The F value was17.935 and p value was zero and it is highly significant. The null hypothesis is rejected at 95% confidence interval. High level of significance was shown by PKD T, TCR T, CLT T, EKM U and EKM T with LAB. There is no significant difference in the enzyme levels of U areas. The CLT T and

EKM T shows a value p is equal to zero with MPM U. In all other cases EKM T shows high level significance with a p value of zero. According to the results, samples from the insecticide regularly applying areas have the highest level of enzyme activity. Mosquitoes cultured in the laboratory condition shows the lowest enzyme activity.

Table: 28a Area wise descriptive profile of laboratory and field collected *Cx. quinquefasciatus*

Area of collection	N	Mean	Std. Deviation	Std. Error	ANOVA	
					F- value	Significance
LAB	372	.218199	.1221209	.0063317	17.935	0.000
MPM U	372	.243005	.1208881	.0062678		
MPM T	372	.254108	.1203403	.0062394		
PKD U	372	.246422	.1214468	.0062967		
PKD T	372	.269586	.1231750	.0063863		
TCR U	372	.249702	.1201171	.0062278		
TCR T	372	.275562	.1239550	.0064268		
CLT U	372	.251804	.1169081	.0060614		
CLT T	372	.289478	.1240531	.0064319		
EKM U	372	.259242	.1215659	.0063029		
EKM T	372	.321632	.1457558	.0075571		
Total	4092	.261704	.1264160	.0019762		

Table: 28b Multiple Comparisons of area wise data

Area		Mean Difference	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
LAB	MPM U	-.0248059	.0090832	.186	-.054058	.004446
	MPM T	-.0359086*	.0090832	.004	-.065161	-.006656
	PKD U	-.0282231	.0090832	.070	-.057475	.001029
	PKD T	-.0513871*	.0090832	.000	-.080639	-.022135
	TCR U	-.0315027*	.0090832	.023	-.060755	-.002250
	TCR T	-.0573629*	.0090832	.000	-.086615	-.028111
	CLT U	-.0336048*	.0090832	.010	-.062857	-.004353
	CLT T	-.0712796*	.0090832	.000	-.100532	-.042027
	EKM U	-.0410430*	.0090832	.000	-.070295	-.011791

	EKM T	-.1034328*	.0090832	.000	-.132685	-.074180
MPM U	MPM T	-.0111027	.0090832	.980	-.040355	.018150
	PKD U	-.0034172	.0090832	1.000	-.032670	.025835
	PKD T	-.0265812	.0090832	.115	-.055833	.002671
	TCR U	-.0066968	.0090832	1.000	-.035949	.022556
	TCR T	-.0325570*	.0090832	.015	-.061809	-.003305
	CLT U	-.0087989	.0090832	.997	-.038051	.020453
	CLT T	-.0464737*	.0090832	.000	-.075726	-.017221
	EKM U	-.0162371	.0090832	.788	-.045489	.013015
	EKM T	-.0786269*	.0090832	.000	-.107879	-.049375
MPM T	PKD U	.0076855	.0090832	.999	-.021567	.036938
	PKD T	-.0154785	.0090832	.834	-.044731	.013774
	TCR U	.0044059	.0090832	1.000	-.024846	.033658
	TCR T	-.0214543	.0090832	.392	-.050707	.007798
	CLT U	.0023038	.0090832	1.000	-.026949	.031556
	CLT T	-.0353710*	.0090832	.005	-.064623	-.006119
	EKM U	-.0051344	.0090832	1.000	-.034387	.024118
	EKM T	-.0675242*	.0090832	.000	-.096777	-.038272
PKD U	PKD T	-.0231640	.0090832	.276	-.052416	.006088
	TCR U	-.0032796	.0090832	1.000	-.032532	.025973
	TCR T	-.0291398	.0090832	.052	-.058392	.000113
	CLT U	-.0053817	.0090832	1.000	-.034634	.023871
	CLT T	-.0430565*	.0090832	.000	-.072309	-.013804
	EKM U	-.0128199	.0090832	.946	-.042072	.016432
	EKM T	-.0752097*	.0090832	.000	-.104462	-.045957
PKD T	TCR U	.0198844	.0090832	.513	-.009368	.049137
	TCR T	-.0059758	.0090832	1.000	-.035228	.023277
	CLT U	.0177823	.0090832	.679	-.011470	.047035
	CLT T	-.0198925	.0090832	.512	-.049145	.009360
	EKM U	.0103441	.0090832	.988	-.018908	.039596
	EKM T	-.0520457*	.0090832	.000	-.081298	-.022793
TCR U	TCR T	-.0258602	.0090832	.141	-.055113	.003392
	CLT U	-.0021022	.0090832	1.000	-.031354	.027150
	CLT T	-.0397769*	.0090832	.001	-.069029	-.010525
	EKM U	-.0095403	.0090832	.994	-.038793	.019712
	EKM T	-.0719301*	.0090832	.000	-.101182	-.042678
TCR T	CLT U	.0237581	.0090832	.241	-.005494	.053010
	CLT T	-.0139167	.0090832	.909	-.043169	.015336
	EKM U	.0163199	.0090832	.782	-.012932	.045572
	EKM T	-.0460699*	.0090832	.000	-.075322	-.016818
CLT U	CLT T	-.0376747*	.0090832	.002	-.066927	-.008422
	EKM U	-.0074382	.0090832	.999	-.036690	.021814
	EKM T	-.0698280*	.0090832	.000	-.099080	-.040576
CLT T	EKM U	.0302366*	.0090832	.036	.000984	.059489
	EKM T	-.0321532*	.0090832	.018	-.061406	-.002901
EKM U	EKM T	-.0623898*	.0090832	.000	-.091642	-.033137

1.4.2.4. Acetylcholinesterase activity:

Acetylcholinesterase in the nerve synapses is the target of organophosphate and carbamate insecticides. Altered or elevated levels of acetylcholinesterase in the nerve synapse are a major mechanism of organophosphate resistance. Activity of acetylcholinesterase was found out in uninhibited and Propoxur-inhibited fractions of mosquito homogenate from the four field strains and was compared with that of laboratory strain. The percentage remaining activity in Propoxur – inhibited fraction was found out by dividing the absorbance for the well with propoxur by that without propoxur for the same insect and multiplying it by 100. A percentage value greater than 30% indicated chance of development of resistance.

The table 29 shows the percentage remaining activity in the Propoxur – inhibited fraction of the *Cx. quinquefasciatus* of field and laboratory sample in the three years. The % remaining activity in propoxur inhibited fraction in the LAB population was less than ten in the years 2014, 15 and 16. In all the five populations, the sample collected from scarcely treated area was less than 30. MPM T population had a value of 32.24±1.8, 31.54±1.2, 33.88±1.4, PKD T had 36.64±1.6, 38.86±2.1, 38.88±2.2, TCR T had 42.36±1.4, 44.54±1.2, 45.88±1.4, CLT T had 38.88±1.5, 44.56±1.4, 45.56±3.2 and EKM T 56.62±2.4, 64.43±1.8, 72.58±2.6% respectively.

Table 29: % Remaining Activity in Propoxur inhibited fraction in the field and laboratory populations of *Cx. quinquefasciatus*

Area of Sample Collection	YEAR		
	2014	2015	2016
LAB	8.86±1.2	6.98±0.08	7.96±1.1
MPM U	12.54±2.2	12.48±1.4	13.56±1.2
MPM T	32.24±1.8	31.54±1.2	33.88±1.4
PKD U	16.54±1.4	15.68±1.5	17.66±1.2
PKD T	36.64±1.6	38.86±2.1	38.88±2.2
TCR U	15.58±2.1	16.88±1.3	17.66±1.2
TCR T	42.36±1.4	44.54±1.2	45.88±1.4
CLT U	16.66±1.2	18.42±1.2	19.54±1.5
CLT T	38.88±1.5	44.56±1.4	45.56±3.2
EKM U	27.36±1.6	28.42±2.2	28.65±2.1
EKM T	56.62±2.4	64.43±1.8	72.58±2.6

Table 30: Year wise descriptive profile of Acetylcholinesterase in field and laboratory *Cx. quinquefasciatus*

Area of sample collection	P value (ANOVA)calculated between years		
	2014/2015	2015/2016	2014/2016
LAB	=0.00	=0.00	<0.05
MPM U	>0.05	<0.05	<0.05
MPM T	>0.05	<0.05	<0.05
PKD U	<0.05	=0.00	<0.05
PKD T	<0.05	>0.05	<0.05
TCR U	<0.05	<0.05	=0.00
TCR T	=0.00	=0.00	=0.00
CLT U	=0.00	<0.05	=0.00
CLT T	=0.00	=0.00	=0.00
EKM U	<0.05	>0.05	<0.05
EKM T	=0.00	=0.00	=0.00
p=0.00 highly significant, p<0.05 significant, p>0.05 not significant			

Table 30 provides data on the % increased activity of propoxur inhibited fraction in the laboratory and field populations. In CLT both U and T shows increased activity, but in other cases the U populations were not

showing such an increase in activity. In the EKM *Culex,* 21.12% increase in the propoxur inhibited fraction was obtained.

Table 31: Increased % remaining activity in Propoxur inhibited fraction in the field and laboratory populations of *Cx. quinquefasciatus*

Area of sample collection	Increased activity % in 2015 from 2014	Increased activity % in 2016 from 2015	Increased activity % in 2016 from 2014
LAB	-21.22	14.04	-10.16
MPM U	-0.48	8.65	8.13
MPM T	-2.17	7.42	5.09
PKD U	-5.20	12.63	6.77
PKD T	6.06	0.05	6.11
TCR U	8.34	4.62	13.35
TCR T	5.15	3.01	8.31
CLT U	10.56	6.08	17.29
CLT T	14.61	2.24	17.18
EKM U	3.87	0.81	4.72
EKM T	13.79	6.44	21.12

1.4.3. WHO Susceptibility Test:

The *Cx. quinquefasciatus* collected from various fields were subjected to WHO susceptibility kit using three different insecticide treated papers Malathion 5%, Cyfluthrin 0.15% and Deltamethrin 0.05%. The mortality rates were recorded and percentage mortality were calculated and tabulated and presented in the table 31. The mortality rate was less in EKM population, for malathion the percentage mortality was 62%, for cyfluthrin 24% and for deltamethrin it was 26.5%. In the case of TCR the mortality rates against malathion, cyfluthrin and deltamethrinwere 77.45, 37 and 37.5 respectively. In the case of PKD, CLT and MPM the malathion, cyfluthrin and deltamethrin mortality was represented as 73, 50, 32.69; 78, 34, 28; and 84, 46, 37.96 respectively.

Table 32: % Mortality of field populations of *Cx. quinquefasciatus* using the WHO Susceptibility kit

Area of sample collection	Insecticides used	No of mosquitoes used as control	No of mosquitoes exposed	No of mosquitoes died	% Mortality
MPM	Malathion 5%	50	100	84	84
	Cyfluthrin 0.15%	50	100	100	46
	Deltamethrin 0.05%	50	108	41	37.96
PKD	Malathion 5%	50	100	73	73
	Cyfluthrin 0.15%	50	100	50	50
	Deltamethrin 0.05%	50	104	34	32.69
TCR	Malathion 5%	50	102	79	77.45
	Cyfluthrin 0.15%	50	100	37	37
	Deltamethrin 0.05%	50	104	39	37.5
CLT	Malathion 5%	50	100	78	78
	Cyfluthrin 0.15%	50	100	34	34
	Deltamethrin 0.05%	50	100	28	28
EKM	Malathion 5%	50	100	62	62
	Cyfluthrin 0.15%	50	100	24	24
	Deltamethrin 0.05%	50	106	25	26.5

1.4.4. Detection of site specific mutation in *kdr* gene:

The DNA isolated from the different locations were amplified the fragment of sodium channel containing *kdr* gene. Allele specific primers used in genotyping of knock down resistant (*kdr*) and knock down susceptible (*kds*) alleles by allele-specific PCR assay (AS-PCR). The AS-PCR assay revealed the presence of leucine-phenylalanine *kdr* mutation in the field strains of *Cx. quinquefasciatus*. PCR assay showed three genotypes, identified by the characteristic 380bp band corresponding to resistant and susceptible specific primers. The 380bp PCR product with both the knock down specific [*kds* (primer3) and *kdr* (primer4)] primers in an individual mosquito indicates heterozygous condition (SR). The appearance of this band only in susceptible-specific primer (*kds* - primer3) indicates homozygous susceptible (SS) and in resistant specific primer (*kdr* - primer4) indicates homozygous resistant (RR).

The result shows (table 32) that out of 20 samples taken CLT and EKM samples have 5 homozygous resistant mosquitoes. From MPM out of 20 samples evaluated 1 homozygous resistant, 4 heterozygous resistant and 15 homozygous susceptible genotypes obtained. The homozygous resistant genotypes obtained from PKD and CLT were 4 and 3 mosquitoes respectively. The number of homozygous susceptible genotypes obtained in the mosquitoes found in MPM, PKD, TCR, CLT and EKM area were 15, 11, 10, 7 and 6 respectively.

Table 33: Genotypes of *Cx. quinquefasciatus* collected from the laboratory and field populations predicted by AS-PCR

Area of sample Collection	Total no of samples	AS PCR		
		SS (500 bp, cq4)	SR (500bp, cq3 & cq4)	RR (500bp, cq3)
LAB	20	20	-	-
MPM	20	15	4	1
PKD	20	10	6	4
TCR	20	11	7	3
CLT	20	7	8	5
EKM	20	6	9	5

1.4.7. Isolation of partial *ace*1 gene and RFLP using Alu1 enzyme:

DNA was isolated from the samples collected from five different locations, and subjected to amplification of partial portion of *ace*1 gene. The amplified portion then digested using Alu1 enzyme to detect the site specific mutation. 15 samples were taken from each area. Three different patterns of bands were formed. A single band in 374bp position indicated there is no mutation and it is homozygous susceptible. A band formed in the 374 position

and one band 272 and another band at 102 indicates that one is heterozygous resistant and the band formed in 272bp and 102bp position indicates that sample was homozygous resistant. After digestion with Alu1 detected the number of bands formed and represented in the table 33. The homozygous susceptible population ie, the number of bands formed in the 374bp in CLT, EKM, MPM, PKD and TCR were 8, 6, 13, 10 and 9 and the heterozygous resistant population ie the bands formed in 374bp, 272bp and 102bp position were 7, 9, 2, 4 and 6 respectively.

Table 34: Genotypes of *Cx. quinquefasciatus* of laboratory and field populations obtained by RFLP using Alu1 enzyme on the partially amplified *ace*1 gene.

Area of sample collection	Total no of samples	No of Bands formed regions after digestion with Alu1		
		SS (374bp)	SR (374bp, 272bp, 102bp)	RR (272bp, 102bp)
LAB	15	15	-	-
MPM	15	13	2	-
PKD	15	10	4	-
TCR	15	9	6	-
CLT	15	8	7	-
EKM	15	6	6	3

1.5. DISCUSSION

Development of insecticide resistance in insect vectors is the most important factor and a serious concern that determines the effectiveness of mosquito control regimes followed across world. Continuous monitoring of insecticide resistance in this context plays a major role in developing new strategies and new products that combat this great menace especially the insect vectors and management of vector borne diseases. The mechanisms of insecticide resistance in vector mosquitoes are being studied worldwide, as they elucidate the pathways of development of resistance and help preventing and delaying insecticide resistance.

The selected insecticide, temephos belongs to the group of organophosphorus insecticides and is a widely used synthetic insecticide in the mosquito control programmes almost throughout Kerala. Hence it is highly imperative to study the progression of development of resistance in native species of mosquitoes to this insecticide. Owing to the extensive use of this chemical pesticide in the field applications throughout Kerala by the local authorities, it became easy to study the development of resistance as the supply of wild population exposed to temephos became abundant with steady supply from the areas selected for sampling.

At the onset, in order to study the resistance status, 0.01ppm temephos was used in the bioassays. Table 1 provides the data on the LT_{50} values of laboratory population and field population. The time taken for the 50% mortality and the resistance ratio of LT_{50} values of field populations to that of laboratory populations provided a clear cut idea about the resistance status. The LT_{50} values of the laboratory population were 37.02, 36.98 and 37.08 minutes in the years 2014, 15 and 16. The MPM *Cx.* shows the values 51.47 (40.71), 52.31 (41.14), 53.56 (41.99) values in parentheses shows the values of populations collected from scarcely treating area. . The larvae collected

from the scarcely treated areas of PKD, TCR, CLT and EKM showed the LT_{50} values of 42.31, 43.44, 43.44, 44.54, 46.64 in 2014; 43.47, 44.88, 45.05, 48.82 in 2015 and 45.64, 45.65, 48.86, 54.46 in 2015 respectively. The treated area of the same locations showed the values of 55.63, 63.51, 69.24, 72.95 in 2014, 65.32, 74.65, 79.32, 85.69 in 2015 and 70.24, 76.64, 84.32, 90.04 in 2016 respectively. The result indicates that in all the populations the value varied from the laboratory populations and the LT_{50} value is higher in EKM samples. Another noted point is that the LT_{50} values show an increase from the year 2014 to 2016 in all cases.

The resistance ratio calculated as per the protocol of Dhang *et al*., 2008 and it supports the bioassay data results. As per Dhang's rule the resistance ratio higher than one indicates resistance. In all the field populations the resistance ratio was higher than one as compared with the laboratory population and the value was high in the case of resistance ratio with regularly insecticide treating location and scarcely insecticide treating location. In TCR, CLT and EKM population the resistance ratio value was higher than two in the year 2016. It indicates the urgent need of resistance management in the field.

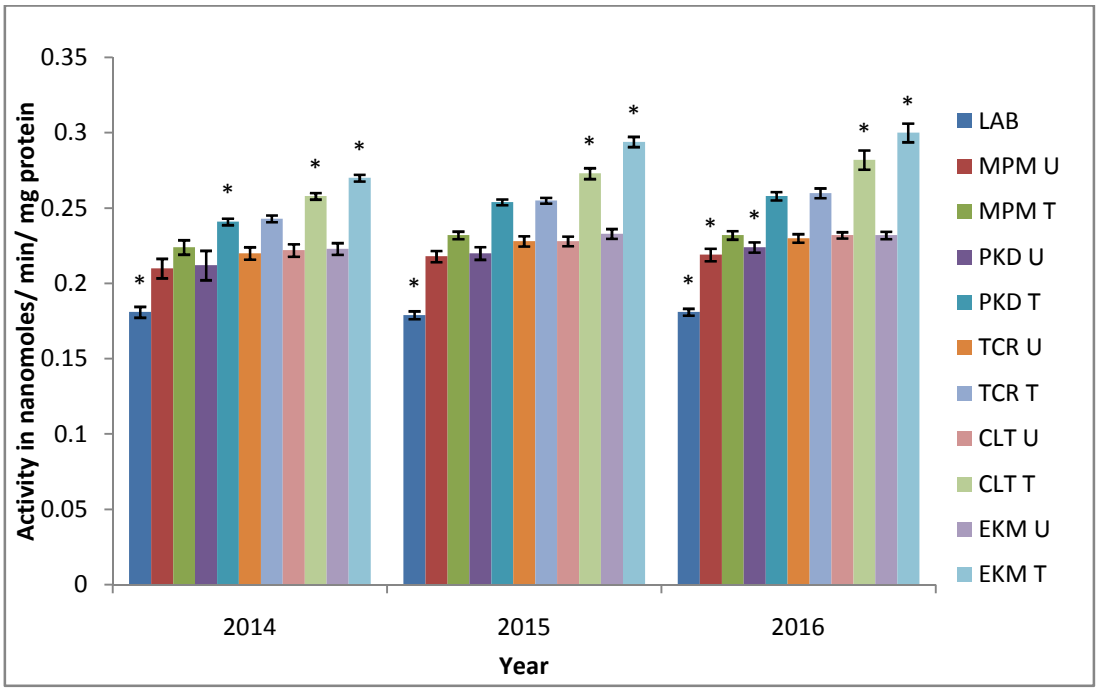
The best method of choice for understanding the mechanism of insecticide resistance among insects is the biochemical estimations. Biochemical assays are sophisticated and highly sensitive, so it analyzes the mechanisms of insecticide resistance with a fair degree of accuracy. Metabolic detoxification is the most common resistance mechanism that occurs in almost all insects. In mosquitoes it occurs due to changes in its enzyme systems that result in a more rapid detoxification of the insecticide than what is normal, preventing the insecticide from reaching the intended site of action (Hemingway and Ranson, 2000).

The metabolic based insecticide resistance mechanisms mainly include non-specific esterase, Glutathione-S-transferase (GSTs) and P450 mediated monooxygenase (MFOs) and they were known to be involved in the detoxification of organophosphate, carbamate and pyrethroid insecticides. In insects, AChE has mainly been studied in relation to insecticide resistance because the enzyme is the target of organophosphate and carbamate insecticides and its insensitivity to insecticides is one of the main factors accounting for resistance; so these enzymes are used as reliable markers to assess the impact of toxic compounds on insects (Kudom *et al*., 2011).

Detoxification mechanism mediated through non-specific esterases (α and β) is one of the major mechanisms of resistance in insects. These esterases detoxify organophosphates, carbamates and synthetic pyrethroid pesticides by two main ways; hydrolysis of the ester bond and binding of the pesticide to the active site of esterase (Crow *et al*., 2007). Most of the insecticide groups contain ester linkages which are susceptible to hydrolysis by esterase. Resistant insects usually show a very high activity of esterases (Yang *et al*., 2004).

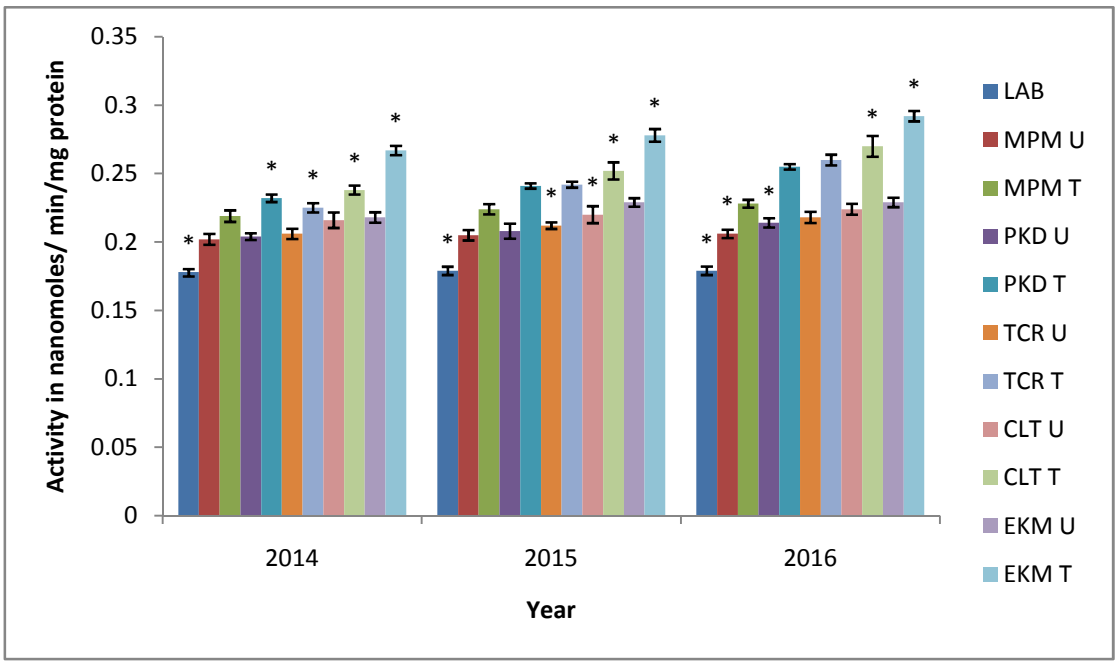
In the present study the data regarding α & β esterase levels (Figure 16 & 17) in the field and laboratory population are given in the graphs and statistical analysis to test the significance were provided in tables locality wise. From the graph it is clear that the carboxylesterase levels were high in the mosquito populations which were collected from regularly insecticide spraying areas. There was a significant increase in α & β esterase activity ($p<0.05$) and the alteration in the activity of α & β esterase levels indicate that the detoxification levels are higher in field population where insecticide application occurs regularly.

Figure 16: α esterase levels in the laboratory and field populations of *Cx.quinquefasciatus* during 2014,15 and 16



Level of significance p<0.05; * = p<0.05

Figure 17: β esterase levels in the laboratory and field populations of *Cx.quinquefasciatus* during 2014,15 and 16



Level of significance p<0.05; * = p<0.05

Esterase based resistance to organophosphorus and carbamate insecticides are common in almost all insects. The esterase either produce broad spectrum insecticide resistance through rapid binding and slow turnover of insecticide or narrow spectrum resistance through metabolism of a very restricted range of insecticides containing a common ester bond. It has been reported that resistance to organophosphate insecticides has been associated with the carboxylesterase activity changes in many insects and the nature of changes varies widely according to the sensitivity and differences in strains (Terriere, 1984). Elevated esterase activity accounts for resistance to organophosphates, carbamate and pyrethroid insecticides (Rao *et al*., 1989).

The increase in enzyme activity also noted and it supports the results that there is high level of change in the detoxification enzymes in the field population. The α esterase activity increase in EKM population was 11.11 fold higher in the year 2016 as compared with the year 2014. In the year 2015the percentage increase was 8.89 from the year 2014 but there is no a higher increase from the year 2015 to 2016 and it was 2.04. The level of increase from the 2015 to 2016 was less as compared with level of increase in 2014 to 2015. More than 5 fold increases was obtained in PKD, TCR, CLT and EKM population during these three consecutive years. But only in the case of PKD, the scarcely treating area showed an increased value of activity more than five. Increased activity of β esterase was shown by TCR and CLT as compared with other areas. The activity increase of TCR *Cx*. population in 2015/2014, 2016/2015, 2016/2014 was 7.556, 7.438 and 15.556 respectively. In CLT and PKD as compared with other localities there is hike in the activity level from 2015 to 2016.

Several studies have shown that insecticide-resistant insects have elevated levels of Glutathione-S-Transferase (GST) activity in crude homogenates, which suggests a role for GSTs in resistance (Grant *et al*., 1991), particularly to DDT (Hemmingway and Ranson, 2000). Elevated GST activities have also been detected in some insect species resistant to

organophosphates (Fournier *et al*., 1992), other organochlorines (Grant *et al*., 1992) and implicated in resistance to pyrethroid insecticides (Kostaropoulos *et al*., 2001).

The GSTs often act as a secondary resistance mechanism in conjunction with a P 450 - or esterase- based resistance mechanism (Hemingway *et al*., 1991). An increased rate of glutathione-dependent dehydrochlorination confers resistance to DDT in *Ae. aegypti* (Grant *et al*., 1991; Lumjuan *et al*., 2005), *An. dirus* (Prapanthadara *et al*., 1996, 2000) and *An. gambiae* (Prapanthadara *et al*., 1993, 1995, Ranson *et al*., 2001).

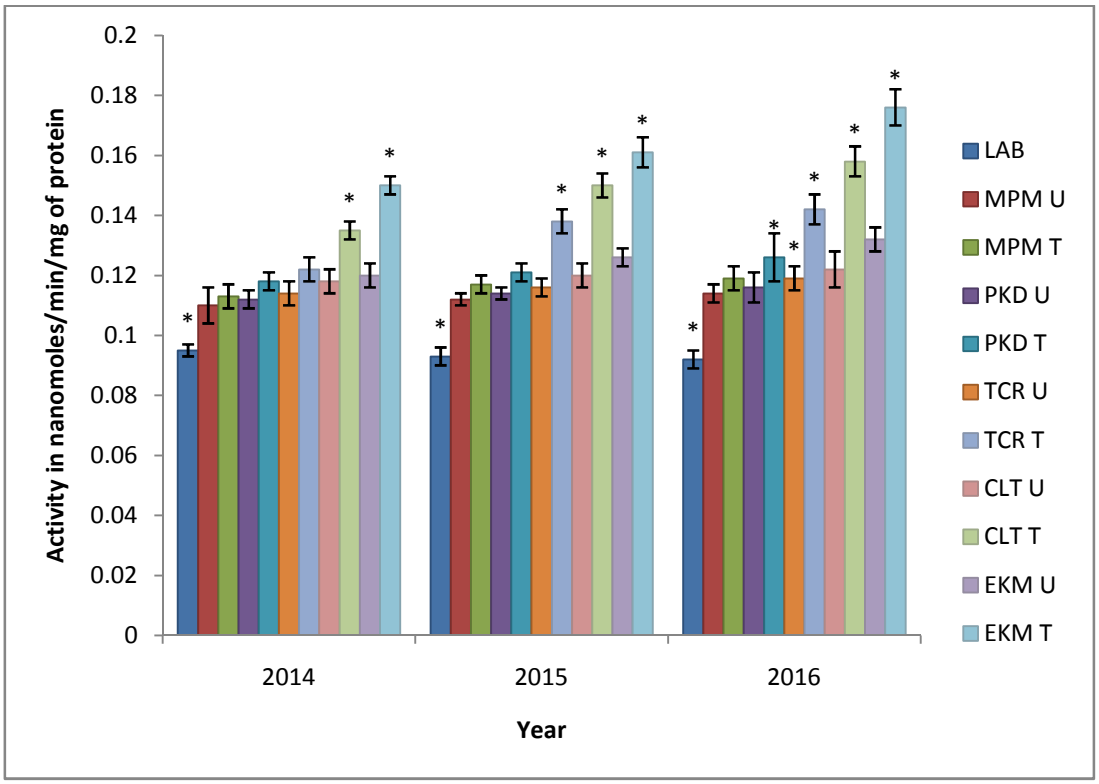
The GST diversity in other species is unknown, and it is expected to become higher in mosquitoes such as *Cx. quinquefasciatus* whose breeding sites are comparatively highly polluted (Hemingway and Ranson, 2000). Whether this diversity is related to insecticide resistance in different mosquito species should be investigated. In some *Anopheline* populations such as the Mexican malaria vector *An. albimanus* (Penilla *et al*., 2006) and the African malaria vector *An. gambiae* (Prapanthadara *et al*., 1995), the GST-based mechanism only confers resistance to DDT, probably as a result of DDT selection pressure on these populations. Interestingly in the Sri Lankan malaria vector *An. subpictus*, the organophosphorus insecticide pressure could have maintained or reselected DDT resistance still observed (Hemingway *et al*., 1991). A similar phenomenon may be conferring DDT resistance in *Cx. quinquefasciatus* from Thailand, where populations had never been exposed to DDT, but have been exposed to multiple toxic chemicals in water (Prapanthadara *et al*., 2000).

The hypothesis whether cytochrome P 450 monooxygenases could be involved in DDT resistance in *Ae. aegypti* (Prapanthadara *et al*., 2002) has been recently supported by microarray experiments in *An. gambiae* (David *et al*., 2005, Vontas *et al*., 2005). Although these findings remain to be demonstrated with functional studies, these experiments suggested that DDT resistance may be the result of over expression and downregulation of several

genes, including genes not formerly associated with insecticide resistance (Vontas *et al.*, 2005). It is noteworthy that the factors responsible for GST regulation involved in resistance seem to be capable to regulate other GST expressions that may not be involves in resistance (Ding *et al*., 2005).

In all the five field populations the GST levels were elevated and were highly significant. The rate of increase in GST activity is higher in TCR (13.115) and CLT (11.11) in the year 2015 and least increase at PKD (2.54). CLT holds the first position in the increased rate of activity from the year 2015 to 2016 and the rate of increase was 5.333. But when comparing the rate of increase from 2014 to 2016 EKM stands in the first position with the value 17.333 followed by CLT (17.037) and TCR (16.393). The result indicates that the field populations were exposed to multiple chemicals in the environment.

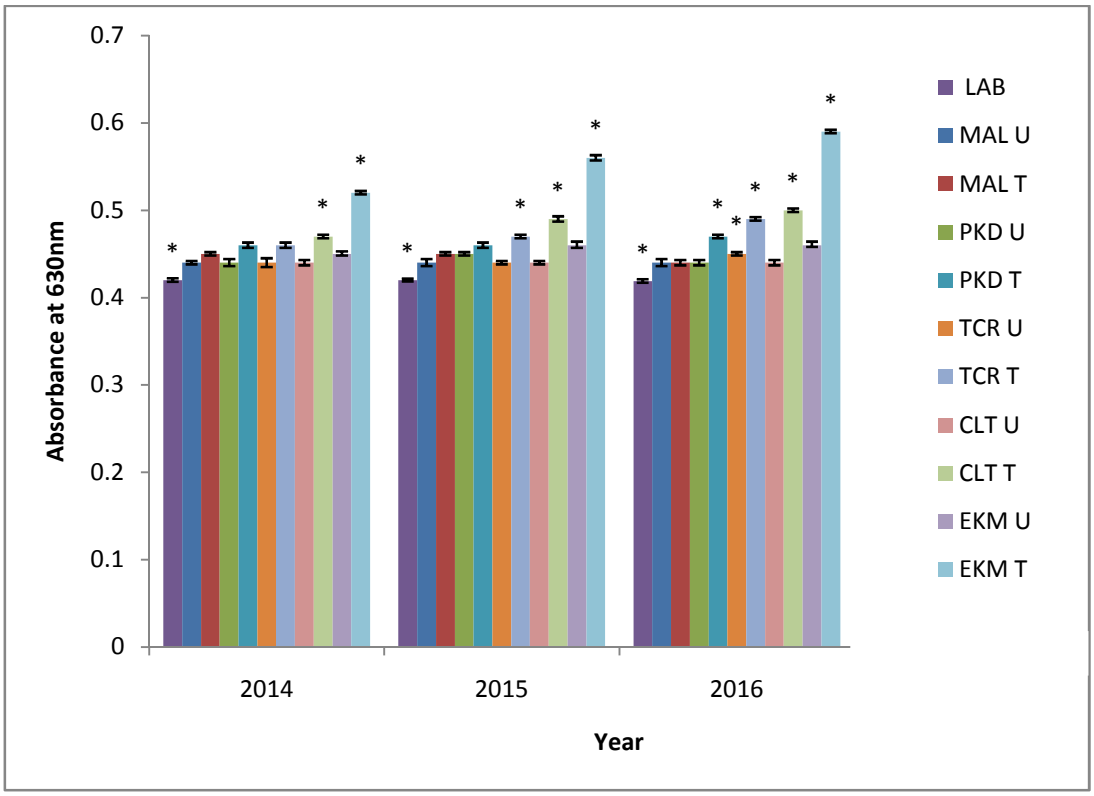
Figure 18: GST levels in the laboratory and field populations of *Cx. quinquefasciatus* during 2014,15 and 16



Level of significance p<0.05; * = p<0.05

P450s are Phase I detoxification heme-thiolate enzymes catalyzing various reactions, but are best known for their monooxygenase activity, introducing reactive or polar groups into xenobiotics or endogenous compounds (Ranson *et al*., 2004 and Scott, 2012). The enzymatic detoxifications due to mixed function oxidases are responsible for the development of resistance in insects. The mixed function oxidase system is responsible for the resistance towards organophosphates, DDT, pyrethroids and growth regulators (Brogdon *et al.,* 1997). The metabolic detoxification of mixed function oxidase (MFO) may cause the development of cross resistance. The elevated levels of MFO in the present study suggested that the detoxification by this enzyme could be implicated in the cross resistance with DDT, pyrethroids and organophosphates.

Figure 19: MFO activity in the laboratory and field populations of *Cx. quinquefasciatus* during 2014,15 and 16



Level of significance p<0.05; * = p<0.05

The activity levels of MFO was significantly higher in the field collected samples than the control group for all the tested populations ($p<0.00001$). There was no increase in the activity in the field populations of MPM and PKD *Cx. quinquefasciatus* from 2014 to 2016. There was no increase from 2014 to 2015 and 4.255 fold increase from the year 2015 to 2016 at TCR. In the case of CLT population there was a hike of 2.174 fold from 2014 to 2015 and no change in the activity from 2015 to 2016. In the EKM population the increase from 2014 to 2015 and 2015 to 2016 were 3.774 and 5.455 respectively. From the results it indicated that the EKM population has cross resistance with pyrethroids, DDT and organophosphates.

Acetylcholinesterase (AChE), responsible for neurotransmitter degradation at the cholinergic nerve synapse, is the target of both organophosphate and carbamate insecticides. Selection of a modified AChE less sensitive to these insecticides has been shown to be a common resistance mechanism. In natural populations of mosquitoes, high level of resistance to carbamate and organophosphates is provided by insensitive acetylcholinesterase.

The value for the well with propoxur divided by that without propoxur multiplied by 100 gives the % remaining activity in propoxur inhibited replicate rate. Populations with more than 70% remaining activity after inhibition can be characterized as homozygous resistance (RR) with respect to altered AChE mechanism. Populations with 30-70% and less than 30% remaining activity can be categorized as heterozygous (RS) and homozygous susceptible (SS) respectively (Bourguet *et al*., 1996). Results of assay conducted to identify the insensitivity of AChE to insecticide inhibition by propoxur are presented in the table 29. In this experiment the all the samples collected from insecticide regularly treating area have a value greater than 30% and it indicates the resistance status of those mosquitoes. All the values

were highly significant with a P-value less than 0.001. The present results have provided strong evidence on the role of insensitive acetylcholinesterase in the development of organophosphate and carbamate resistance in EKM *Cx. quinquefasciatus.*

While calculating ANOVA between the different areas of sample collection, the p-value is less than 0.05 in all cases. It proves that there is significant change in acetylcholiesterase activity between the areas. But while calculating the year wise profile it seems that only TCR T, CLT T and EKM T have the p value zero between 2014/15, 2015/16 and 2014/16; which means that there is significant change in the acetylcholinesterase activity. And the hike in enzyme levels with years proves that the TCR, CLT and EKM collected mosquitoes are developing resistance towards oganophospahe. And it implies the need of alternate control measures.

The % increase in activity was higher in CLT in the year 2015 as compared with 2014. In the year 2016, the activity was higher at EKM field collected *Cx. quinquefasciatus* as compared with the year 2015. From 2014 to 2016 there is a hike in the activity % of enzymes at EKM. The activity of both CLT collected populations showed similar pattern of increase from the year 2014 to 2016 and the values were 17.287 for scarcely treating area and 17.181 for regularly treating area.

Susceptibility of the species to the organophosphate Malathion and the pyrethroids Cyfluthrin and Deltamethrin was found out by WHO adult bioassays using insecticide impregnated papers having diagnostic doses of the insecticides (Malathion – 5%, Cyfluthrin – 0.15% and Deltamethrin – 0.05%). According to WHO criteria for resistance (WHO 1998), mortalities below 80% represented definite, strong resistance, whereas mortalities ranging from 80 to 98% represented varying degrees of resistance; generally described as tolerance, and those above 98% represented definite susceptibility.

As per the results of the WHO bioassay mortality of 62% - 84% was shown in Malathion, with the lowest mortality (62%) shown by the EKM population and the highest (84%) shown by MPM. The CLT, PKD and TCR population showed a mortality of 78, 73 and 77.45 respectively. Very high resistance was shown towards pyrethroids, with mortality ranging from 24% to 50%. In the test with Cyfluthrin, the lowest mortality (24%) was shown by the EKM population followed by CLT (34%), and TCR (37%), MPM (46%) while the highest mortality was shown by the PKD (50%). Of all the three papers tested, resistance was shown to Deltamethrin also, where the lowest mortality was 26.5% (EKM) and the highest mortality was 37.96% (MPM). In all the three insecticides tested, *Cx. quinquefasciatus* collected from MPM showed the least resistance while the highest resistance was shown by mosquitoes collected from EKM followed by those collected from CLT and TCR.

Knock-down resistance due to a point mutation (designated the L to F *kdr* mutation) in the voltage gated sodium channel is a common mechanism of resistance to pyrethroids. Simple and reliable techniques are in great need to detect and monitor pyrethroid resistance among mosquito populations in the field. Allele specific polymerase chain reaction (AS-PCR) method is used to detect the L to F *kdr* mutation in the mosquito *Cx. quinquefasciatus*.

After optimizing experimental conditions, AS-PCR could effectively distinguish individual mosquitoes that were homozygous or heterozygous for the mutations.

The results indicate that all the mosquito populations showing resistance towards pyrethroids. The CLT population have 7 homozygous susceptible, 8 heterozygous resistant and 5 homozygous resistant mosquitoes out of the 20 mosquitoes taken for the experiment. The EKM population have a higher number of heterozygous resistant mosquitoes and MPM have the

least number of resistant mosquitoes. The results indicate that the pyrethroid usage is high at EKM and CLT areas.

Acetylcholinesterase, a key enzyme of the central nervous system, is the target of both organophosphate and carbamate insecticides. Acetylcholinesterase, the target site for organophosphates and carbamates, is a synaptic enzyme that hydrolyzes then neurotransmitter acetylcholine to terminate nerve impulses, thereby blocking nervous transmission and leading to the death of the insect. Selection of a modified acetylcholinesterase less sensitive to these insecticides has been shown to be a common resistance mechanism in mosquitoes (Alout *et al*., 2009). Most other insects rather than fruit flies, studied so far possess two *ace* genes, *ace*-1 and *ace*-2, and when insecticide insensitivity has been confirmed functionally it has been attributed to a point mutation in the *ace*-1 gene. To date, only few positions, all lining the active site of AChE1, have been shown to be involved in insensitivity, suggesting a high structural constraint of this enzyme (Oakeshott *et al*., 2005).In mosquitoes, only three amino acid substitutions involved in *ace*1 resistance have been identified so far: G119S, F290V and F331W. The G119S substitution was selected in several species including *An. gambiae, An. albimanus*, *Cx. vishnui* and *Cx. pipiens* and was shown to be widespread in *Cx. pipiens* natural populations (Weill *et al*., 2003, Labbé *et al*., 2007).

The PCR diagnostic assay that was performed in the present study detected *ace*1 mutations (G119S) in some of the mosquito populations. Nevertheless, very low frequencies of homozygote resistance were found. Expectedly, high frequencies of *ace*1 mutations were found in mosquitoes in areas where the bioassay test showed the mosquitoes to be resistant to organophosphate and carbamate insecticides, suggesting the involvement of the mutation in the resistance of the mosquito population to the insecticides. The low frequency of homozygote resistance can be explained by the high

fitness cost that is associated with *ace*1 mutation, such as long development time and decreased male reproductive success (Raymond *et al*., 2001). Despite *ace*1 mutations being reported to provide cross resistance to organophosphates and carbamates (Alout *et al*., 2008), the resistance level greatly varied between the two classes of insecticides. However, some studies have suggested that *ace*1 mutations have a greater impact on carbamate than organophosphate resistance.

Number of bands formed while using Alu1 restriction enzyme confirms the level of resistance in each population. Only from the EKM population we got the homozygous resistant mosquitoes. Of the 15 mosquitoes from EKM, 6 are homozygous susceptible and heterozygous resistant. *Culex* collected from CLT has 8 SS and 7SR and from TCR have 9SS and 6SR genotypes. PKD and MPM *Culex* showed 10SS, 5SR and 13 SS, 2SR genotypes of the 15 mosquitoes used for the study respectively. The genotypes indicate the urgent need of opting alternate control measures against the field populations of *Culex* mosquitoes.

In the present study only at EKM we got homozygous resistant mosquitoes. In all the other populations heterozygous resistant mosquitoes are present. The least number of resistant mosquitoes are seen in MPM and it supports the results of bioassay and biochemical assays.

As a conclusion of the present study, the results from the biochemical assay showed an association between enzyme levels and the degree of insecticide resistance among the *Culex* mosquitoes. This may suggest the involvement of metabolic resistance mechanism in the study populations. In addition, target site mutations (*kdr* and *ace*1) were also observed in *Cx. quinquefasciatus* from the study populations. This is the first description showing evidence of the existence of multiple insecticide resistance mechanisms in *Cx. quinquefasciatus* in Kerala. The pattern of resistance and

the mechanisms involved can be expected to have a number of implications on resistance management strategies.

Various insecticide selection pressures particularly from agriculture and domestic use of insecticides were suspected to be the cause of resistance to the insecticides. It also appears that the presence of urban pollutants in mosquito breeding sites probably has a direct or indirect impact on mosquito resistance. For this reason, proper management of waste, particularly in urban areas and effective regulation of use of pesticides appear to be critical in resistance management programs. One way is to reduce the mosquito population, especially in urban areas where most of the important larval habitats have been shown to be anthropogenic (Kudom *et al*., 2011). Such habitats can easily be managed through proper waste management, proper construction of drains and the change of the inhabitants' behaviour through proper education. Hence proper management and control measures are necessary for the control of vector mosquitoes.

2.1. INTRODUCTION

Mosquitoes form the most serious threat to public health wherein they spread several acute, chronic and fatal diseases such as dengue, malaria, Japanese encephalitis, yellow fever and filariasis in both animals and human beings. *Anopheles, Culex, Aedes* and *Mansonia* mosquitoes are known to transmit chronic diseases to more than 700 million people every year. Insect-transmitted diseases are the major sources of infections, discomforts and in some cases, even deaths worldwide. Key components that determine the occurrence of vector- borne diseases include: (1) the abundance of vectors and intermediate and reservoir hosts (2) the prevalence of disease- causing pathogens suitably adapted to the vectors and the availability of human or animal host (3) the local environmental conditions, especially temperature and humidity and (4) the resilience behavior and immunity status of the human population.

The only way to prevent virus borne disease transmission is to eliminate or control the disease-carrying mosquito, which is achieved by usage of chemical and botanical insecticides. Synthetic insecticides are used as larvicides, repellents and adulticides to control mosquito vectors. The choice of larvicides for the control of mosquitoes is dependent upon the species and habits of the mosquitoes and the hazards it may pose to human beings, animals, fish, aquatic organisms and other wild life. Larvicides are mostly used in mosquito breeding zones. Insecticides are used on house walls or bed nets and repellents are used as personal application to reduce incidence of insect bites and their infection. First used synthetic insecticide dichloro diphenyl trichloro ethane (DDT) was developed by Paul Miller in 1874 and it is banned in most developed countries. Major chemical insecticides are organochlorides such as aldrin, chlordane, DDT, endrin, dieldrin, mirex and toxaphene. Organophosphates such as malathion and chloropyrifos function

as nerve poisons. The insect growth regulator methoprene is a mimic of juvenile hormone and interfere with metamorphosis and emergence. Increased use of these chemicals leads to many environmental and public health issues. Many insects have been reported to develop resistance to these chemical pesticides and there is growing concern about the health issues and risks of environmental hazards. Environmental protection agencies have banned or placed severe restrictions on the use of many pesticides which were formerly used in mosquito control programmes and there are now fewer adulticides available than there have been for the last 20 years (Carvalho *et al*., 2003).

Biological control techniques including the direct introduction of parasites, pathogen and predators to function as natural enemies to target mosquitoes have been researched extensively. Aerial predators such as dragonflies, birds and bats receive much attention, but do not specialize in adult mosquitoes. Using Purple Martin, a small bird which feed on winged insects especially mosquitos is an excellent example of mosquito control using natural enemies of mosquitoes. Most control efforts have been directed at the larval stage of mosquitoes owing to many reasons. In the life history of mosquitos, the larval stages which are confined to the aquatic environment occupy almost 60-80 % of its life span thus making the control measures easy to be executed at various larval stages when compared to adult stage.

Aquatic predators are effective biocontrol agents and include predatory fish that feed on mosquito larvae. Mosquito fish; *Poecelia reticulata* and *Gambusia affinis* have been shown to be effective in controlling mosquitoes and some cyprinids (carps and minnows) and killifish (Patten *et al.,* 1975) found to feed on mosquito larvae. Tilapia also consumes mosquito larvae. Dragon flies are natural predators of mosquitoes both in their larval stage and as adults, depending on the species. Dragon fly may exist as a larvae or nymph anywhere from several weeks to more than one year, during which

time it feeds on other larval insects including mosquito larvae. Dragon flies are often referred to as “mosquito hawks” for their supposed ability to kill thousands of mosquitoes. Healthy wetlands keep the mosquito population down naturally due to the presence of this natural predator. Some other biocontrol agents that have lesser degrees of success include the predator mosquito- *Toxorhynchites* and predator crustaceans - mesocyclops, copepods and nematodes etc.

Suspensions based on many microorganisms are eco-friendly and act with high degree of specificity. Microbial pathogens of mosquitoes include viruses, bacteria, fungi, protozoa, nematodes and microsporidia. Dead spores of varieties of the natural soil bacterium *Bacillus thuringiensis,* especially *B. israelensis* (*Bti*) and *B. sphaericus* can also be used to a great extent. *Bti* is used to interfere in the digestive system of larvae. In addition to this, viruses are also used as control agents. Important insect specific viruses are nuclear polyhedrosis viruses (NPV) and granulosis viruses (GV). Apart from these, fungi like *Metarhizium anisopliae* and *Beauveria bassiana* are presently being used for killing adult mosquitoes. The fungal spores disseminate through wind and reach the target pest and the mycelium spreads throughout the body and kills the pests. Application of microorganism over a prolonged period as a biocontrol agent also met with the phenomenon of development of a strong resistance in the insect body.

In most of the mosquito control regimes followed around world, depend upon extensive and seemingly irrational way of using synthetic insecticides owing to the reasons of having immediate control of mosquito populations. Indiscriminate use of various chemical and synthetic insecticides has led to various health and environmental issues in addition to development of resistance in target insect vectors.

The development of resistance due to the use of chemical/synthetic

insecticides could be reduced to an extent by the use of botanical insecticides. The pharmacological and insecticidal properties of plants have been studied and identified all around the world, especially in our country where plant specimens are abundant and the identified ones have consequently been used for the control of insect pests/vectors traditionally. The secondary metabolites present in these plants having insecticidal properties can be utilized for the control of a wide range of insect vectors. Many plant derived compounds have ovicidal, larvicidal, adulticidal and repellent properties against the mosquito vectors. Due to environmental concern over the use of synthetic insecticides for vector control and widespread insecticide resistance and also lack of alternative, cost-effective and safe insecticides, interest on possible use of environment friendly natural products such as extracts of plant or plant parts has tremendously increased and is being extensively used for the control of insect vectors.

Phytochemicals are preferred because of their eco-safety characteristic, target-specificity, non-development of resistance, reduced number of applications, higher acceptability and suitability for application in rural areas. Botanicals can indeed be used as an alternative to synthetic insecticides or along with other insecticides under integrated vector control programs. The plant product or phytochemicals used as an insecticide for killing larvae or adult mosquitoes or as repellents for protection against mosquito bites. Phytochemicals are obtained from the whole plant or specific part of the plant through extraction using different types of solvents such as aqueous, methanol, chloroform, benzene, acetone etc., depending on the polarity of the phytochemical. Some phytochemicals act as toxicant (insecticide) both against adult as well as larval stages of mosquitoes while others interfere with growth and growth inhibitors or with the reproduction process or even produce an olfactory stimulus, thus acting as repellent or attractant.

It is no surprise that many herbal products have been used as natural insecticides even before the discovery of synthetic insecticides. Botanicals such as *Pyrethrum*, *Chrysanthemum*, *Derris*, *Quassia*, *Nicotine*, *Hellebore*, *Azadirachtin* and *Turpentine* have already proven their insecticidal capabilities in the pre-DDT era (Shaalan *et al*., 2005). Plants from Annonaceae, Asteraceae, Lamiaceae, Meliacaea and Rutaceae families are reported as the most promising botanical insecticides of the present time. Many traditional medicinal plants have been used for the control of *Cx. quinquefasciatus* (Kuppusamy and Murugan, 2006). Currently, more than 1,005 plant species have been identified with insecticidal properties, of which 384 are anti-feedants, 297 acts as repellents and 27 as attractants and possess growth inhibitors (Jayaraj, 1993). The methanol extracts from three important medicinal plants namely *Andrographis paniculata*, *Cassia occidentalis* and *Euphorbia hirta* mixed with water, crude hexane, ethyl acetate, benzene, exhibit strong repellent and ovicidal properties (Panneerselvam and Murugan, 2013). Ramkumar *et al.*, (2014) reported the adulticidal and smoke toxicity activity of acetone, benzene, chloroform, ethyl acetate and methanol leaf extracts of *Cipadessa baccifera* against *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus*. The leaf extract of *Clausena dentata* has been observed to exhibit remarkable adulticidal properties against *Ae. aegypti* and *Cx. quinquefasciatus* vector mosquitoes (Govindaraju *et al*., 2014).

OBJECTIVES OF THE STUDY

1. To determine the larvicidal activity of acetone, methanol and defatted methanol extracts of *Melaleuca leucadendron* (L) leaves and *Anamirta cocculus* (L) Wight and Arn seeds extract on *Culex quinquefasciatus* Say and *Aedes albopictus* Skuse.

2. To find out the larval extension duration using the ethyl acetate fractions of *M. leucadendron* leaves and *A. cocculus* seeds extract on *Cx. quinquefasciatus* and *Ae. albopictus.*

3. To identify the larvicidal activity of column fractions of *M. leucadendron* leaves and *A. cocculus* seeds extract on *Cx. quinquefasciatus* and *Ae. albopictus.*

4. To find out the repellent and adulticidal activity of the oils of *M. leucadendron* and *A. cocculus* against *Cx. quinquefasciatus* and *Ae. albopictus.*

5. To identify the compounds present in the essential oil of *M. leucadendron* using GCMS.

6. To identify the compounds present in the most active fractions of column chromatography using NMR and LCMS.

2.2. REVIEW OF LITERATURE

In view of public health importance, mosquitoes identify themselves as vectors of capital diseases like malaria, filariasis, dengue, Japanese encephalitis etc. in nearly all tropical and subtropical countries in the world. Their high potential for reproduction, dispersal and the ability to exploit even adverse environmental conditions contribute significantly them to rapidly building up their population (Berlin, 1972). About 3000 species of mosquitoes have been recorded worldwide until now, out of which more than 100 species are reported to be dynamic of transmitting diseases to human beings (Reuda, 2008). Mosquito-borne diseases infect over 700,000,000 people every year globally and are actively rampant in more than 100 countries across the world. The data is alarming in the Indian scenario with about 40,000,000 individuals affected by mosquito transmitted diseases every year (Meenakshi *et al*., 2014). WHO has officially declared mosquitoes as "public enemy number one". It is important to known that malaria, spread by mosquitoes, causes one to two million deaths annually.

Lymphatic Filariasis has been reported to affect at least 120 million people in 73 countries including Africa, India, Southeast Asia and Pacific Islands. As per previous reports, filariasis in India constitutes around 40 percent of global filariasis burden with the estimated annual economic loss of about 720 crores (Ghosh *et al*., 2012). Japanese encephalitis accounts for the annual incidence of 30,000-50,000 with a mortality rate estimate of 10,000 cases (Bagavan and Rahuman, 2010).

Global pandemic of dengue begun in South East Asia after World War-II and has intensified during last 15 years. Epidemics caused by multiple serotypes are more frequent and the geographic distribution of dengue and Dengue Haemorrhagic Fever (DHF) has been expanded (Gubbler, 1992). In developing countries such as India, the mosquito borne diseases not only

cause high level of morbidity and mortality but also inflict great economic loss and social disruption (Kanika Tehri and Naresh Singh, 2015). An outbreak of Chikungunya virus disease emerged in the southwest Indian Ocean Islands in 2005, spread out to India, and resulted in an ongoing outbreak that has involved >1.5 million patients, including travellers who have visited these areas (Taubitz *et al.,* 2007).

Over centuries, scientists have been experimenting with various methods, which include use of synthetic insecticides, to encounter threats from mosquito borne diseases. The commodious, disproportionate and repeated use of organic insecticides such as carbamates, organophosphates and organochlorines has led to severance of natural biological control systems. This has eventually led to revitalization and development of resistance in target species and destruction of non-target flora and fauna inhabiting the same aquatic habitat. The remnants of the pesticides in the field are known to exhibit bio-magnification by entering into the ecosystem and circulating through the food web, ultimately triggering environmental imbalance. The long standing ill-effects of intense insecticide usage have thus expedited the need for research and development on environmentally safe and bio-degradable method for controlling mosquitoes.

Although, biological control activities have an important role to play in modern vector control programs, it lacks the provision for a complete solution by itself. Irrespective of the less harmful and eco-friendly methods suggested and used in the control programs, the onus falls back upon the use of chemical control methods in instances of epidemic outbreak and sudden increase of adult mosquitoes. Insecticides are known for their speedy action and effective control during epidemics and automatically become a more reliable choice for speedy counter measures. As a result, they are preferred as an effective control agent to reduce the mosquito population irrespective of their side

effects. Recent studies stimulated the investigation of insecticidal properties of plant-derived extracts; and concluded that they are environmentally safe, degradable and target specific (Senthil Nathan and Kalaivani, 2005).

Phytochemicals derived from various botanical sources have provided numerous beneficial uses ranging from pharmaceuticals to insecticides. Synthetic organic insecticides, although highly efficacious against target species such as mosquitoes can be detrimental to a variety of animal life including man (Matsumura, 1975). In addition to adverse environmental effects from conventional insecticides, major mosquito disease vector and pest species have become physiologically resistant to many of these compounds (Brown, 1963). These factors have created the need for environmentally safe, degradable and target-specific insecticides against mosquitoes. The search for such compounds has been directed extensively into the plant kingdom. Traditionally plant parts, plant based products and secondary metabolites of plant origin have been used in human communities for many centuries in managing insect infestations. Several secondary metabolites present in plants serve as a defense mechanism against insect attacks. Historically, the commercial development of botanical insecticides is credited to a lady of Ragusa, Dalmatia, who noticed dead insects on a discarded bouquet of pyrethrin flowers. She began milling pyrethrum into powder and thus the pyrethrin industry was born (Hartzell and Wilcoxon, 1944). Since then, pyrethrins from *Chrysanthemum* flowers and many synthetic derivatives stand prominent as effective pesticides. Apart from this, various botanical insecticides like Pyrethrum, Derris, Quassia, Nicotine, Hellebore, Anabasine, Azadirachtin, d-limonene camphor, Turpentine, *etc*., were in use in different countries for the controlling insect pests/vectors (Shaalan *et al*., 2005).

One of the earliest reports of the use of plant extracts against mosquito larvae is credited to Campbell *et al.,* (1993) who found that plant alkaloids like nicotine, anabasine, methyl anabasine and lupinine extracted from the Russian weed *Anabasis aphylla*, killed larvae of *Cx. pipiens*, *Cx. territans*, and *Cx. quinquefasciatus*. Wilcoxon *et al.,* (1940) reported that extracts derived from the male fern, *Aspidium filix-mas*, yielded a toxic constituent, filicin, a phloroglucinol propyl ketone, which proved toxic to *Cx. quinquefasciatus*. Wilcoxon and Hartzell (1941) evaluated extracts from 150 species of plants for their toxicity to mosquitoes and found several to be very effective. Jacobson (1958) reported the larvicidal, adulticidal and repellent properties of *Aconitum barbatum*, *Ambrosia psilostachya*, *Backhousia myrifolia*, *Erythrophelum couminga*, *Piscidiapis cipula* etc. against *Anopheles* mosquitoes.

The insecticidal effects of plant extracts depend upon the plant species, mosquito species targeted, geographical varieties, plant parts used, extraction methodology adopted and the polarity of solvents used during extraction. Among these, the plant species and plant parts used significantly influence the efficacy of botanical mosquitocides. Different plant parts such as leaf, root, stem, fruit, fruit peel, seeds, rhizome, flowers, bark etc. have been reported to be used as a source of botanicals in mosquito control with varying efficacy. The search for an eco-safe, low cost and a highly potential insecticide for the control of mosquitoes require the preliminary screening of plants to evaluate their insecticidal activities. Plant based products donot have any hazardous effect on ecosystem as they are biodegradable in nature. Recent research has proved that, the effectiveness of plant derived compounds, such as saponine, steroids, isoflavonoids, essential oils, alkaloids and tannins in functioning as potential mosquito larvicides. Plant secondary metabolites and their synthetic derivatives provide alternative source in the control of mosquitoes (Shivakumar *et al.,* 2013).

Plant extracts affect insect behaviour, growth, health and many possess abilities of repellence, feeding deterrence and so on. Phytochemicals with mosquitocidal potentials are now recognized as potent alternative to replace synthetic insecticides in mosquito control programs due to their excellent ovicidal, larvicidal, adulticidal and repellent properties. As with toxicity, the growth inhibition from phytochemicals may also be species specific (Novak, 1985). About 2000 species of terrestrial plants have been reported for their insecticidal properties (Feinstein, 1952). Several plants have been reported as mimics of insect ecdysone and juvenile hormone (Grainge and Ahmed, 1988). The phytochemicals of the plants serve as huge storage of compounds like alkaloids, flavonoid, saponins and tannins are known to possess medicinal and pesticidal properties (Azmathullah *et al*., 2011, Daniela *et al*., 2013). Bioactive organic compounds produced by plants can act as growth inhibitors, repellents, oviposition deterrants or toxin and food deterrents (Ezeonu *et al.,* 2001, Carlini and Grossi-de-Sa, 2002). Thus, crude plant extracts has been screened as natural and biodegradable form to control pest and vectors of infectious diseases (Omena *et al.,* 2007).

In certain instances, the same phytochemical toxin from a single plant species exhibits various degrees of toxicity to different mosquito species. Minijas and Sarda (1986) showed that crude extracts containing saponin from fruit pods of *Swartzia madagascariensis* produced higher mortality rate in larvae of *An. gambiae* than in larvae of *Ae. aegypti* and no mortality was induced in larvae of *Cx. quinquefasciatus*. Sujatha *et al*., (1988) also observed differential susceptibilities with petroleum ether extracts of fruit pods of *Acorus calamus*, *Ageratum conyzoides*, *Annona squamosa, Bambusa rundanasia*, *Madhuca longifolia* and *Citrus medico* against larvae of 3 species of mosquitoes. Of the six extracts *Bambusa arundanasia* was most toxic against *An. stephensi* and *Acorus calamus* extract, was found most effective against *Cx. quinquefasciatus. Citrus medico* extracts affected only in *An.*

stephensi larvae whereas *Madhuca longifolia* extracts had no effect on this species. Similarly, when extracts of the pond weeds *Myriophyllum* and *Potamogeton* were assayed against larvae of *An. occidentals* and *Cx. pipiens* showed more resistance to both extracts (Graham and Schooley, 1984). Saxena and Sumithra (1989) found the leaf extract of *Ipomea carneafistolosa* most effective against *An. stephensi* when they tested the extract against larvae and pupae of *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus*.

The susceptibility of mosquitoes to phytochemicals differs according to their life stages. Osmani and Sighamony (1980) found that the oil of lemongrass (*Cymbopogon citratus*) and oil of geranium (*Pelargonium roseum*) were poor ovicides and had no effect on first instar larvae, but did cause significant growth inhibition and mortality in later developmental stages of *Ae. aegypti*. Butanol extract of soapberry plant, *Phytolaccado decandra*, was very toxic to second and third instar larvae of *Ae. aegypti* and *Cx. pipiens*, but the eggs and pupae were unaffected and adults died only after ingestion of the concentrated extract (Spielman and Lemma, 1973). Likewise, the ethanolic extracts of *Haplophyllum tuberculatum* did not produce any ovicidal effect, but killed first instar larvae of *Cx. quinquefasciatus* (Mohsen *et al.,* 1989). The latex from *Calotropis procera* showed complete ovicidal and larvicidal effects on *Aedes, Anopheles* and *Culex* but had no adulticidal activities against these genera.

The bioactivity of plant extracts also depends upon the nature of solvent used for the extraction process. The active constituent responsible for the needed bioactivity is extracted in greater measures only with certain solvents. When *Macrocystis pyrifera* and *Artamesia cana* were extracted with water and with organic solvents, the later extract produced higher mortality in *Cx. quinquefasciatus* (Sherif and Hall, 1985) and the effect could have been due to the polarity range of the solvents. Hartzell (1944) tested acetone

extracts and water extracts of certain plant products against *Cx. quinquefasciatus* larvae and found acetone to be a better solvent. *Derris elliptica* root extracts, when bioassayed against fourth instar larvae of *Ae. aegypti*, exhibited a response indicating that absolute ethyl alcohol was the most potent among the liquid extracts, and among the crude residues acetone extract was the most effective (Ameen *et al.,* 1983). The commercial oil of *Linumus itatissimum* had no apparent toxic effects on *Cx. pipiens* larvae, but the crude methanolic extract showed limited toxicity (Banu and Nurul-Huda, 1983). Dhillon *et al*., (1982) found petroleum ether extracts of *Chlorella ellipsoidea* and *Rhizoctonium heireoglyphicurn* eluted with petroleum ether, benzene or methanol all induced mortality in *Ae. aegypti* and *Cx. quinquefasciatus*, with the methanol fraction being the most active. Bioassay of *Aloe pluridens* roots extracted with solvents of increasing polarity showed that the petroleum ether extract possessed the maximum insecticidal activity against *Ae. aegypti* larvae (Confalone *et al.,* 1988). Qureshi *et al*., (1986) assayed alcoholic extracts and petroleum ether extracts of *Ervatamia coronaria* against fourth instar larvae of *Ae. aegypti* and, found the alcoholic extracts to be highly toxic whereas the petroleum ether extract had no larvicidal activity. But in the case of *Acorus calamus*, when the rhizomes were extracted with different solvents like petroleum ether, ether, chloroform and alcohol the best results against *Cx. quinquefasciatus* larvae were obtained with the petroleum ether extract (Chavan *et al*., 1979). According to the bioassay results against *Ae. aegypti* larvae with different extracts of *Lithospermum arvense,* the active principle was concentrated mainly in the hexane-soluble portion (Madrigal *et al*., 1979).

The activation of plant secondary substances by light and their subsequent photosensitizing effects on insects, especially mosquito larvae, is an important factor contributing to the enhancement of toxicity. Certain plant derivatives showed enhanced action in the presence of light. Arnason *et al*.,

(1981) observed light has often been forgotten or under estimated factor in the study of insects. Until recently, little attention has been paid to its role in plant-insect reactions. Polyacetylenes and thiophenes that occur in certain plants of the *Asteraceae* family had shown the greatest potential as photoactive pest control agents. The common marigold, *Tagetes* sp. yielded a highly active polyacetylene alpha-terthienyl from its roots, which proved very toxic to *Ae. aegypti* larvae (Arnason *et al*., 1981, Kagan *et al.,* 1987). The activity increased with light indicating a phototoxic action of alpha-terthienyl. Berberine, an isoquinoline alkaloid present in many different plant families, is also photo-activated. The larval, pupal and adult survival of *Ae. atropalpus* was affected by berberine treatment with toxicity of the alkaloid increasing after exposure to light. Philogene *et al*., (1984) speculated that the fluorescent nature of berberine could be the reason for its photodynamic activity. Rose bengal, a xanthene-derivative, also causes enhanced mortality in mosquito larvae by photosensitized oxidation reactions. Its primary mode of action depends on the absorption of visible light energy, causing photo-oxidative toxicity (Pimprikar *et al*., 1979).

Plant chemicals, by and large act as general toxicants and a few however, and show selective interference with growth and reproduction. Precocene from *Ageratum* was noted for its unique action of interfering with growth by transgressing certain stages of development. In mosquitoes, it prevented pupal formation and adult emergence when newly hatched young larvae were exposed (Cupp *et al.,* 1977). When females were treated with precocene after blood feeding, it inhibited trypsin synthesis, retarding ovarian maturation and resulting in abnormal oviposition (Kelly and Fuchs, 1978). Some other plant chemicals, such as aristolochic acid from *Aristolochia bracteata,* inhibited reproduction, inducing sterility in mosquitoes (Saxena *et al.*, 1979). Biotin from plants, aflatoxin from *Aspergillus flavus* and

pactamycin and porfiromycin from lower plants has also sterilized mosquitoes (Borkovec, 1987).

Although numerous plants have shown tendencies to interfere with growth and reproduction, neem (*Azadirachta indica*) occupies an important place in the biological combat against mosquitoes, because of its strong action in inducing toxicity through inhibition of growth and reproduction. Although the exact mode of action of Azadirachtin and other components present in neem seed kernels is not clearly understood, it seems likely that there is interference in hormonal balance. Zebitz (1984) suggests that azadirachtin acts as an anti-ecdysteroid or affects the neuro-endocrine control of the ecdysteroids. The unique mode of action of Azadirachtin, by its controlling effect on hormones and its favorable toxicological and selective properties from the ecological perspectives, provides a basis for emergence of a promising phytochemical in mosquito control.

The use of phytochemicals as repellents, ovipositional deterrents and anti-feedants has been evaluated against both agricultural pests and medically important insect species (Jacobson, 1958). Thorsell *et al.*, (1970) reported that extracts from 3 plant species, *Ledum palustre*, *Lycopersicon lycopersicum* and *Myrica gale,* exhibited repellency to *Ae. aegypti* adults. The essential oils of certain plants often exhibit repellent actions to mosquitoes, as shown with the leaf oil of *Ocimum suave* (Chogo and Crank, 1981).

In addition to repellency, phytochemicals can influence the ovipositional behavior of mosquitoes. Consoli *et al*., (1989) found ethanolic, hexanic and lyophilized extracts of 8 plants (*Allium sativum*, *Anacardium occidentale*, *Bidens segetum*, *Caesalpinia peltophoroides*, *Jatropha curcas, Mihania schenckii*, *Poinciana anaergia* and *Spathodea campanulata*) deter oviposition of *An. fluviatilis*. Acetone extracts of 4 species of the *Labiatae* family were reported ovipositional deterrents for *Ae. aegypti* (Sharma *et al.*,

1981b), with one species, *Lavendula gibsonii*, having also an ovicidal and general repellent effect on *Ae. aegypti* (Sharma *et al.*, 1981a). The factors of species specificity and the solvent used for extraction are components that can affect ovipositional deterrence from phytochemicals. The methanolic extract of the *Lemna minor* also deterred oviposition in *Ae. aegypti*, but the pentane extract showed no ovipositional deterrent activity. Judd and Borden (1980) theorized from the results of significant ovipositional deterrent activity of the aqueous and methanolic extracts that the active principles are of polar nature.

Several indigenous plants in India and subtropical parts of Asia, such as *Ocimum basilicum*, *Ocimum sanctum*, *Azadirachta indica*, *Lantana camara*, *Vitex negundo* and *Cleome viscose* were studied for their larvicidal action on the field collected fourth instar larvae of *Cx. quinquefasciatus* (Kalyanasundaram and Das, 1985). Chavan (1984), Zebitz (1984, 1986), Schmutterer (1990), Murugan and Jeyabalan (1999) reported that *Leucas aspera*, *Ocimum sanctum*, *Azadirachta indica*, *Allium sativum* and *Curcuma longa* had a strong larvicidal, anti- emergence, adult repellency and anti-reproductive activity against *An. stephensi.* Some botanical extracts such as nicotine obtained from *Nicotiana tabacum* leaves, alkaloidal anabasin and lupinine extracted from *Anabasis aphylla*, rotenone from *Derris elliptica* and pyrethrum from *Chrysanthemum cinerariifolium* flowers have been used as natural insecticides even before the discovery of synthetic organic insecticides (Campbell *et al.*, 1993).

Some of the reports using extracts of different plants like *Cleome viscosa, Ocimum basilicum* and *Vitex negundo* (Kalyanasundaram and Babu, 1982), *Delonix regia* and *Oligochaeta ramose* (Saxena and Yadav, 1982), Neem product (Rao, 1987), *Quassia amara, Anacardium occidentale* and *Theveti aneriifolia* (Evans and Kaleysa Raj, 1988), *Andrographis paniculata* and *Swietenia mahagoni* (Anuradha *et al.,* 1995), *Acalypha indica* and

Ervatamia divaricata (Daniel *et al.,* 1995) *Abrus precatorius, Argemone mexicana, Aristolochia bracteata*, *Artemisia nilagirica, Carica papaya, Coldenia procumbens, lndoneesiella echioides, Jatropha curcas, Pergularia extensa, Toddalia asiatica, Withania somnifera, Wrightia tinctoria* (Karmegam *et al.,* 1996) prove the effectiveness of plant extracts,against the larvae of *Cx. quinquefasciatus.*

The leaf and seed extract of plant *Agave Americana* (Dharmshaktu *et al*., 1987) has mosquito larvicidal properties and the extract of *Tagetes minuta* flowers has mosquito larvicidal activity against *Ae. aegypti* (Green *et al*., 1991). The methanolic fraction of leaves of *Mentha piperita*, *Phyllanthus niruri*, *Leucas aspera* and *Vitex negundo* were against larvae of *Cx. quinquefasciatus* (Pandian *et al*., 1994). The methanolic extracts of *Solanum surattense*, *Azadirachta indica* and *Hydrocotyle javanica* exhibited larvicidal activity against *Cx. quinquefasciatus* (Muthukrishnan *et al*., 1997). Muthukrishnan and Pushpalatha (2001) evaluated the larvicidal activity of extracts from *Calophyllum inophyllum* (Clusiaceae), *Rhinacanthus nasutus* (Acanthaceae), *Solanum suratense* (Solanaceae) and *Samadera indica* (Simaroubaceae), *Myriophyllum spicatum* (Haloragaceae) against *An. stephensi*. In addition, *Pelargonium citrosum* (Jeyabalan *et al*., 2003), *Dalbergia sissoo*(Ansari *et al*., 2000a) and *Mentha piperita* (Ansari *et al*., 2000b) were shown to contain larvicidal and growth inhibitory activity against *An. stephensi.* Jang *et al*., 2002 evaluated the larvicidal activity of methanol extracts of *Cassia obtusifolia*, *Cassia tora* and *Viciatetra sperma* against early fourth stage larvae of *Ae. aegypti* and *Cx. pipiens.*

Bagavan *et al*., 2008 evaluated the acetone, chloroform, ethyl acetate, hexane and methanol extracts of *Acalypha indica*, *Achyranthes aspera*, *Leucas aspera*, *Morinda tinctoria* and *Ocimum sanctum* leaves against the 4th instar larvae of *Cx. quinquefasciatus* and *Ae. aegypti.* Laboratory evaluation

of a phytosteroid compound of mature leaves of Day Jasmine (Solanaceae: Solanales) against larvae of *Cx. quinquefasciatus* (Diptera: Culicidae) and non-target organisms was done by Ghosh *et al*., 2008. Methanolic extracts of *Cassia fistula* leaves were tested for larvicidal and ovicidal activities against *Cx. quinquefasciatus* and *Ae. aegypti* by Govindarajan and team in 2008. They also reported the larvicidal, ovicidal and ovipositional attractant activity of *Acalypha indica* leaves with different solvents like benzene, chloroform, ethyl acetate and methanol against *An. stephensi*.

The larvicidal, ovicidal and repellent activities of *Pemphis acidula* Forst. (Lythraceae) against filarial and dengue vectors were described by Samidurai *et al*., 2009. Govindarajan *et al*., 2011a investigated the larvicidal efficacy of different extracts of *Ficus benghalensis* against *Cx. quinquefasciatus*, *Ae. aegypti* and *An.stephensi*. Niraimathi *et al*., 2010 observed that larvicidal activity of *Sida acuta* was effective against 3rd instar larvae of *An. subpictus* and *Cx. tritaeniorhynchus*. Govindarajan *et al*., 2011b described the ovicidal and repellent activities of methanol leaf extract of *Ervatamia coronaria* and *Caesalpinia pulcherrima* against *Cx. quinquefasciatus, Ae. aegypti* and *An. stephensi*.

Plant derived essential oils can be considered as a valuable alternative for insect control (Govindarajan *et al*., 2008). A mosquito repellent is a substance applied to skin, clothing or other surfaces which discourages insects (and arthropods in general) from landing or climbing on that surface. There is also mosquito repellent products available based on sound production, particularly ultrasound (inaudibly high frequency sounds). The best or most suitable plant based repellents should invariably be non-toxic, non-irritating and long lasting and eco-friendly in nature. Plants of terrestrial origin have been reported to be an important source of mosquito repellents (Hwang *et al*., 1985). The essential oils from medicinal herbs in Lebanon proved as an

environmentally safe measure to control the sea side mosquito (Knio *et al.,* 2008). Studies have shown that low concentrations of plant-derived oils are effective in controlling Coleopterans and *Gelechiid* moths. In addition, it has been reported that the essential oils extracted from *Achillea millefolium* and *Anethum graveolens* possessed insecticidal activities against beetles, cockroaches and mosquitoes (Song *et al*., 2016). However, few studies on plant-derived oils and their related compounds have been conducted to replace synthetic insecticides and acaricides for controlling storage insect and mite pests. Several essential oils from herbs act as *Culex* and *Aedes* larvicides (Sukumar *et al.,* 1991). Essential oils from *Cannabis sativa* (Thomas *et al.,* 2000) and *Taget spatula* (Dharmaggada *et al.,* 2005) were reported to have activity against *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus*. Prajapati *et al.,* (2005) studied the larvicidal, adulticidal, oviposition deterrent and repellent activities of essential oils from 10 medicinal plants against *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus*. The undiluted oils of *Cymbopogon nardus* L., *Pogostemon cablin* Benth., *Syzigium aromaticum* Linn. And *Zanthoxylum limonella* Alston were the most effective and provided two hours of complete repellency (Trongtokit *et al*., 2005).

The essential oils of *Melaleuca* have a strong repellent effect on ant *Wasmannia auropunctata* which is a pest of both the forest plantations and fruit crops **(**Menendez *et al*., 1992). Alonso *et al*., 1996 showed that undiluted oil of Cajuput imposed 100% mortality in cowpea of Cuba and has distinct repellent effects. Cajuput oil is also known to prevent the infection of the plant of the herpes simplex type 1 on plant and inhibits the growth of fungi, bacteria and yeast (Dubey *et al*., 1983, Dhirendra *et al*., 1989, De Colmenares *et al*., 1998, Farag *et al*., 1998, Nawawi *et al*., 1999). Ursolic acid isolated from the chloroform extract and piceatannol and oxyresveratrol isolated from the methanolic extract are identified from Cajuput oil as active compounds. The alpha-terpineol and linalool, the contents of it exhibit antimicrobial

activity. The essential oil from *M. leucadendron leaves* are green in color with a camphor odor and is medicinally farming such as antiseptic, stomachic stimulant, analgesic, anti-rheumatic, expectorant and for treatment of intestinal worms **(**Usher, 1974, Kitanov *et al*., 1992). *M. leucadendron* showed 100% repellency against *Cx. quinquefasciatus* and 80.9% repellency against *Ae. aegypti* at its 8th hour of exposure. Adulticidal activities of the essential oils observed after 24hour exposure showed 100% adult mortality, indicating their potential as biocontrol agents for mosquitoes (Pushpalatha and Anju Viswan, 2013). Amer and Mehlhorn (2006), reported forty one essential oils against different species of mosquitoes and found out five most effective essential oils viz; *Litsea cubeba, M. leucadendron, M. quinquenervia, Viola odorata* and *Nepeta cataria* which induced 100% repellency over a protection period of 480 min against *Ae. aegypti, An. stephensi* and *Cx. quinquefasciatus.*

Anamirta cocculus (L.) Wight & Arn is a wild woody climber belonging to the family Menispermaceae, distributed throughout India as well as South-East Asia. *A. cocculus* either alone or in combination with other mosquito control agents, taking into consideration of the cost effectiveness and availability of the plant materials, can lead to the evolution of a better mosquito control agent (Harve and Kamath, 2004). Phenolic compounds, alkaloids and glycosides present in the seeds possess medicinal properties including antibacterial activity (Okwu, 2004, Afolabi *et al*., 2007). Its seeds are known as Indian fish berry or crow killer and are being exploited by humans for hunting and fishing. The seeds are also utilised in eradicating the unwanted wild fishes from aquaculture ponds (Jothivel and Paul, 2008a, b). The possibility of the presence of other secondary metabolites in these seeds can not be ruled out as it has been used in traditional medicine for treating various diseases (Mutheeswaran *et al*., 2011). In addition to the antimicrobial activity, alkaloids, glycosides and phenols present in *Anamirta* seed extracts

are potent and are reported to cause mortality even in ticks also (Kumar *et al*., 2011). Picrotoxin (cocculin) is the major reported toxic component of the *Anamirta* seed and is composed of poisonous picrotoxinin and the bitter non-poisonous picrotin. Altogether five sesquiterpene lactones viz., picrotoxinin, methyl picrotoxate, picrotin, dihydroxypicrotoxinin and picrotoxic acid have been isolated from the seeds of *A. cocculus*. The rich presence of fats and fatty acids in the seeds of *A. cocculus* might be rendering the extracts a waxy-solid consistency as well as an acidic nature. The traditional use of *A. cocculus* as a remedy for barber's itch, scald-head itch and other unyielding skin diseases indicates its antimicrobial role. Due to the presence of various bioactive compounds in *A. cocculus*, antibacterial, antifungal and anti-inflammatory properties have been attributed to it. It is primarily a piscicidal plant which produces various primary and secondary metabolites (Satya and Paridhavi, 2012). Presence of flavonoids in the methanol extracts of *A. cocculus* seeds helps in the potential usage of these compounds for antimicrobial action. Alkaloid derivatives of plant origin are widely used as antimicrobial agents. Presence of heterocyclic nitrogen containing alkaloids in benzene, petroleum ether and methanol extracts indicates the pharmacological significance of *A. cocculus* (Umer *et al*., 2015). The acetone extract of *A. cocculus* fruit did not exhibit any malformation in the treated concentrations as the larvae never survived beyond III instar stage so as to develop any malformations and the plant extract caused complete death of the treated larval populations (Pushpalatha *et al*., 2015).

The present study aims to explore the larvicidal, adulticidal and repellent activity of *M. leucadendron* and *A. cocculus* extracts against *Cx. quinquefasciatus* and *Ae. albopictus* and also to study the potential compounds from the plants for an alternative to the synthetic insecticides to control mosquito vectors.

2. 3. MATERIALS AND METHODS

2.3.1. TEST ORGANISM

The mosquito species *Culex quinquefasciatus* Say and *Aedes albopictus* Skuse were used for the study.

2.3.1.1. *Culex quinquefasciatus* Say (Plate 2.1)

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Diptera
Family	:	Culicidae
Genus	:	*Culex*
Species	:	*quinquefasciatus*

Cx. quinquefasciatus, the southern house mosquito is the most widely distributed mosquito in India (Borah *et al.,* 2010). It is a medium sized, brown colored, night-time active, opportunistic blood feeder and vector of many pathogens, several of which affect humans. *Cx. quinquefasciatus* is the principal vector of bancroftian filariasis. This mosquito species is also a potential vector of several arboviruses like West Nile virus (WNV), Rift Valley fever virus, JE virus, avian pox and protozoa like *Plasmodium relictum* that causes bird malaria. Furthermore, it can transmit several other arboviruses in the laboratory conditions. It acts as an important "urban bridge vector" which bridges different reservoir/amplifier hosts to humans because of its encounter with different vertebrates. *Cx.quinquefasciatus* also creates an ecological bridge between urban, peri urban and rural areas owing to its presence and adaptability in diverse ecological niches. *Cx. quinquefasciatus* emerged as a smart vector because of the adaptive fitness, ecological

plasticity, invasive behaviour, host specificity and high reproductive potential along with expanded immune gene repertoire property at the genetic level. *Cx. quinquefasciatus* possesses the necessary potential to initiate and facilitate the disease transmission by establishing an effective vector-host transmission cycle for diverse pathogens in different environments (Bhattacharya and Basu, 2016).

Breeding Sites of *Culex* Mosquito:

Culex, the domestic pest mosquitoes breed in association with human habitations. They preferred to breed in polluted waters, such as sewage and sullage/grey water systems including water collections from household sinks, showers, baths and septic tanks. Their egg-raft found on the surface of water in rain barrels, neglected bird baths, swimming pools, clogged rain gutter, tin cans, old tires, car bodies, cisterns, roof gutters and any other containers which hold water. They can also breed in comparatively clean water collections if such types of polluted water bodies are absent.

Life Cycle of *Culex* Mosquito:

Culex mosquitoes like other insects also passes through different stages like egg-raft, larva, pupa and adult in its life cycle.

Egg-raft: *Culex* genus mainly lays their eggs in the form of raft. Each raft may contain 200-300 or more eggs, loosely cemented together. Their egg numbers per egg-raft depend upon the species and the quality and quantity of blood meal taken by them. A single gravid female may lay up to 5 egg rafts in its life time. The egg normally hatches at the optimum temperature of 25°C to 30°C within 24 to 30 hours after being oviposited (NVBDCP, 2012).

Larva: There are four larval stages (L1, L2, L3 and L4) and each stage has the larval duration of about 24 to 26 hours at optimum temperature and

changed by the molting process. After hatching, the youngest stage is called first instar larva (L1). All the instars are voracious eaters, taking anything and everything of microscopic size into the buccal cavity by instant vibration of its feeding brushes. They are mainly bottom feeders but may feed from the surface also. These larvae show distinctive swimming style and hence known as "wrigglers". They have a distinctive tube for breathing which extends from the end of their body.

Pupa: At the end of its 4th instar stage they moult into to a comma shaped pupa. Pupae do not feed but are very active, respiring through its pair of breathing trumpets. The pupal period is approximately 36 hours at 27 ^{0}C and then they transform into adults. The entire cycle from egg to emergence of adult is completed in 10- 14 days.

Adult: The pupa finally moults into adult leaving the life stages confined to aquatic habitat. Male adult mosquitoes primarily feed on nectar and do not bite humans while the female mosquito after mating requires a blood meal for their ovary development. Adult mosquitoes of both sexes require carbohydrate foods. They generally feed during the evening and morning. The life span of female and male *Culex* mosquito is about more than a month and 1 to 2 weeks respectively.

2.3.1.2. *Aedes albopictus* Skuse

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Diptera
Family	:	Culicidae
Genus	:	*Aedes*
Species	:	*albopictus*

Ae. albopictus also called as Asian tiger mosquito. It is a small, dark mosquito with a white dorsal stripe and banded legs. They are strongly attracted to humans for feeding, but will also feed on cats, dogs, squirrels, deer and other mammals as well as birds. They bite outdoors and indoors, but are usually found outside. *Ae. albopictus* is most well-known for transmitting dengue and chikungunya viruses. It can also transmit dog heartworm parasites. *Ae.albopictus* is a very aggressive day-time biter. This mosquito has a rapid bite that allows it to escape most attempts by people to swat it. Because these mosquitoes are produced in nearly any sort of water-filled container, they often become very common and bothersome, even in neighborhoods where there are normally few mosquitoes.

Breeding Sites of *Aedes* Mosquito:

The Asian tiger mosquito lays its eggs on the inner sides of water-holding receptacles in urban, suburban and rural areas as well as in nearby edges of forested areas. *Ae. albopictus* is closely associated with vegetated areas in and around homes. The immature forms (larvae and pupae) are found in artificial containers with water such as tires, flower pots, plates under potted plants, cemetery urns/vases, buckets, tin cans, clogged rain gutters, ornamental ponds, drums, water bowls for pets, birdbaths etc. In some instances this species has been found in catch basins. Larvae can also be found in natural habitats such as tree holes, rock holes, hollow bamboo stumps and leaf axils. It is a forest dwelling species and has adequately adapted itself to urban environments as well. Actually it is a native of Asia, and it has spread to most tropical parts of the globe. In Southern India, especially in Kerala state, it breeds abundantly in the latex collecting cups associated with rubber plantations.

Life Cycle of *Aedes* Mosquito:

The life cycle is completed through four distinct stages: egg, larva, pupa and adult.

The Egg: Eggs are small, about 1mm in length, torpedo shaped and deeply black in colour, although when they are freshly laid they are transluscent and pure white. The eggs are capable to remain viable for weeks or even months and can withstand prolonged desiccation. The head of the larva occupies nearly one-third of the egg volume. While the siphon and other terminal parts fill most of the remaining posterior pole. The egg has very characteristic polygonal markings lined with a network of milky white membraneous channels filled with air at the base of the exochorion. Beneath exochorion is a dense, tough and hard endochorion membrane which gives the egg its shape and rigidity.

The Larva: As in the case all other mosquitoes there are four larval instars, each instar ending in a moult or ecdysis. The first instar larva hatches out from the egg by first widening the transverse crack at the dorsal side anteriorly. Finally the larva breaks the egg by means of a horne-shaped egg breaker. Initially the freshly hatched larva is unable to move but shortly the young larva wriggles its way to the water surface to make the contact with free air. It takes about 2-3days at 27-29ºC to hatch out from the egg. However the larva may take few minutes to several hours to emerge at the surface. The first instar larva is identifiable owing to the presence of the egg breaker. The larva carries a characteristic siphon which carries the trachea.

The second and third instar larvae can be identified by their size and general appearance. Little change occurs until fourth instar when the development of future pupal structure begins. The compound eyes or the optic lobes begin to form in the late third instar. The imaginal buds at the bases of

the pleural hairs become apparent in the second instar and are quite conspicuous in the third though their development is a feature of the fourth instar. The gonads are already visible in the 6th abdominal segment in the third instar, when the buds of the developing gonoxites also become apparent.

During the fourth instar period a condition is reached in which the larva is little more than the sculptured body of the future adult disguised in the larval skin. Such a condition is termed as pre pupa. The most important factor that influence the larval duration is the water temperature.

The Pupa: The main characters of the pupae are the presence of the respiratory trumpets, the arrangements and characters of the hairs, particularly those in abdominal region and the characters of the tall fins or paddles. The pupal body consists of a large globular anterior region, the cephalothorax and a narrower abdomen which is normally kept flexed under the former to propel the insect when swimming.

The Adult: Adults mosquitoes are easily identifiable by the stripe of silvery white scales on the dorsum of thorax. It is a domesticated mosquito and breeds almost exclusively in artificial containers in and around human habitations. The female lays their eggs singly on the water at the margin, or on the sides of the container above the water line. The adult mostly prefer human blood and it is a day biter. The adult quietly hails to settle for feeding, preferring to bite around the ankles, under shirt sleeves or in the back of the neck.

Maintenance of mosquitoes in the laboratory

The larvae or pupae were collected from the field were brought to the laboratory and transferred to small sized plastic trays containing water. The pupae moulted were collected and kept for emergence in mosquito emergence cage. The freshly emerged adults were identified and maintained at room

temperature. The adult females were given blood meals from an immobilized hen and the males were fed by 10% sucrose solutions. Bowls with water were provided in the cage for oviposition. The freshly laid eggs were collected from the cage and transferred in a tray and allowed to hatch. The freshly hatched larvae were fed by fine powder of dog biscuit and yeast in the ratio 3:1. The water was changed in every alternative day. The feeding was continued till the larvae transformed in to pupae. The freshly moulted larvae were selected for bioassay tests.

2.3.2. PLANTS SELECTED FOR THE STUDY:

Two plants were selected for the present study; *Melaleuca leucadendron* L. and *Anamirta cocculus* (L.) Wight & Arn

2.3.2.1 *Melaleuca leucadendron* L.

Latin name	:	*Melaleuca leucadendron* (L)
Sanskrit/Indian name	:	*Kayaputi, Kajuput*
Common name	:	Cajeput, white tea tree, swamp tea tree etc..
Kingdom	:	Plantae
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Order	:	Myrtales
Family	:	Myrtaceae
Genus	:	*Melaleuca*
Species	:	*leucadendron*

Melaleuca leucadendron (L) grows to a tree of nearly 20 m height and has a long flexible trunk with irregular ascending branches covered with a pale thick, lamellated bark, it is soft and spongy and from time to time throw off its outer layer in flakes; leaves entire, linear, lanceolate ash colour,

alternate on short foot stalks; flowers sessile white on long spike. The leaves have a very aromatic odour and the oil is distilled from the fresh leaves and twigs, and is volatile and stimulating with an aroma like camphor, rosemary or cardamom seeds. Taste bitter, aromatic and camphoraceous. Traces of copper have been found in it, hence the greenish tint. It should be stored in amber colored bottles in cool place. Cajuput oil is fluid, clear inflammable burns without residue and highly volatile.

The essential oil of the Cajuput Tree is obtained by using steam to distill the plant leaves. The principal constituent of oil is cineol, which should average 45-55%. Solid terpineol is also present. Aldehydes such as valeric, butyric and benzoic are also present. Traditionally, the leaves of this plant have been used for healing skin cuts, burns and infections. Recent studies have revealed its antifungal property, making it an important ingredient in treating scalp infections like dandruff. Cajuput oil is also helpful in treating respiratory problems. Common sore throats, coughs, runny nose and severe respiratory ailments such as asthma, tuberculosis and bronchitis can be treated with Cajuput oil. Used as a stimulatory expectorant in chronic laryngitis and bronchitis, as an antiseptic in cystisis, as an antihelminthic against round worms and also used in chronic rheumatism. The oil has antibacterial, antifungal and antiviral properties, which quickly heal superficial wounds. Used in the manufacture of Anti-dandruff hair oil and Anti-dandruff shampoo. Used externally for psoriasis and other skin infections.

2.3.2.2 *Anamirta cocculus* (L.) Wight and Arn.

Latin name	:	***Anamirta cocculus***
Common name	:	levant berries, fish berries, kallakkayaa, pettumarunnu
Kingdom	:	Plantae

Division	:	Magnoliophyta
Class	:	Magnoliopsida
Order	:	Rananculales
Family	:	Menispermaceae
Genus	:	*Anamirta*
Species	:	*cocculus*

Anamirta cocculus (L) Wight and Arn. is large woody climbing shrub with vertically furrowed ash colored bark and glabrous young parts. Leaves large, simple, alternate, long petiole, petioles thickened at the base and apex broadly ovate, subcoriaceous, cordate or truncate at the base, tufts of hairs in the axils of the nerves except the basal ones, flowers greenish in long panicles, drooping from the nodes of the old wood, fruits druped kidney shaped turning red on ripening.

The berries / fruits of plant are slightly bigger than full-sized pea and are round shaped. They are wrinkled and blackish brown in colour. The kernel is white, bitter in taste and oily. The seed contains approximately 1.5 percent of poisonous crystalline Picrotoxin (sesquiterpene glycoside $C_{30}H_{34}O_{13}$). Picrotoxin is also present in leaves. The seeds contain two alkaloids menispermine and paramenispermine. Seeds yield fat with a pale yellow colour. *A. cocculus* contain palmitic acid, stearic acid, oleic acid, linoleic acid and also the saponified matter contains sitosterol.

2.3.3.. PREPARATION OF PLANT EXTRACTS

Fresh leaves of *M.leucadendron* and seeds of *A.cocculus* were collected from Calicut University campus. The leaves were thoroughly washed with water and shade dried in the room temperature. The dried materials of selected plants were powdered using a mixer grinder and sifted

through a fine mesh of sieve. The powders were packed in airtight ziplog bags (half a kg capacity) and stored at -20ºC.

A known weight of powdered material was taken in a thimble (Whatman No.1 filter paper) and loaded in the Soxhlet extractor. The soxhlet extractor is placed in to the flask containing analytical grade acetone or methanol. It is placed in a water bath and allowed to heat at a temperature of 60^0 C. The solvent vapour travels up the distillation arm and floods in to the chamber housing the thimble of dried plant materials. The condenser ensures that any solvent vapours cools and drips back down in to the chamber housing the dried powders some of the compounds in the plant materials slowly dissolve in the warm solvents. When Soxhlet chamber is almost full, the chamber is automatically emptied by siphon side arm with the solvent running back to the distillation flask. The cycle is repeated for fifteen times. After fifteen cycles the desired tire is taken concentrated in the rotary evaporator. The filtrate was taken and transferred in to a pre weighed petridish. The yield of the material was calculated from the dried extract. A part of the extract taken and 10% stock solutions were prepared and stored in air tight amber coloured glass containers and kept it in a refrigerator.

Defattation of methanol extracts:

Methanol extract is then defatted with equal volume of analytical grade petroleum ether (60^0 - 80^0 C). 100 ml of crude methanol extract is taken in a separating funnel and 100 ml of petroleum ether was added to it and mixed well and left for separation. The distinct layers were then separated slowly and collected in to conical flasks. After the collection the solution is placed in to pre- weighed petri plate and allowed to dry. From the dried extract, 1% stock solution was prepared and refrigerated in air tight amber coloured bottles.

Partial purification with ethyl acetate:

Defatted methanol fraction was then subjected to fractionation by using ethyl acetate and double distilled water.

Cold Extraction method:

Powders of *A. cocculus* and *M. leucadendron* were packed as 20g packets using Whatman No.40 filter paper and held by stapler pin. The thimbles were placed carefully in to one litre conical flask thus leaving 3 packets per flask. Five such conical flasks were arranged. Then methanol for *A. cocculus* and acetone for *M. leucadendron* was added to each conical flask in such a way that the packets were submerged in the solvent. The mouth of the conical flasks were tightly plugged with non-absorbent cotton, wrapped with aluminium foil and paraffin wax paper and secured using rubber band. Whenever needed methanol was added to the conical flasks. After 48h, the extracts obtained from the conical flasks were filtered through Whatman No.40 filter paper. Then the collected extracts were reduced in vacuum rotary evaporator and collected miscella were transferred to amber coloured bottle, tightly covered, labelled and stored in a deep freezer at -20ºC.

Fractionation of *A. cocculus* extracts:

Fractionation of miscella was done by following column chromatography techniques. A glass column of 50cm length was used. Silica gel powder (60/120 mesh) was packed in the column and loaded with 5g of miscella.

Then the miscella was eluted with different solvents (HPLC grades) and solvent systems such as n-heaxane, ethyl acetate and acetone and combination of solvents at different ratio viz., n-hexane, ethyl acetate, hexane: ethyl acetate (1:1), hexane: ethyl acetate (1:2), hexane: ethyl acetate (1:3), hexane: ethyl acetate (3:1), hexane: ethyl acetate (2:1) and acetone.

Fractionation of *M.leucadendron* extracts:

Fractionation of miscella was done by following column chromatography methods. A glass column of 50cm length was used. Silica gel powder (60/120 mesh) was packed in the column and loaded with 5g of miscella.

Then the miscella was eluted with different solvents (HPLC grades) and solvent systems such as n-heaxane, ethyl acetate and chloroform and combination of solvents at different ratio viz., n-hexane and ethyl acetate (50:50), ethyl acetate and chloroform (50:50) and chloroform and n- hexane (50:50).

100 ml of each solvents and combinations of solvents were used for elution. The elutes collected were dried at room temperature and the extracts were further diluted and used in bioassays.

2.3.4. EXTRACTION OF ESSENTIAL OIL

Fresh leaves of *M. leucadendron* collected from the field, washed with distilled water and essential oils were extracted by steam distillation in Clevenger apparatus (Craveiro *et al.,* 1976). Fresh leaves were cut into small pieces and added to the round bottom flask of Clevenger apparatus up to one third levels. Double distilled water is added to the flask up to two third level of the flask. It is placed in the mantle and heated up to a temperature of 100^0C. The oil formed and deposited on the side tube and collected in to the vials and kept in refrigerator for further use.

2.3.5. BIOASSAY

Different extracts of selected plant materials were tested against 1^{st} to 4^{th}instar larvae of *Cx. quinquefasciatus* and *Ae. albopictus*. The appropriate volume of 10% stock solution was diluted to 250ml of fresh water in a disposable glass to obtain desired concentration of the test medium. 10 larvae were released in to each glass containing test medium. Controls with 250ml of

fresh water and with different concentrations of acetone and methanol were also set. Triplicates were also maintained with each set. Observations were taken after 1hour, and the mortality of the larvae at the end of 24 hour, 48 hour and 72hours were recorded and percentage mortality is estimated. LC_{50} were calculated using a Probit programme developed by Finney, 1971.

2.3.6. EFFECT ON LARAVAL DURATION

Different concentrations of EA fraction of the selected plant extracts were set and exposed the first instar larvae in to it to find out any effect on larval duration. Observations were made every day to check moulting into the next stage and/or the death or emergence of the treated larvae.

2.3.7. ADULTICIDAL BIOASSAY

Sugar fed adult mosquitoes (4-6 days old) was used for bioassay. Different concentrations of the essential oils were impregnated on filter papers of $1cm^2$ size. The bioassay was conducted in a cylindrical glass tube (15cm X 5cm) following the method of WHO (1981). The experiment was carried out in triplicate for each essential oil. For each replicate two tubes were used; one was used to expose the mosquitoes to the essential oil and another to hold the mosquitoes before and after the exposure period. Each tube was closed at one end with a wire mesh screen. Twenty sucrose fed mosquitoes were released in to the tube, and the mortality rate was observed every 15 min for 3h exposure. At the end of the exposure period, the mosquitoes were transferred in to the holding tube. A cotton pad soaked in 10% sugar solution was placed in the tube during the holding period. Mortality of mosquitoes recorded after 24h.

2.3.8. REPELLENCY ASSAY

Repellent activity of volatile oils was evaluated using human bait technique. Each test was conducted for a period of 8 hrs, depending on the

response. *Ae. albopictus* was tested between 07.00 h and 15.00 h while *Cx. quinquefasciatus* was tested between 17.00 h and 01.00 h. Each oil was tested 100, 70, 50 and 10 percent concentrations. An arm of a human volunteer was covered with a paper sleeve with 3x10cm window and 0.1ml of desired concentrations of the oil was applied. The uncovered arm was exposed into a standard mosquito emergence cage having 100 hungry 4 to 5 days old female mosquitoes for one minute. Prior to the commencement of each exposure, the mosquitoes were tested for their readiness to bite by placing an untreated bare hand of a volunteer for 30 seconds. An arm of human volunteer without any oil application was kept as control. The number of incidence of landing without biting and those of biting ones were recorded at each interval until the rate of bite fell into 1 to 1.5 per minute. The duration between the application of repellent and the commencement of bite was recorded as the protection time. The percentage of repellency was calculated at the end of every test using the formula mentioned by Tawastsin *et al.,* (2001) viz; (C-T/C) x 100 where, C is total number of mosquitoes landing or biting the control area and T is total number of mosquitoes landing or biting the treated area.

2.3.9. GCMS ANALYSIS

The essential oil taken using the Clevenger apparatus were given for the GCMS Analysis at Kottakkal Arya Vaidya Sala, Kottakkal, Malappuram, Kerala. They used Agilent Technologies 6850 Network GC system equipped with HP5MS column (30m x 0.25mm and film thickness 0.25um) and MS 5975 CVLMSD with triple axis detector.

2.3.10.NMR SPECTRA ANALYSIS

To identify the compounds present in the fractions we have carried out the structural elucidation of the compounds isolated from chromatographic studies. Preliminary structural analysis using LC-MS, NMR spectroscopic techniques was carried out in collaboration with Department of Chemistry, Annamalai University.

The Nuclear magnetic resonance (NMR) spectroscopy is most frequently used technique by the chemists and biochemists to analyze the properties of organic molecules. It is a research technique that exploits the magnetic properties of certain atomic nuclei. It determines the physical and chemical properties of atoms or molecules in which they contained. The samples in the range of small compounds were analyzed using 1-dimensional (^{1}H) proton or carbon-13 (^{13}C) NMR spectroscopy. The large macromolecules or nucleic acids analyzed using the 3 or 4-dimensional techniques. The impact of NMR spectroscopy on the sciences has been substantial because of the range of information and the diversity of samples, including solutions and solids (Johnson, 1999). The NMR spectra (^{1}H &^{13}C NMR) were recorded at 400/100MHz using DMSO-d^{6} as a solvent system and tetramethylsilane (TMS) used as an internal standard (Hari and Thriveni, 2016).

2.3.11.LCMS ANALYSIS

The mass spectrometry used to calculate the mass to charge measurement after assigning the most abundant ion 100%. It is valuable analytical tool for the organic chemist who deals with compounds of molecular weight below 600 and must be appreciable volatility (Ernest *et al.*, 1957). The peak of a mass spectrum with the greatest intensity is called the base peak. The molecular ion is often, but not always, the base peak. The complexity of fragmentation patterns has led to mass spectra being used as "fingerprints" for identifying compounds (Mc Lafferty, 1959).

2.3.12.STATISTICAL ANALYSIS

Statistical analysis was performed using Statistical package SPSS 20.0

2.4. RESULTS

2.4.1. PERCENTAGE YIELD OF SELECTED PLANTS

The percentage yield of acetone and methanol extracts of the selected plants; *M. leucadendron* and *A. cocculus* is provided in table 1 and 2. The yields of the methanol and acetone extracts of *A. cocculus* are 28 % and 30% respectively. The methanol extract appeared in light brown colour and that with acetone was yellowish brown and both presented a waxy solid appearance. When the extract dissolved in water it gives a light milky appearance to the water. The percentage yield of methanol and acetone extract of *M. leucadendron* was 26 % and 28% respectively. The colour of both the acetone and methanol extracts was greenish brown and it appeared in powder form. The extracts completely dissolve in water.

Table 1: Physico-chemical properties of *A. cocculus* seed extracts

Purticulars	Methanol Extract	Acetone Extract
Yield (%)	28	30
Colour	Light brown	Yellowish brown
Consistency	Waxy Solid	Waxy Solid

Table 2: Physico-chemical properties of *M. leucadendron* leaf extracts

Purticulars	Methanol Extract	Acetone Extract
Yield (%)	26	28
Colour	Greenish brown	Greenish brown
Consistency	Powder	Powder

2.4. 2. CRITICAL LETHAL CONCENTRATIONS:

The data on 24 hr, 48 hr and 72 hr LC_{50} and LC_{90} (ppm) values of the acetone extract of *A. cocculus* leaf tested against *Cx. quinquefasciatus* are provided in table 3, 4 and 5 respectively.

The values of 24 hr LC_{50} for the different instars of the larvae *Cx.quinquefasciatus* ranged from 67.77 ppm to 107.86ppm for all the four larval stages. The 48 and 72 hr LC_{50} values of the acetone extract of the *A. cocculus* ranged from 48.36 to 96.89 ppm and 35.06 to 82.7 ppm for all the four larval stages. The LC_{90} values for I, II, III & IV instar larvae were 107.86, 120.41, 148.80, 153.84 ppm for 24 hrs, 97.89, 105.51,139.02,144.43 ppm for 48 hr and 89.33, 91.42, 123.45 and 134.4 ppm for at 72 hrs respectively.

Table 3: 24 hr LC_{50} (ppm) and associated statistics of *A. cocculus* acetone extracts tested against different instar stages of *Cx. quinquefasciatus*

Instar	LC50 (LFL-UFL)	LC90 (LFL-UFL)	χ^2
1	67.77 (59.59- 77.07)	107.86 (96.03- 124.74)	3.42
2	83.95 (76.76-93.58)	120.41 (106.79-140.29)	2.84
3	100.08 (91.8-110.64)	148.80 (119.62-168.42)	4.22
4	107.86 (96.03-124.74)	153.84 (139.79-178.47)	6.54

Table 4: 48 hr LC_{50} (ppm) and associated statistics of *A. cocculus* acetone extracts tested against different instar stages of *Cx. quinquefasciatus*

Instar	LC50 (LFL-UFL)	LC90 (LFL-UFL)	χ^2
1	48.36 (39.46- 56.50)	97.89 (87.33-112.51)	1.62
2	64.71 (59.73- 71.13)	105.51 (95.59- 118.97)	3.84
3	91.05 (76.8-107.65)	139.02 (122.55-163.56)	4.42
4	96.89 (88.07-108.8)	144.43 (127.11-170.37)	1.65

Table 5: 72 hr LC_{50} (ppm) and associated statistics of *A. cocculus* acetone extracts tested against different instar stages of *Cx. quinquefasciatus*

Instar	LC50 (LFL-UFL)	LC90 (LFL-UFL)	χ^2
1	35.06 (24.43- 43.65)	89.33 (79.73-102.16)	2.24
2	48.36 (39.46 – 56.50)	91.42 (83.29-102.36)	6.82
3	71.44 (61.02- 89.94)	123.45 (109.38 – 144.08)	5.32
4	82.70 (69.89-105.84)	134.40 (118.66- 157.77)	4.56

The data on LC_{50} (ppm) and LC_{90} (ppm) values of acetone extracts of *A.cocculus* tested against *Ae. albopictus* are described in Table 6, 7 and 8. The 24 hr LC_{50} (ppm) values of acetone extracts of *A. cocculus* were 64.71, 81.13, 96.89, 105.57 ppm and LC_{90} (ppm) values were 105.57, 118.48, 139.02, 144.43 ppm for I to IV instar larvae respectively (table 6). The 48 hr LC_{50} (ppm) and LC_{90} (ppm) values were 44.12, 59.41, 83.95, 94.79 ppm and 96.89,

107.21, 134.41, 139.02 ppm and for 72 hr LC_{50} (ppm) and LC_{90} (ppm) values were 31.93, 40.94, 54.51, 74.53 ppm and 87.3, 82.72, 106.86, and 130.36 ppm respectively for the I, II, III and IV instar larvae of *Ae. albopictus* (tables 7& 8).

Table 6: 24 hr LC_{50} (ppm) and associated statistics of *A. cocculus* acetone extracts tested against different instar stages of *Ae. albopictus*

Instar	LC50 (LFL-UFL)	LC90 (LFL-UFL)	χ^2
1	64.71 (59.73-71.13)	105.57 (95.58- 118.97)	2.81
2	81.13 (74.26- 90.24)	118.48 (109.09-123.16)	6.2
3	96.89 (88.07-108.82)	139.02 (122.55- 163.56)	7.42
4	105.57 (95.59-118.97)	144.43 (127.11- 170.37)	4.44

Table 7: 48 hr LC_{50} (ppm) and associated statistics of *A. cocculus* acetone extracts tested against different instar stages of *Ae. albopictus*

Instar	LC50 (LFL-UFL)	LC90 (LFL-UFL)	χ^2
1	44.12 (40.68- 47.93)	96.89 (88.07 –108.81)	3.24
2	59.41 (51.38- 73.11)	107.21 (97.02- 122.64)	4.88
3	83.95 (76.76- 93.58)	134.41 (118.66-157.78)	6.32
4	94.79 (82.33-108.51)	139.02 (122.55-163.56)	8.46

Table 8: 72 hr LC_{50} (ppm) and associated statistics of *A. cocculus* acetone extracts tested against different instar stages of *Ae. albopictus*

Instar	LC50 (LFL-UFL)	LC90 (LFL-UFL)	χ^2
1	31.93 (28.31-35.31)	87.3 (79.69- 97.51)	2.68
2	40.94 (35.59-48.27)	82.72 (69.89- 105.84)	3.86
3	64.51 (51.66 – 98.29)	106.86 (95.02- 123.64)	2.46
4	74.53 (66.11- 84.73)	130.36 (115.24-152.71)	6.82

Tables 9, 10 and 11 provide the data on LC_{50} (ppm) and LC_{90} (ppm) values of methanol extracts of *A. cocculus* against *Cx. quinquefasciatus.* The LC_{50} (LC_{90}) values for I, II, III & IV instars larvae for 24 hrs were 83.95 (172.64), 96.89 (184.36), 126.37 (197.56) and 132.34 (202.54) and that of 48 and 72hrs were 76.37 (158.84), 87.43 (178.42), 107.86 (173.91), 116.64 (188.44) and 64.71 (136.46), 78.07 (142.54), 91.41 (144.43), 96.89 (159.51) respectively.

Table 9: 24 hr LC_{50} (ppm) and associated statistics of *A. cocculus* methanol extracts tested against different instar stages of *Cx. quinquefasciatus* larvae

Instar	LC50 (LFL-UFL)	LC90 (LFL-UFL)	χ^2
1	83.95 (76.76- 93.58)	172.64 (128.46-199.88)	4.56
2	96.89 (88.06- 108.8)	184.36 (140.56-200.64)	8.98
3	126.37 (113.82-143.93)	197.56 (139.79-174.87)	3.48
4	132.34 (118.54-145.65)	202.54 (189.86-212.65)	4.56

Table 10: 48 hr LC_{50} (ppm) and associated statistics of *A. cocculus* methanol extracts tested against different instar stages of *Cx. quinquefasciatus* larvae

Instar	LC50 (LFL-UFL)	LC90 (LFL-UFL)	χ^2
1	76.37 (70.09- 84.69)	158.84 (148.86- 202.52)	6.86
2	87.43 (76.69- 97.51)	178.42 (150.54- 204.24)	3.62
3	107.86 (96.02-124.74)	173.91 (151.75-207.55)	5.45
4	116.64 (96.22-136.72)	188.44 (160.56- 210.42)	2.22

Table 11: 72 hr LC_{50} (ppm) and associated statistics of *A. cocculus* methanol extracts tested against different instar stages of *Cx. quinquefasciatus* larvae

Instar	LC50 (LFL-UFL)	LC90 (LFL-UFL)	$\chi 2$
1	64.71 (59.73- 71.13)	136.46 (118.78- 169.98)	6.64
2	78.07 (72.51- 87.31)	142.54 (128.86-164.46)	8.42
3	91.41 (83.29- 102.36)	144.43 (127.11- 170.37)	4.86
4	96.89 (88.07- 108.80)	159.51 (140.14- 189.92)	7.42

The tables 12, 13 and 14 exhibit the data on LC_{50} (ppm) and LC_{90} (ppm) values of methanol extracts of *A. cocculus* tested against different instars of *Ae. albopictus*. The 24 hr LC_{50} (ppm) values for I, II, III and IV instars larvae were 81.13, 91.42, 117.93, 124.42 ppm respectively. The 48 and

72 hrs LC_{50} values were 74.33, 83.94, 105.51, 112.46 ppm and 58.33, 76.33, 87.3, 94.52 ppm for the I to IV instar larvae *Ae. albopictus* respectively. The 24 hr LC_{90} values of I, II, III & IV instar larvae were 118.88, 174.65, 185.82, 192.54ppm and that of 48 hrs were 106.54, 164.54, 168.84, 176.54 ppm and for 72 hrs were 96.64, 146.98, 136.84 and 142.56ppm respectively.

Table 12: 24 hr LC_{50} (ppm) and associated statistics of *A. cocculus* methanol extracts tested against different instar stages of *Ae.albopictus*

Instar	LC50 (LFL-UFL)	LC90 (LFL-UFL)	χ^2
1	81.13 (74.26-90.24)	118.88 (94.24 -132.44)	4.86
2	91.42 (83.29- 102.36)	174.65 (138.46-198.86)	11.24
3	117.93 (81.47-143.74)	185.82 (146.57-222.63)	8.42
4	124.42 (84.32 – 146.52)	192.54 (150.45 -189.98)	6.55

Table 13: 48 hr LC_{50} (ppm) and associated statistics of *A. cocculus* methanol extracts tested against different instar stages of *Ae. albopictus*

Instar	LC50 (LFL-UFL)	LC90 (LFL-UFL)	χ^2
1	74.33 (68.29-82.32)	106.54 (92.86- 118.28)	4.86
2	83.94 (76.75- 92.95)	164.54 (140.46-188.84)	10.8
3	105.51 (94.42- 118.82)	168.84 (148.88- 204.52)	6.88
4	112.46 (98.02- 124.42)	176.54 (150.54 -208.84)	2.64

Table 14: 72 hr LC_{50} (ppm) and associated statistics of *A. cocculus* methanol extracts tested against different instar stages of *Ae. albopictus*

Instar	LC50 (LFL-UFL)	LC90 (LFL-UFL)	χ^2
1	58.33 (39.29-72.32)	96.64 (72.86- 118.24)	7.46
2	76..33 (68.28- 8.31)	146.98 (112.62- 168.85)	3.45
3	87.3 (79.69- 97.51)	136.84 (119.86- 164.62)	4.65
4	94.52 (81.14- 102.22)	142.56 (126.54- 180.22)	6.44

The data on 24 hr, 48 hr and 72 hr LC_{50} (ppm) and LC_{90} (ppm) of the defatted methanol fraction of *A. cocculus* tested against different instars of C*x. quinquefasciatus* are presented in tables 15, 16 and 17. The 24 hr LC_{50} on I, II, III and IV instar larvae of *Cx. quinquefasciatus* were 81.11, 87.33, 102.8 and 112.48 ppm and LC_{90} values were 118.86, 120.24, 132.64 and 144.64 ppm respectively (table 15). The 48 and 72 hrs LC_{50} (LC_{90}) values were 78.65 (106.64), 81.13 (118.42), 91.02(126.42), 105.54 (126.66) ppm (table 16) and 64.95 (98.86), 77.88 (106.66), 86.15(118.98), 98.84 (132.24) ppm for the different instars (table 17).

Table 15: 24 hr LC_{50} (ppm) and associated statistics of *A. cocculus* defatted methanol extracts tested against different instar stages of *Cx. quinquefasciatus*

Instar	LC50 (LFL-UFL)	LC90 (LFL-UFL)	χ^2
1	81.11 (74.25-90.23)	118.86 (93.32 -134.54)	2.43
2	87.33 (79.69- 97.51)	120.24 (100.04-142.65)	3.42
3	102.8 (92.8-112.64)	132.64 (116.54-145.68)	4.58
4	112.48 (78.24-140.64)	144. 64 (108.88- 162.44)	9.12

Table 16: 48 hr LC_{50} (ppm) and associated statistics of *A. cocculus* defatted methanol extracts tested against different instar stages of *Cx. quinquefasciatus*

Instar	LC50 (LFL-UFL)	LC90 (LFL-UFL)	χ^2
1	78.65 (72.57- 87.31)	106.64 (96.67- 118.94)	4.66
2	81.13 (74.55- 90.29)	118.42 (89.88- 132.46)	2.68
3	91.02 (76.69-107.7)	126.42 (83.32 -144.52)	6.66
4	105.54 (90.86- 115.58)	126.68 (104.42-146.64)	7.34

Table 17: 72 hr LC_{50} (ppm) and associated statistics of *A. cocculus* defatted methanol extracts tested against different instar stages of *Cx. quinquefasciatus*

Instar	LC_{50} (LFL-UFL)	LC_{90} (LFL-UFL)	χ^2
1	64.95 (48.76- 83.58)	98.86 (73.32 -124.54)	8.42
2	77.88 (52.65- 84.64)	106.66 (78.88- 118.84)	2.66
3	86.15 (69.54- 120.98)	118.98 (80.4-143.64)	7.87
4	98.84 (78.85- 118.82)	132.24 (114.54-144.62)	4.64

The tables 18, 19 and 20 reveal the data on LC_{50} and LC_{90} (ppm) values of defatted methanol fraction of *A. cocculus* tested against different instars of *Ae. albopictus*. The LC_{50} value for I, II, III & IV instars larvae for 24 hrs were 72.46, 86.95, 98.88 and 109.54 ppm (table 18) respectively. The 48 and 72 hrs LC_{50} values were 64.79, 76.37, 87.3, 91.42 ppm and 53.36, 74.34, 78.61, 86.88 ppm for I, II, III and IV instars of *Ae. albopictus* respectively (tables 19 &20). The LC_{90} values for I, II, III & IV instar larvae at 24 hrs were 158.54, 124.24, 138.86, 152.65 and that of 48 hrs 144.54, 114.56, 124.54, 136.64 ppm (tables 18, 19). The LC_{90} for 72 hrs are 126.46, 105.57, 112.76, 122.24 ppm (table 20) respectively.

Table 18: 24 hr LC_{50} (ppm) and associated statistics of *A. cocculus* defatted methanol extracts tested against different instar stages of *Ae. albopictus*

Instar	LC50 (LFL-UFL)	LC90 (LFL-UFL)	χ^2
1	72.46 (66.63- 80.13)	158.54 (136.52-175.54)	3.64
2	86.95 (72.46-92.58)	124.24 (102.04-142.64)	3.54
3	98.88 (88.08- 108.8)	138.86 (124.46- 148.86)	2.22
4	109.54 (96.66- 120.65)	152.65 (138.88- 170.02)	4.64

Table 19: 48 hr LC_{50} (ppm) and associated statistics of *A. cocculus* defatted methanol extracts treated against different instar stages of *Ae. albopictus*

Instar	LC50 (LFL-UFL)	LC90 (LFL-UFL)	χ^2
1	64.79 (59.73- 71.13)	144. 54 (109.98- 160.44)	5.48
2	76.37 (70.08- 84.69)	114.56 (108.84-120.24)	2.24
3	87.3 (79.79- 96.89)	124.54 (102.28- 138.86)	3.12
4	91.42 (83.29- 102.35)	136.64 (122.64- 142.86)	2.87

Table 20: 72 hr LC_{50} (ppm) and associated statistics of *A. cocculus* defatted methanol extracts treated against different instar stages of *Ae. albopictus*

Instar	LC50 (LFL-UFL)	LC90 (LFL-UFL)	χ^2
1	53.26 (49.33- 58.05)	126.46 (112.82-144.95	6.66
2	74.34 (68.28- 82.32)	105.57 (95.65- 117.89)	1.26
3	78.61 (72.06- 87.32)	112.76 (104.24 -122.44	2.46
4	86.88 (78.06- 98.9)	122.24 (106.24-138.64)	3.24

The data on 24, 48 and 72 hrs LC_{50} and LC_{90} values of *M. leucadendron* acetone extracts tested against different instars of *Cx. quinquefasciatus* are given in the tables 21, 22 and 23. The 24 hr LC_{50} (LC_{90}) values for I, II, III and IV instar larvae of *Cx. quinquefasciatus* were 88.61 (122.42), 91.42 (138.82), 109.98 (150.54) and 122.88 (166.54) (table 21) and that of 48 and 72 hrs LC_{50} (LC_{90}) values for I, II, III and IV instar larvae of *Cx. quinquefasciatus* were 78.61 (124.98), 86.54 (138.84), 96.42 (140.46), 104.66 (154.88) ppm and 69.64 (108.86), 78.98 (116.68), 90.09 (126.66), 98.88 (139.89) ppm (tables 22 & 23) respectively.

Table 21: 24 hr LC_{50} (ppm) and associated statistics of *M. leucadendron* acetone extracts treated against different stages of *Cx. quinquefasciatus*

Instar	LC50 (LFL-UFL)	LC90 (LFL-UFL)	χ^2
1	88.61 (72.05-97.31)	122.42 (106.76-140.49)	3.42
2	91.42 (83.29- 102.35)	138.82 (127.75- 152.52)	2.86
3	109.98 (96.64-120.96)	150.54 (140.44- 162.22)	5.65
4	122.88 (108.96-132.68)	166.54 (148.88- 178.96)	4.26

Table 22: 48 hr LC_{50} (ppm) and associated statistics of *M. leucadendron* acetone extracts treated against different stages of *Cx. quinquefasciatus*

Instar	LC50 (LFL-UFL)	LC90 (LFL-UFL)	χ^2
1	78.61 (72.05-87.31)	124.98 (106.65- 134.42)	3.45
2	86.54 (78.88- 94.42)	138.84 (122.54- 146.64)	5.63
3	96.42 (80.88- 104.68)	140.46 (129.92- 152.54)	6.76
4	104.66 (88.89- 110.98)	154.58 (144.16-160.64)	2.89

Table 23: 72 hr LC_{50} (ppm) and associated statistics of *M. leucadendron* acetone extracts treated against different instar stages of *Cx. quinquefasciatus*

Instar	LC50 (LFL-UFL)	LC90 (LFL-UFL)	χ^2
1	69.64 (58.68- 71.12)	108.86 (96.66-114.54)	2.46
2	78.98 (70.02-84.63)	116.68 (104.56-118.26)	3.64
3	90.09 (82.04- 98.98)	126.66 (110.08-132.24)	2.26
4	98.88 (90.76- 108.84)	139.89 (129.98-151.16)	4.08

The tables 24, 25 and 26 present the data on the activity of acetone extracts of *M. leucadendron* on *Ae. albopictus.* The 24hr LC_{50} and LC_{90} values on the different instars were 84.34, 87.3, 105.64, 118.65 and 123.68, 136.57, 150.31, 162.84 ppm (table 24) respectively for all the four instars of *Ae. albopictus*. The 48 hr and 72 hrs LC_{50} (LC_{90}) values for I, II, III and IV instars of *Ae. albopictus* were as follows: 76.87 (120.42), 82.77 (132.46), 92.54 (146.64), 100.08 (152.24) and 70.02 (106.54), 76.54 (112.82), 89.56 (120.42), 96.64 (132.36) ppm (table 25 & 26) respectively.

Table 24: 24 hr LC_{50} (ppm) and associated statistics of *M. leucadendron* acetone extracts treated against different instar stages of *Ae. albopictus*

Instar	LC50 (LFL-UFL)	LC90 (LFL-UFL)	χ^2
1	84.34 (68.29-92.32)	123.68 (108.8- 143.54)	4.62
2	87.3 (79.69- 97.51)	136.57 (122.56- 148.82)	4.65
3	105.64 (94.68- 117.87)	150.31 (118.28- 194.56)	2.85
4	118.65 (102.88-128.88)	162.84 (139.79- 174.87)	3.88

Table 25: 48 hr LC_{50} (ppm) and associated statistics of *M. leucadendron* acetone extracts treated against different instar stages of *Ae. albopictus*

Instar	LC50 (LFL-UFL)	LC90 (LFL-UFL)	χ^2
1	76.87 (67.76- 89.92)	120.42 (105.56- 138.84)	1.18
2	82.77 (70.75-92.87)	132.46 (120.24-147.54)	3.36
3	92.54 (82.24- 102.45)	146.64 (138.86-154.54)	2.87
4	100.08 (84.56- 112.42)	152.24 (145.56-158.84)	9.64

Table 26: 72 hr LC_{50} (ppm) and associated statistics of *M. leucadendron* acetone extracts treated against different instars of *Ae. albopictus*

Instar	LC50 (LFL-UFL)	LC90 (LFL-UFL)	χ^2
1	70.02 (59.98- 81.12)	106.54 (94.46-112.45)	4.46
2	76.54 (64.47- 84.48)	112.82 (104.56- 120.46)	2.98
3	89.56 (75.57- 95.64)	120.42 (108.76-129.42)	5.24
4	96.64 (82.26- 108.85)	132.36 (121.16-140.12)	6.4

The data on the 24, 48 and 72 hrs of LC_{50} and LC_{90} values of methanol extracts of *M. leucadendron* tested against different instars of *Cx. quinquefasciatus* were rendered in tables 27, 28 and 29. The larvicidal activity in terms of LC_{50} (LC_{90}) tested against the I, II, III and IV instar larvae for 24 hr, 48 hr and 72 hr were 99.86 (144.43), 121.42 (153.84), 138.89 (186.82), 151.09 (191.18) ; 84.52 (135.02), 102.09 (140.46), 120.46 (154.09), 136.22

(175.09) and 75.24 (142.46), 88.89 (152.18), 110.24 (164.66), 116.88 (170.77) ppm respectively.

Table 27: 24 hr LC_{50} (ppm) and associated statistics of *M. leucadendron* methanol extracts treated against different instar stages of *Cx. quinquefasciatus*

Instar	LC50 (LFL-UFL)	LC90 (LFL-UFL)	χ^2
1	99.86 (89.98- 101.89)	144.43 (127.01- 168.37)	4.48
2	121.42 (104.6- 129.98)	153.84 (139.79- 174.76)	7.67
3	138.89 (129.98-148.82)	186.82 (166.57- 202.46)	6.56
4	151.09 (122.55- 163.56)	191.18 (161.99- 221.12)	8.98

Table 28: 48 hr LC_{50} (ppm) and associated statistics of *M. leucadendron* methanol extracts treated against different instar stages on *Cx. quinquefasciatus*

Instar	LC50 (LFL-UFL)	LC90 (LFL-UFL)	χ^2
1	84.52 (75.68-96.54)	135.02 (124.54-143.57)	2.68
2	102 .09 (93.8- 112.64)	140.46 (126.02- 164.37	11.4
3	120.46 (108.86-129.46)	154.09 (120.46- 163.58)	9.8
4	136.22 (127.36-145.44)	175.09 (154.74- 208.58)	7.9

Table 29: 72 hr LC_{50} (ppm) and associated statistics of *M. leucadendron* methanol extracts treated against different instar stags of *Cx. quinquefasciatus*

Instar	LC50 (LFL-UFL)	LC90 (LFL-UFL)	χ^2
1	75.24 (70.12- 79.98)	142.46 (128.02-160.36)	2.65
2	88.89 (81.62- 94.54)	152.18 (128.55-166.54	4.48
3	110.24 (101.45-116.76)	164.66 (159.98-169.96)	6.42
4	116.88 (109.98- 125.54)	170.77 (164.46- 176.76)	4.46

The tables 30, 31 and 32 provide the data on the methanol fractions of *M. leucadendron* leaves tested against different instars of *Ae. albopictus.* The LC_{50} value for I, II, III and IV instars larvae for 24 hrs duration were 97.56, 110.4, 126.54 and 139.01 ppm (table 30) respectively. The 48 and 72 hrs LC_{50} values were 80.84, 106.22, 116.64, 128.22 ppm and 72.12, 84.85, 106.98, 114.43 ppm for the I, II, III and IV instars respectively (tables 31 and 32). The 24 hrs LC_{90} values for I, II, III and IV instar larvae were 139.01, 151.74, 173.09, 185.82 ppm and for 48 hrs were 130.54, 138.82, 156.56, 164.42 ppm and for 72 hrs 136.66, 148.54, 160.66 and 168.82 ppm respectively.

Table 30**: 24 hr LC_{50} (ppm) and associated statistics of *M. leucadendron* methanol extracts treated against different instar stages of *Ae*. *albopictus***

Instar	LC50 (LFL-UFL)	LC90 (LFL-UFL)	χ^2
1	97.56 (89.98- 107.74)	139.01 (122.54- 163.57)	4.46
2	110.4 (102.54- 119.98)	151.74 (132.69- 178.75)	5.64
3	126.54 (112.89- 132.76)	173.09 (151.74- 207.55)	3.86
4	139.01 (122.55- 163.56)	185.82 (156.57- 212.63)	4.58

Table 31: **48 hr LC_{50} (ppm) and associated statistics of *M. leucadendron* methanol extracts treated against different instar stages of *Ae*. *albopictus***

Instar	LC50 (LFL-UFL)	LC90 (LFL-UFL)	χ^2
1	80.84 (76.54- 85.54)	130.54 (122.86-138.46)	3.84
2	106.22 (98.65- 113.24)	138.82 (129.95-140.85)	6.42
3	116.64 (110.45-121.42)	156.56 (142.22- 165.54)	7.22
4	128.22 (120.46- 136.54)	164.42 (148.82-170.94)	2.56

Table 32: 72 hr LC_{50} (ppm) and associated statistics of *M. leucadendron* methanol extracts treated aginst different instar stages of *Ae. albopictus*

Instar	LC_{50} (LFL-UFL)	LC_{90} (LFL-UFL)	χ^2
1	72.12 (61.54-80.92)	136.66 (129.98-142.52)	2.86
2	84.85 (64.56- 98.82)	148.54 (140.98-156.54)	8.86
3	106.98 (100.54- 112.6)	160.66 (151.56-166.92)	5.4
4	114.43 (102.28- 120.41)	168.82 (162.24-171.98)	2.65

The tables 33, 34 and 35 provide the data on LC_{50} and LC_{90} values of defatted methanol fraction of *M. leucadendron* tested against different instars of *Cx. quinquefasciatus.* The 24 hr LC_{50} (ppm) of I, II, III and IV instar larvae were 90.82, 106.56, 124.54, 140.56 ppm and that of LC_{90} values were 140.46, 151.09, 168.86, 182.28 ppm (table 33) respectively. The 48 and 72 hrs LC_{50} (LC_{90}) values of the defatted methanol fraction of *M. leucadendron* were 80.12 (125.22), 95.14 (138.42), 108.22 (149.66), 115.45 (168.84) ppm and 68.46 (135.22), 76.67 (142.24), 94.65 (150.76), 108.22 (158.82) ppm for all the four instars (tables 34 & 35) respectively.

Table 33: 24 hr LC_{50} (ppm) and associated statistics of *M. leucadendron* defatted methanol extracts treated against different instar stages of *Cx. quinquefasciatus*

Instar	LC50 (LFL-UFL)	LC90 (LFL-UFL)	χ^2
1	90.82 (82.24- 96.56)	140.46 (132.82-146.56)	3.45
2	106.56 (99.98-112.89)	151.09 (122.55- 163.56	4.86
3	124.54 (114.54-132.22)	168.86 (160.42-174.56))	2.87
4	140.56 (130.22-142.22)	182.28 (172.26-190.45)	2.65

Table 34: 48 hr LC_{50} (ppm) and associated statistics of *M. leucadendron* defatted methanol extracts treated against different instar stages of *Cx. quinquefasciatus*

Instar	LC50 (LFL-UFL)	LC90 (LFL-UFL)	χ^2
1	80.12 (70.46-90.02)	125.22 (118.98-132.46)	4.22
2	95.14 (90.41- 99.89)	138.42 (130.42-144.56)	5.62
3	108.22 (99.89-118.22)	149.66 (139.98-156.56)	6.87
4	115.45 (104.46-124.24)	168.84 (154.56-178.25)	2.56

Table 35: 72 hr LC_{50} (ppm) and associated statistics of *M. leucadendron* defatted methanol extracts treated against different instar stages of *Cx. quinquefasciatus*

Instar	LC50 (LFL-UFL)	LC90 (LFL-UFL)	χ^2
1	68.46 (59.98- 76.54)	135.22 (126.6-146.64)	6.25
2	76.67 (70.12- 82.24)	142.24 (132.21-152.54)	2.56
3	94.65 (86.62-101.24)	150.76 (138.88-159.98)	3.86
4	108.22 (98.89- 115.98)	158.82 (148.86-167.95)	4.42

The 24 hr, 48 hr and 72 hr LC_{50} and LC_{90} (ppm) values and its associated statistics of the defatted methanol fraction of *M. leucadendron* tested against different larval instars of *Ae. albopictus* are provided in tables 36, 37 and 38. The 24 hr LC_{50} and LC_{90} values for the different instars of *Ae. albopictus* were 88.86, 96.56, 112.24, 130.32 and 136.62, 145.46, 158.09, 170.14 ppm (table 36) respectively. Table 37 and 38 exhibit the data on 48 and 72 hrs LC_{50} for I, II, III and IV instar larvae of *Ae. albopictus* when treated with defatted methanol *M. leucadendron* extracts and the values of LC_{50} (LC_{90}) ppm were 75.66 (115.11), 84.68 (135.56), 90.65 (150.49), 110.24 (157.72) ppm and 66.66 (121.14), 72.24 (132.22), 88.45 (146.56) and 99.15 (150.05) ppm respectively.

Table 36: 24 hr LC_{50} (ppm) and associated statistics of *M. leucadendron* defatted methanol extracts treated against different instar stages of *Ae. albopictus*

Instar	LC50 (LFL-UFL)	LC90 (LFL-UFL)	χ^2
1	88.86 (79.98-96.66)	136.62 (128.86-142.52)	1.65
2	96.56 (90.44-105.65)	145.46 (132.84-146.52)	3.26
3	112.24 (104.56-118.86)	158.09 (132.51- 163.56	1.88
4	130.32 (120.26-140.56)	170.14 (158.98- 180.62)	2.15

Table 37: 48 hr LC_{50} (ppm) and associated statistics of *M. leucadendron* defatted methanol extracts treated against different instar stages of *Ae. albopictus*

Instar	LC50 (LFL-UFL)	LC90 (LFL-UFL)	χ^2
1	75.66 (68.82- 82.15)	115.11 (104.48-128.89)	3.75
2	84.68 (75.57- 92.25)	135.56 (112.28-145.68)	5.46
3	90.65 (81.91- 98.98)	150.49 (142.25- 157.75)	6.82
4	110.24 (98.82-118.65)	157.72 (150.75-167.72)	5.45

Table 38: 72 hr LC_{50} (ppm) and associated statistics of *M. leucadendron* defatted methanol extracts treated against different instar stages of *Ae. albopictus*

Instar	LC50 (LFL-UFL)	LC90 (LFL-UFL)	χ^2
1	66.66 (60.88- 74.44)	121.14 (114.71-128.14)	2.24
2	72.24 (62.22- 81.12)	132.22 (126.62-138.88)	3.68
3	88.45 (79.12- 98.24)	146.56 (140.12-152.22)	4.64
4	99.15 (90.15- 109.26)	150.05 (141.98- 159.88)	3.72

The different column fractions of *A. cocculus* eluted using column chromatographic techniques were tested against III instar larvae of *Cx. quinquefasciatus* and *Ae. albopictus* (tables 39 &40). The data on 24hr LC_{50} (ppm) of the eight different fractions of *A. cocculus* tested against III instar larvae of *Cx. quinquefasciatus* were provided in the table 39. The 24 hr LC_{50} values ranged from 10.1 ppm to 28.86 ppm for the different column fractions viz; Hexane, Ethyl acetate (EA), Hexane : EA (3:1), Hexane : EA (2:1), Hexane : EA (1:1), Hexane : EA (1:2), Hexane : EA (1:3) and acetone.

Table 39: 24 hr LC_{50} and LC_{90} (ppm) and associated statistics of different column fractions of *A. cocculus* tested against III instar larvae of *Cx. quinquefasciatus*

Column Fractions:	LC_{50} (LFL-UFL)	LC90 (LFL-UFL)	χ^2
Hexane	10.1 (5.56-16.98)	45.76 (36.67-58.6)	8.2
Ethyl acetate (EA)	22.4 (16.54- 32.25)	58.66 (50.54-65-65)	5.4
Hexane: EA(3:1)	16.84 (9.88- 23.32)	48.98 (34.46-60.09)	7.5
Hexane: EA (1:3)	28.86 (18.54-40.12)	70.92 (63.33-78.85)	4.4
Hexane: EA(2:1)	19.96 (17.98- 27.18)	36.1 (9.98- 43.36)	4.2
Hexane: EA(1:2)	24.98 (16.66-32.24)	72.12 (66.65-78.83)	3.1
Hexane: EA(1:1)	20.44 (16.42-25.62)	68.82 (58.98-76.67)	5.6
Acetone	15.12 (8.8-22.42)	34.42 (26.66-45.56)	6.4

24 hr LC_{50} and LC_{90} (ppm) values obtained after the treatment of the eight column fractions of *A. cocculus* with III instar of *Ae. albopictus* is provided in table 40. As in the case of *Cx. quinquefasciatus* the activity of *A. cocculus* was same as that for *Ae. albopictus*. The 24 hr LC_{50} values for the different column fractions ranged between 9.81 ppm and 24.22 ppm for all the eight column fractions viz; Hexane, Ethyl acetate (EA), Hexane : EA (3:1), Hexane : EA (2:1), Hexane : EA (1:1), Hexane : EA (1:2), Hexane : EA (1:3) and acetone.

Table 40: 24 hr LC_{50} and LC_{90} (ppm) and associated statistics of different column fractions of *A. cocculus* against III instar of *Ae. albopictus*

Column Fractions:	LC_{50} (LFL-UFL)	LC90 (LFL-UFL)	χ^2
Hexane	9.81 (4.46-13.22)	29.76 (16.57-38.16)	5.2
Ethyl acetate (EA)	19.4 (14.24- 28.28)	50.56 (42.54-60-25)	6.5
Hexane: EA(3:1)	14.44 (8.28- 20.64)	46.98 (36.46-50.04)	2.4
Hexane: EA (1:3)	24.22 (17.44-30.12)	65.12 (60.44-70.25)	4.6
Hexane: EA(2:1)	17.96 (14.98- 24.28)	34.19 (29.28- 42.36)	3.8
Hexane: EA(1:2)	22.22 (16.66-28.42)	66.42 (60.15-73.85	3.6
Hexane: EA(1:1)	18.64 (14.42-24.62)	60.12 (55.98-72.62)	5.4
Acetone	12.36 (7.46-20.12)	32.18 (26.68-40.12)	2.9

The table 41 and 42 present the values in ppm for 24 hr LC_{50} and LC_{90} of the different column fractions of *M. leucadendron* tested against III instar larvae of *Cx. quinquefasciatus* and *Ae. albopictus*. The 24 hr LC_{50} of Hexane, EA and Chloroform fractions of *M. leucadendron* against the III instar larvae of *Cx. quinquefasciatus* were 23.67, 42.4 and 14.12 ppm respectively (table 41). 24 hr LC_{50} (LC_{90}) values (ppm) obtained for the Hexane, EA and Chloroform fractions of *M. leucadendron* tested against the III instar larvae of *Ae. albopictus* were 20.42 (50.76), 40.38 (82.66) and 11.66 (38.76) ppm respectively.

Table 41: 24 hr LC_{50} and LC_{90} (ppm) and associated statistics of different column fractions of *M. leucadendron* against III instar of *Cx. quinquefascatus*

Column Fractions:	LC_{50} (LFL-UFL)	LC_{90} (LFL-UFL)	χ^2
Hexane	23.67 (15.56-36.48)	55.76 (46.62-68.24)	5.2
Ethyl acetate	42.4 (36.54- 54.28)	98.66 (80.54-112-44)	6.4
Chloroform	14.12 (6.56-22.98)	39.76 (30.67-48.6)	4.2

Table 42: 24 hr LC_{50} and LC_{90} (ppm) and associated statistics of different column fractions of *M. leucadendron* against III instar of *Ae. albopictus*

Column Fractions:	LC_{50} (LFL-UFL)	LC_{90} (LFL-UFL)	χ^2
Hexane	20.42 (12.68-32.48)	50.76 (44.65-62.22)	4.4
Ethyl acetate	40.38 (32.22- 52.24)	82.66 (70.54-92-42)	5.2
Chloroform	11.66 (5.44-18.44)	38.76 (28.88-42.55)	3.8

2.4.3. EXTENSION OF LARVAL DURATION:

The table 43 presents data on extension of larval duration of *Cx. quinquefasciatus* when treated with ethyl acetate fraction of *A. cocculus* and *M. leucadendron.* On treatment with 25 ppm of the EA fraction of *M. leucadendron,* it took 45±2 days to molt into pupal stage and the control set completed this molting to pupae within 12±1 days. The data on extension of larval duration for the concentrations 50, 75 and 100 ppm of the EA fraction of *M. leucadendron* were 38±1, 35±1 and 34±1 days respectively. Table 44 shows the statistical data of the larval duration extension activity of ethyl

acetate fraction of *A. cocculus* and *M. leucadendron.* The t-score was high in *M. leucadendron* when compared with *A. cocculus* ethyl acetate fractions and hence the test proves that the *M. leucadendron* ethyl acetate fractions were statistically, highly significant against *Cx. quinquefasciatus* than *A. cocculus.*

Table 43: Extension of larval duration after treatment with Ethyl acetate fraction of *A. cocculus* and *M. leucadendron* commenced with I instar larvae of *Cx. quinquefasciatus*

Plant extract	Concent-ration (ppm)	Duration in Days (number of larvae alive)				Total no of days
		I	II	III	IV	-
M.leucadendron	25	14(24)	10(20)	9(18)	12(15)	45 ±2
	50	12(21)	11(18)	7 (15)	8(14)	38 ±1
	75	10(20)	9(15)	10(14)	6 (13)	35±1
	100	11(18)	7(14)	9 (13)	7(11)	34±1
A.cocculus	25	5(6)	4(4)	3(3)	4(2)	16±2
	50	5(4)	4(4)	4(3)	3(1)	15±1
	75	4(5)	4(4)	3(3)	3(2)	14 ±1
	100	4(4)	3(3)	4(3)	3(1)	14±1
Control	-	3(30)	4(30)	3(30)	2(30)	12 ±1

Table 44: Data observed on t- Test of Ethyl acetate treated against *Cx. quinquefacsiatus* with control sample

Plants	Concentration (ppm)	T-Score	P-Value	Significance status at 95% Confidence Interval
M.leucadendron	25	80.83	<0.0001	Extremely statistically significant
	50	100.69	<0.0001	Extremely statistically significant
	75	89.078	<0.0001	Extremely statistically significant
	100	85.2056	<0.0001	Extremely statistically significant
A.cocculus	25	9.7980	<0.0001	Statistically significant
	50	11.6190	<0.0001	Statistically significant
	75	7.7460	<0.0001	Statistically significant
	100	7.7460	<0.0001	Statistically significant

The table 45 exhibits the data on the extension of larval duration when treated with different concentrations of ethyl acetate fractions of *A. cocculus* and *M. leucadendron*. The duration (in days) taken in each instar and the total duration taken for completion is provided in the table. The values in parenthesis show the number of larvae alive during the experiment. The concentration of 25, 50, 75 and 100 ppm of the EA fraction of *M. leucadendron* and *A. cocculus* extend the larval duration of 45, 40, 34 and 33 days and 15, 14, 13 and 14 days respectively. The larvae in the control set completed its duration in 12±2 days. Table 46 presents the T-test values of the comparison of ethyl acetate treated fractions with control fractions. The t-score was higher in *M. leucadendron* fractions than in *A. cocculus* fractions.

Table 45: Extension of larval duration after treatment with Ethyl acetate fraction of *A. cocculus* and *M. leucadendron* commenced with I instar larvae of *Ae. albopictus*

Plant extract	Concentration (ppm)	Duration in Days (number of larvae alive)				Total no of days
		I	II	III	IV	-
M.leucadendron	25	8(25)	7(19)	14(17)	16(16)	45 ±1
	50	6(22)	7(20)	13(16)	14(15)	40 ±2
	75	7(19)	5(16)	10(14)	12(13)	34 ±1
	100	7(18)	7(14)	10 (13)	9(12)	33±2
A.cocculus	25	5(9)	4(8)	4(4)	3(2)	15±1
	50	4 (9)	3 (7)	3(4)	4(2)	14±1
	75	3(7)	3(5)	3(3)	4(2)	13±1
	100	4 (5)	4 (5)	3(4)	3(2)	14±1
Control	-	3(30)	2(30)	3(29)	3(29)	12 ±2

Table 46: Data observed on t- Test of Ethyl acetate treated against *Ae. albopictus* with Control sample

Plants	Concentration (ppm)	T-Score	P-Value	Significance status at 95% Confidence Interval
M.leucadendron	25	80.83	<0.0001	Extremely statistically significant
	50	54.22	<0.0001	Extremely statistically significant
	75	53.88	<0.0001	Extremely statistically significant
	100	40.66	<0.0001	Extremely statistically significant
A.cocculus	25	7.34	<0.0001	Statistically significant
	50	4.89	<0.0001	Statistically significant
	75	2.44	= 0.0173	Statistically significant
	100	4.89	<0.0001	Statistically significant

2.4.4. ADULTICIDAL ACTIVITY OF ESSENTIAL OILS OF THE SELECTED PLANTS:

The tables 47 and 48 present data on adulticidal activity of the essential oils of the selected plants tested against *Cx. quinquefasciatus* and *Ae. albopictus*. The time taken for 100% mortality of *Cx. quinquefasciatus* and *Ae. albopictus* when treated with different concentrations the essential oils of *A. cocculus* and *M. leucadendron* were recorded and estimated. Three different concentrations (10, 50 and 100 %.) were used for the adulticidal bioassay. The control was maintained using coconut oil and there was no mortality while using coconut oil as control. Treatment with a concentration 100, 50 and 10 % essential oil produces 100 % mortality in of 35±2, 75±4 and 120±2 minutes and 40±3, 85±3 and 130±4 minutes for *Cx. quinquefasciatus* and *Ae. albopictus* respectively. The 100% *M. leucadendron* oil killed *Ae. albopictus* in 30 min while the 100% *A. cocculus* took 40 min to kill 100%

adults in the experiment. The 100% *M. leucadendron* oil took only 15 min for the complete mortality of *Cx. quinquefasciatus.* The 100% *A. cocculus* took 35 min to kill 100% *Cx. quinquefasciatus.* The 50% and 10% *M. leucadendron* oil killed 100% *Cx. quinquefasciatus* and *Ae. albopictus* in 45, 60 and 88, 120 min respectively. The 50% *A. cocculus* killed *Cx. quinquefasciatus* in 75 min and *Ae. albopictus* in 85 min. It took 120 and 130 min for complete mortality of *Cx. quinquefasciatus* and *Ae. albopictus* when 10% *A. cocculus* was used.

Table 47: Adulticidal activity of different concentrations of *A. cocculus* oil against *Cx. quinquefasciatus* and *Ae. albopictus*

Mosquito Species	**Concentration (%)**	**Time (min) taken for 100% mortality of adult mosquitoes**
Cx. quinquefasciatus	100	35±2
	50	75±4
	10	120±2
Ae. albopictus	100	40±3
	50	85±3
	10	130±4
There is no death while using coconut oil as control		

Table 48: Adulticidal activity of different concentrations of *M. leucadendron* essential oilagainst *Cx. quinquefasciatus* and *Ae. albopictus*

Mosquito Species	Concentration (%)	Time (min) taken for 100% mortality of adult mosquitoes
Cx. quinquefasciatus	100	15±2
	50	45±3
	10	88±2
Ae. albopictus	100	30±5
	50	60±5
	10	120±5
There is no death while using coconut oil as control		

2.4.5. REPELLENT ACTIVITY OF ESSENTIAL OILS OF THE SELECTED PLANTS:

Table 49 and 50 shows the data on the repellent activity of *M. leucadendron* against *Cx. quinquefasciatus* and *Ae. albopictus*. 100% *M. leucadendron* essential oil provides 8 hours protection for both *Cx. quinquefasciatus* and *Ae. albopictus.* The 50% concentrated essential oil provides the protection of 8 hrs from *Cx. quinquefasciatus* and 6 hrs and 30 min from *Ae. albopictus.* 7 hours protection from *Cx. quinquefasciatus* and 6 hours protection from *Ae. albopictus* was provided by 10% essential oil. The Tukey's test proves that the values were highly significant and *M. leucadendron* essential oil has the repellent activity against mosquitoes.

Table 49: Repellent activity of *M. leucadendron* essential oil against *Cx. quinquefasciatus* and *Ae. albopictus*

Mosquito Species	Concentration	Complete protection time (min)	
		Treated	Control
Cx. quinquefasciatus	100	480±5	15±5
	50	480±10	
	10	420±15	
Ae. albopictus	100	480±10	10±5
	50	390±15	
	10	360±20	

Table: 50 Data on Tukey test calculated using repellent activity of *M. leucadendron* against *Cx. quinquefasciatus* and *Ae. albopictus*

Mosquito Species	Comparison	Significant at (P <0.05)	t value
Cx. quinquefasciatus	Control and 100ppm	Yes	735.230
	Control and 50ppm	Yes	735.230
	Control and 100ppm	Yes	640.361
Ae. albopictus	Control and 100ppm	Yes	743.135
	Control and 50ppm	Yes	600.833
	Control and 10ppm	Yes	553.399

2.4.6. GCMS ANALYSIS:

Table 51 describes the compounds present in the essential oil of *M. leucadendron* when analyzed using GCMS. γ –Selinene was found to be the most abundant component in the essential oil of *M. leucadendron* and the percentage of abundance was 39.66%. The retention time for γ –Selinene was 31.17 min. α- Pinene and Carophylline was present in 9.59 and 9.07 with retention times 5.59 and 24.04 min respectively. 5.97% of Terpineol and 3.2% Limonene were also present with the retention times 14.62 and 8.41. 3.11 % of α- Gurjunene was present in the essential oil with 27.09 min retention time. Other compounds present in the essential oil were Ledol, Terpene-4-ol, P-Cymene, β- Pinene, β- Eudesmol, Linalool and Spathulenol. And they were having the % abundance (retention time) in the order 2.63 (2.63), 1.86 (13.97), 1.73(8.18), 1.35(6.71), 1.21(32.86), 1.15(10.86) and 1.08 (25.66) respectively.

Table: 51 GCMS data of compounds present in the essential oil of *M. leucadendron* with retention time and % of abundance

Name of Compound	% of abundance	Retention time (RT)
γ –Selinene	39.66	31.17
α- Pinene	9.59	5.59
Carophylline	9.07	24.04
Terpineol	5.97	14.62
Limonene	3.2	8.41
α- Gurjunene	3.11	27.09
Ledol	2.63	2.63
Terpene-4-ol	1.86	13.97
P- Cymene	1.73	8.18
β- Pinene	1.35	6.71
β- Eudesmol	1.21	32.86
Linalool	1.15	10.86
Spathulenol	1.08	25.66

2.4.7. NMR AND LCMS ANALYSIS:

The hexane fraction of *A. cocculus* and chloroform fraction of *M. leucadendron* have shown more activity against III instar larvae of both *Ae. albopictus* and *Cx. quinquefasciatus*. These fractions were subjected to NMR and LCMS analyses.

LCMS and NMR data of *A. cocculus*:

Fraction I

The fraction I was subjected to ^{1}H and ^{13}C NMR and LCMS analysis. The ^{1}H and ^{13}C NMR as well as LCMS data are given in **Plate 2.4 A, 2.4 B and 2.4C.**

Compound 1

It is interesting to note that the structure isolated resembles the structure of 4, 6a-dihydroxy-1a1-methyl-2-oxo-5-(prop-1-en-2-yl)octahydro-1a1H-oxireno[2',3':1,2]indeno[7,1-bc]furan-6-carbo- xylic acid. **IUPAC Name:** 4, 6a-dihydroxy-1a1-methyl-2-oxo-5-(prop-1-en-2-yl) octahydro-1a1H- oxireno [2',3':1,2]indeno[7,1-bc]furan-6-carboxylic acid, **Chemical Formula**: $C_{15}H_{18}O_{7.}$

^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 1.18 (s, 3H, H(31), H(32) and H(33). 1.54 (d, 1H, H(24) J=1, 7 Hz). 1.95 (d, 1H, H(23), J=1.5Hz). 1.84 (s, 3H, H(35), H(36) and H(37). 2.23 (d, 1H, H(29) J=2). 2.29 (t, 1H, H(28) J=1.9Hz). 3.59 (s, 1H, H(34). 5.27 (s, 1H, H(26). 7.20 (s, 1H, H(39). (**Plate No**. **2.4 A**).

^{13}C NMR (100 MHz, $CDCl_3$), δ (ppm): 15.2(C15), 23.0 (C20), 30.9 (C20), 34.06 (C2), 44.4 (C10), 48.6 (C3), 51.5 (C4), 61.58 (C5), 72.3 (C1),

76.7 (C8), 77.1 (C7), 112.9 (C21), 130.0 (C19), 174.5 (C11), 179.5 (C16) (**Plate No**. **2.4 B**).

Mass spectra ESI $[M-H]^-$ 309.13 (Actual Mol. Wt. 310.11)

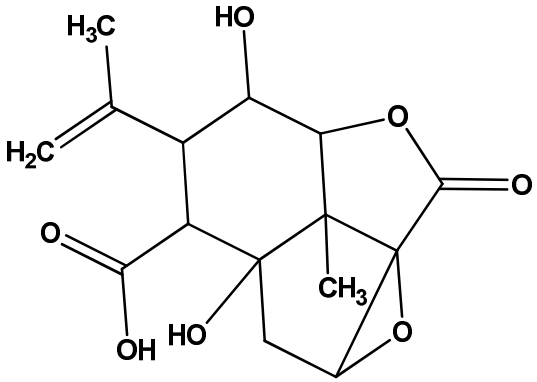


Fig.1 The structure of the compound isolated from the fraction I of *A. cocculus.*

Fraction II

The fraction II was subjected to ^{1}H and ^{13}C NMR and LCMS analysis. The ^{1}H and ^{13}C NMR as well as LCMS data are given in **Plate 2.5A, 2.5 B and 2.5 C**.

Compound 2

It is interesting to note that the structure isolated resembles the structure of 11-hydroxy-1, 2, 10-trimethoxy-6, 6-dimethyl-5, 6, 6a, 7-tetrahydro-4H-dibenzo [de, g] quinolin-6-ium. **IUPAC Name:** 11-hydroxy-1, 2, 10-trimethoxy-6, 6-dimethyl-5, 6, 6a, 7-tetrahydro-4H-dibenzo [de, g] quinolin-6-ium, **Chemical Formula**: $C_{21}H_{26}NO_4^+$.

^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 1.05 (s, 6H, H(27), H(28), H(29), H(50), H(51), H(52). 1.96 (t, 2H, H(32), H(33) J= 6.7Hz. 3.11 (d, 2H, H(36), H(37) J= 2.3Hz. 3.79 (t, 2H, H(30) and H(31) J=3.5Hz. 1.32 (s, 9H, H(40), H(41), H(42), H(43), H(44), H(45), H(46), H(47), H(48), H(49). 5.27

(t, 1H, H(35) J=1.6Hz. 4.49 (s, 1H, H(43). 6.99 -7.325 (Ar. C-H). (**PlateNo**. **2.5A**).

^{13}C NMR (100 MHz, $CDCl_3$), δ (ppm): 29.80(C20), 29.80(C20 and C25), 31.94 (C15), 34.0(C1 and C6), 46.7 (C12), 43.2 (C4), 50.7 (C3), 61.6 (C11), 113.0-139 (Ar.-C) (**PlateNo**. **2.5 B**).

Mass spectra ESI $[M-H]^-$ 355.21 (Actual Mol. Wt. 356.19) (**PlateNo**. **2.5 C**).

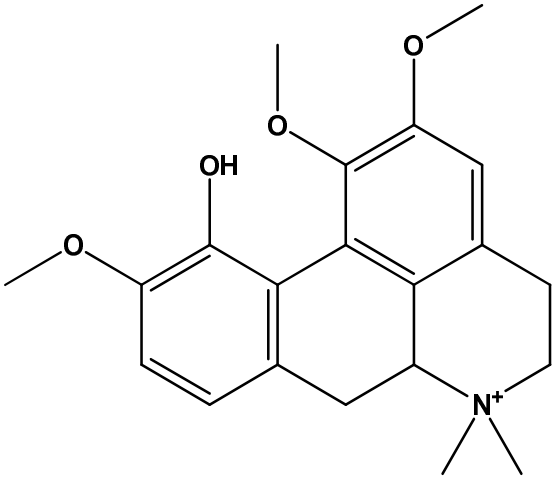


Fig. 2 The structure of the compound isolated from the fraction II of *A.cocculus.*

LCMS and NMR data of *M. leucadendron*

Fraction II

The fraction II was subjected to ^{1}H and ^{13}C NMR and LCMS analysis. The ^{1}H and ^{13}C NMR as well as LCMS data are given in **Plate 2.6 A, 2.6 B and 2.6 C**.

Compound 3

It is interesting to note that the structure isolated resembles the structure of (E)-3, 7-dimethylocta-2, 6-dienal. **IUPAC Name:** (E)-3, 7-dimethylocta-2, 6-dienal, **Chemical Formula**: $C_{10}H_{16}O$.

^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 1.837 (s, 3H, H(12), H(13) and H(14), 1.935 (s, 3H, H(25), H(26), H(27), 2.927 (t, 2H, H(16), H(17), J=1.2Hz), 3.644 (t, 2H, H(18), H(19), J=1.0), 2.250 (s, 3H, H(22), H(23), H(24), 4.923 (t, 1H, H(15) J=2.1Hz), 5.037 (d, 1H, H(20), J=1.0 Hz), 7.314 (s, 1H, H(20) (**PlateNo**. **2.6A**).

^{13}C NMR (100 MHz, $CDCl_3$), δ (ppm): 22.81 (C1), 29.55 (C11), 28.44 (C10), 44.13 (C4), 52.82 (C5), 112.10 (C3), 139.83 (C7), 174.46 (C8) (**PlateNo**. **2.6B**).

Mass spectra ESI $[M-H]^-$151.27 (Actual Mol. Wt. 152.23) (**PlateNo**. **2.6C**).

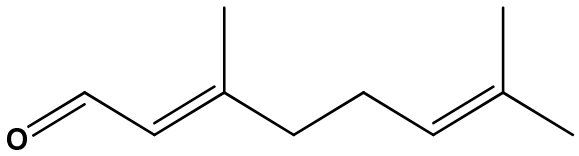


Fig. 3 The structure of the compound isolated from the fraction II of *M. leucadendron.*

Fraction VII

The fraction VII was subjected to ^{1}H and ^{13}C NMR and LCMS analysis. The ^{1}H and ^{13}C NMR as well as LCMS data are given in **Plate 2.7A, 2.7 B and 2.7 C**.

Compound 4

It is interesting to note that the structure isolated resembles the structure of 1,1,4,7-tetramethyl-1a,2,3,4,4a,5,6,7b-octahydro-1H-cyclopropa[e]azulene. **IUPAC Name:** 1,1,4,7-tetramethyl-1a,2,3,4,4a,5,6,7b-octahydro-1H-cyclopropa[e]azulene, **Chemical Formula**: $C_{15}H_{24.}$

^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 1.013 (d, 3H, H(16), H(17) and H(18) J=1.1Hz), 1.100 (s, 6H, H(26), H(27), H(28), H(29), H(30) and H(31), 1.285 (m. 1H, (H24) J=0.4Hz, 1.355 (d, 1H, H(25) J=5.6Hz, 1.477 (m, 2H, H(20), H(21) J=1.8Hz, 1.561 (m, 1H, H(19) J=1Hz, 0.983 (s, 3H, H(37), H(38) and H(39), 1.784 (m, 2H, H(34), H(35) J=1.1Hz), 1.912 (m, 1H, H(36) J=1.8), 4.030 (t, 2H, H(32), H(33) J=2.1Hz) (**PlateNo**. **2.7A**).

^{13}C NMR (100 MHz, $CDCl_3$), δ (ppm): 25.73(C15), 27.89 (C1), 28.90 (C8 and C9), 29.24 (C3), 31.84 (C7), 33.70 (C6), 34.78 (C5), 35.99 (C13), 36.34 (C2), 41.59 (C12), 46.84 (C14), 76.43 (C10) and 77.06 (C11) (**PlateNo**. **2.7B**).

Mass spectra ESI $[M-H]^-$ 203.37 (Actual Mol. Wt. 204.35) (**PlateNo**. **2.7 C**).

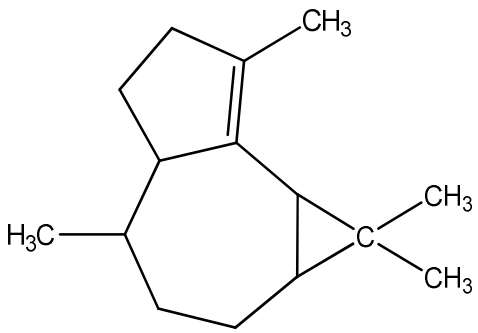


Fig. 4 The structure of the compound isolated from the fraction VII of *M. leucadendron*

2.5 DISCUSSION

In almost all the tropical and subtropical countries the major public health problem is vector borne diseases especially mosquito borne diseases. Vector control is facing a threat due to the emergence of resistance in vector mosquitoes to conventional synthetic insecticides. There has been a paradigm shift towards botanicals to overcome the problems associated with the use of synthetic compounds in mosquito management. Botanical insecticides may serve as suitable alternative to synthetic insecticides in future as they are relatively safe and easily degradable thus making them an ecofriendly alternative. Botanicals can be used as larvicides, adulticides, and repellents for the personal protection against mosquito bites. The studies on the potential of plant derived substances to be used as an effective control agent against mosquitoes have increased many folds, however very few have moved from laboratory to the field due to the instability of these phytochemicals on exposure to sunlight and heat when compared to the synthetic insecticides.

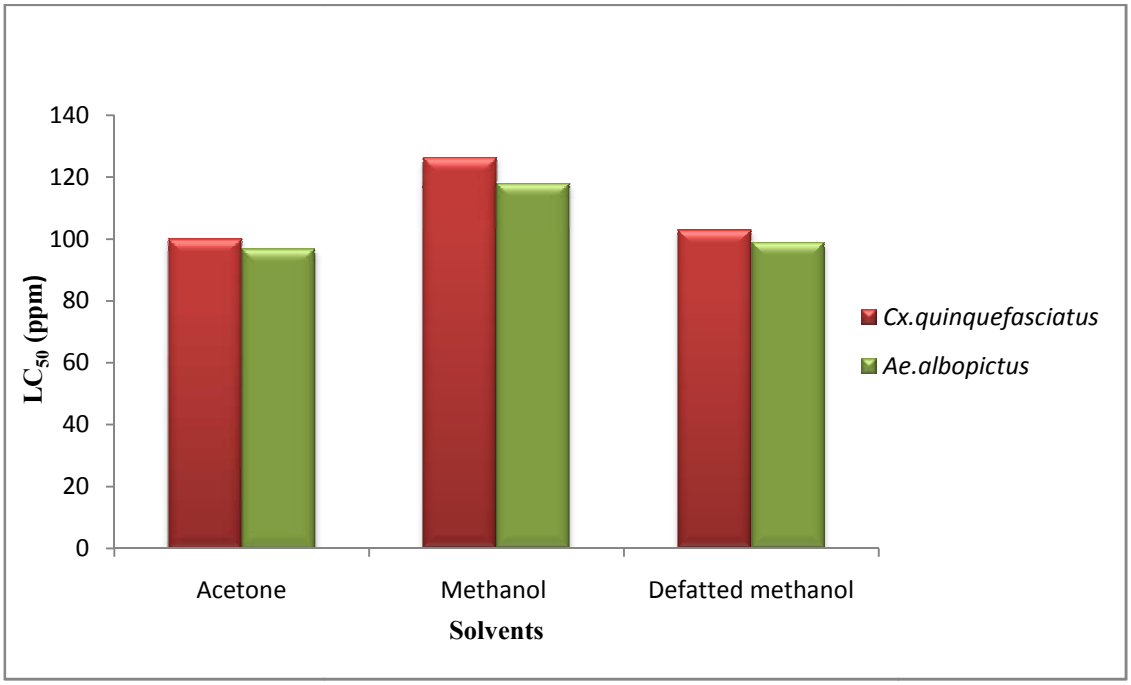
The activity of plant extracts is attributed to a single compound or a mixture of compounds, and the activity is enhanced when these compounds are applied after isolation. Identification, isolation and mass synthesis of bioactive compounds of plant origin and its effective use against mosquito are imperative for the management of mosquito borne diseases. Generally, the preliminary studies are carried out to identify the activity of selected botanicals and in the next stage, the bioactive compounds in the extract which shows the most desired activity is selected for further purification and isolation to pure form. Identifying plant based insecticides that are efficient, suitable and adaptive to local ecological conditions and biodegradable with widespread mosquitocidal property will work as a new weapon in the arsenal of insecticides and in the future may provide as a suitable alternative product to fight against mosquito menace and mosquito-borne diseases.

The present study investigated initially, the larvicidal efficacy of two plant extracts *A. cocculus* (seed) and *M. leucadendron* (leaf) against *Ae. albopictus* and *Cx. quinquefasciatus*. Larviciding is a successful way of reducing mosquito populations in their breeding place before emerging into adults. During this immature stage, mosquitoes are relatively immobile; remaining more concentrated than they are in the adult stage (Rutledge *et al*., 2003). The results presented in tables 1 to 12 show the larvicidal activity of acetone, methanol, and defatted methanol extracts of *A.cocculus* and *M. leucadendron* against I, II, III and IV instars of larvae of *Ae. albopictus* and *Cx. quinquefasciatus.* The comparison between the LC_{50} values indicated that the *A. cocculus* seed extracts showed more larvicidal activity than *M. leucadendron* extracts. The *A. cocculus* treated larvae exhibited more restlessness, convulsions and sluggishness as compared with the *M. leucadendron* treated larvae and the restless activity is more in *Ae. albopictus* than in *Cx. quinquefasciatus*. The sluggish and peculiar coiling movement in treated larvae might be due to the neuronal or muscular disturbances caused by active ingredients released by the extracts in to the water. After exposure to *A. cocculus* extracts, the larvae showed abnormal motions, tremors and convulsions followed by paralysis and finally settle at the bottom of the container. The result is in agreement with the reports of Sagar and Seghal (1997) against *Cx. quinquefasciatus.*

A. cocculus acetone extracts shows more activity than methanol and defatted methanol extracts. And its activity was higher in *Ae. albopictus* as compared with *Cx. quinquefasciatus.* The larvicidal activity is seen increasing with the concentration and time of exposure in every case. The 72 hour LC_{50} value is less as compared with the 24 hour LC_{50} value and in every treatment both extracts showed more activity towards the 1^{st} instar larvae. In both cases the acetone extracts showed more activity than methanol and defatted methanol extracts. In all the cases the control did not show any mortality. As

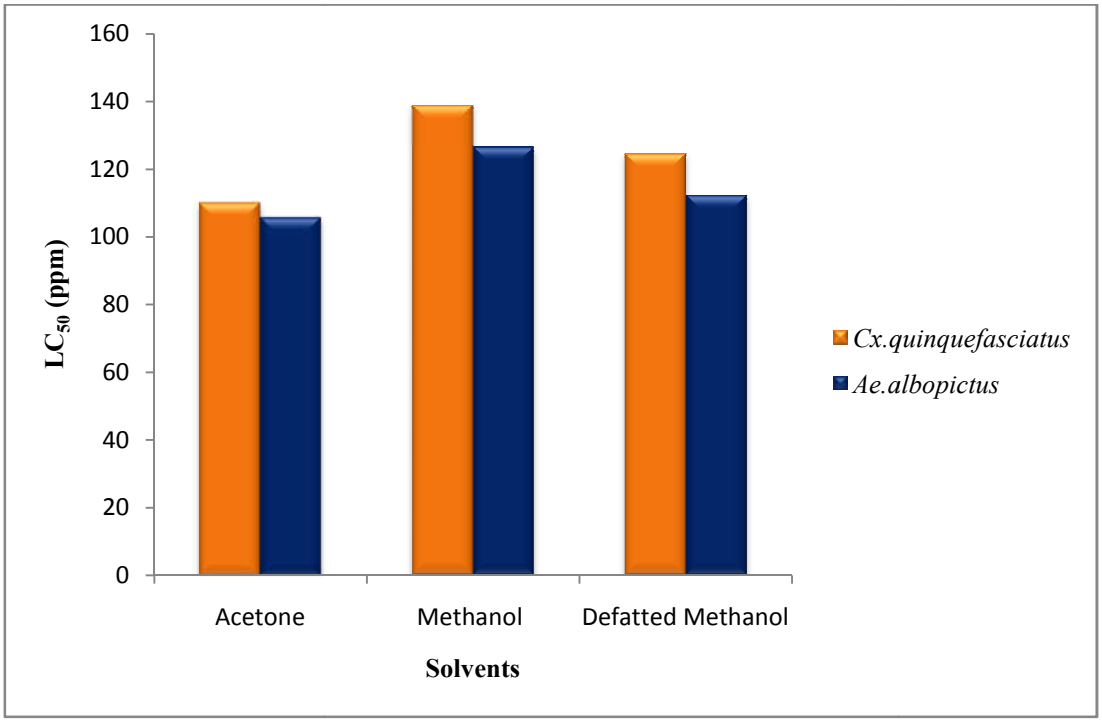
the concentration and time of exposure increases the larvicidal effect also increases.

Figure 5: LC_{50} (ppm) values of different extracts *A. cocculus* against III instar larvae of *Cx. quinquefasciatus* and *Ae. albopictus*



In the case of treatment with *M. leucadendron,* the most active fraction was acetone as in the case of *A. cocculus*. The activity was higher in *Ae. albopictus* as compared with *Cx. quinquefasciatus.* The larvicidal activity increases with the concentration and time of exposure in every case. The 72 hour LC_{50} value is less as compared with the 48hr and 24hr LC_{50} values in every treatment. All extracts showed more activity towards the 1^{st} instar larvae as in the case of *A. cocculus*, the acetone extracts showed more larvicidal activity than methanol and defatted methanol extracts in *M. leucadendron*. In all the cases the control did not show any mortality and the concentration and time of exposure directly related with the activity of plant extracts.

Figure 6: LC_{50} (ppm) values of different extracts *M. leuacadendron* against III instar larvae of *Cx. quinquefasciatus* and *Ae. albopictus*

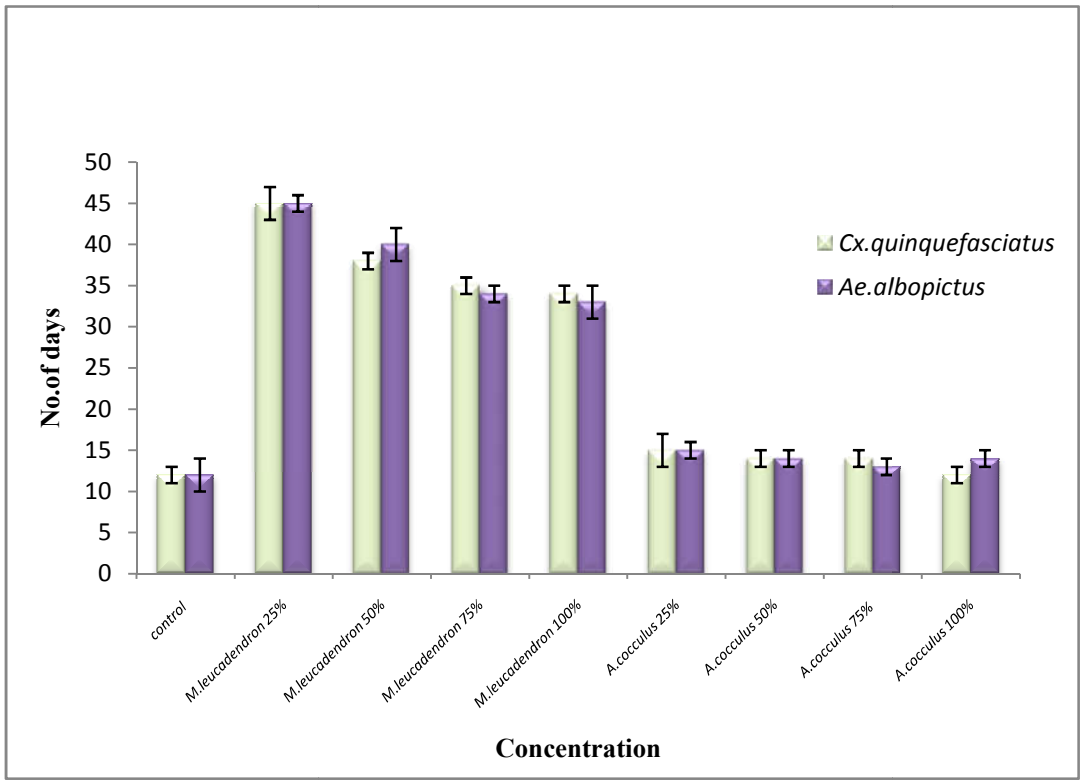


The treatment of ethyl acetate extract of *M. leucadendron* resulted in prolongation of larval period accompanied with decrease in larvicidal activity. F-value for comparison of different concentrations of *M. leucadendron* and control group was 5432.07. The p-value is <0.00001 and hence the result is highly significant at $p<0.05$. The prolongation of larval duration while applying ethyl acetate extract of *M. leucadendron* shows the effect on the normal development of the mosquito species; similar cases are reported in the usage of aqueous and ethanol extracts of *Pseudocalymma* (Carlos *et al*., 2014), when using acetone extract of *Azadirachta indica* on larvae and pupae of *Cx. pipiens* and *Ae. aegypti* (Sagar and Sehgal, 1997). Singh, 1996 used methanol extract of neem seed to increase the larval instar duration in *Cx. quinquefasciatus*. Ndungu'u *et al.,* 2004 reported the growth inhibition effects of root bark extracts of five *Meliaceae* species against *An. gambiae.*

The insect growth regulatory effect is attributed to compounds that mimic juvenile hormone in arthropods (Mulla, 1991) delaying/prolonging their development or causing malformations that lead to the death of mosquitoes. Larval stage extension while using neem against *An. stephensi* was reported by (Murugan *et al*., 1996). Shalaan *et al.,* 2005 said that the secondary metabolites of many plant species show effect on growth and development in various life stages of mosquitoes, causing delay or extension of larval and pupal development, molting and inhibition, morphological abnormalities and mortality, especially during the molting process and melanization.

In the present study, lengthening of larval duration while treating with ethyl acetate fraction of *M. leucadendron* indicates the activity of the bio-active compounds present in the plant with the normal hormonal activity coordination of the metabolic process of the larval stages. Prolongation of development of mosquito larvae treated with plant extracts were generally attributed to interference of the active ingredients with the endocrine system of the mosquito (Zebitz, 1986). The *M. leucadendron* ethyl acetate fraction shows more days of development or elongation of larval duration as compared with *A. cocculus* extract and control population. There is no much change in the duration of larval life span of *A. cocculus* and control population. The moratlity rate is high in the case of *A. cocculus* treated population as compared with that of *M. leucadendron* treated ones. In *M. leucadendron* treated cases both in *Cx. quinquefasciatus* and *Ae. albopictus* the larval mortality is very low. Approximately 50% of the treated larvae changed in to pupal stage.

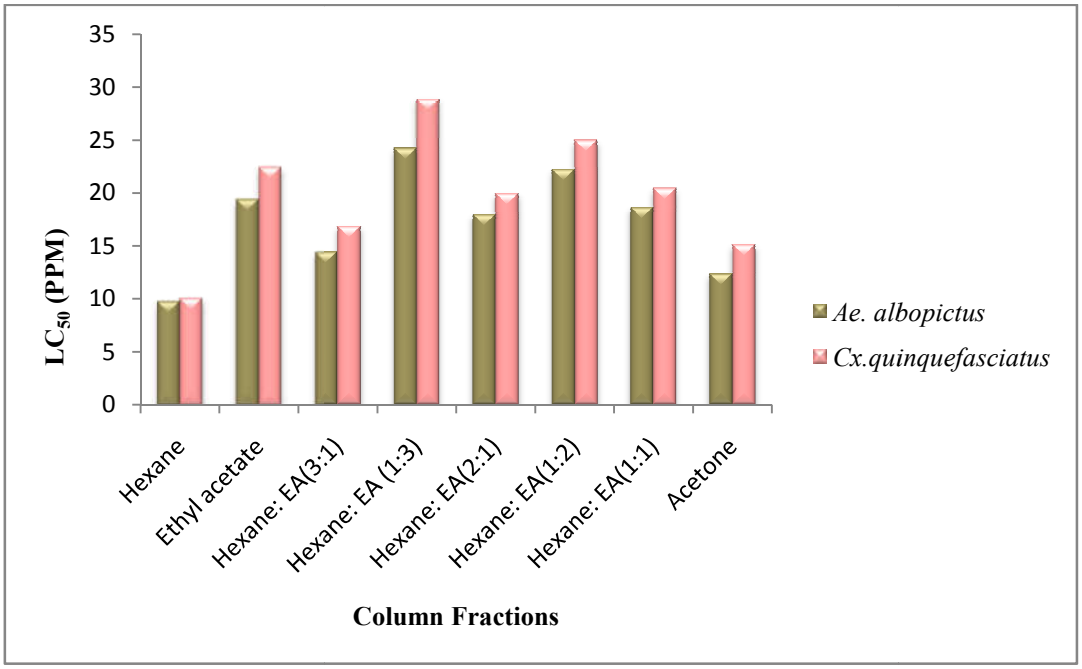
Figure 7: Extension of larval duration while treating with different concentrations of Ethyl acetate fractions of *A. cocculus* and *M. leucadendron*



Eight fractions of *A. cocculus* seed extracts were taken using the column chromatography and analysed their larvicidal activity against 3rd instar larvae of both *Cx. quinquefasciatus* and *Ae. albopictus*. The mortality rates were recorded and the LC_{50} values were calculated. The calculated values show that the hexane fraction of *A. cocculus* shows more activity than the other seven fractions. A slight difference is seen in the activity against *Cx. quinquefacsiatus* and *Ae. albopictus*. With all the eight fractions, *Ae. albopictus* showed less LC_{50} values for mortality as compared with *Cx. quinquefasciatus,* which may be due to its breeding habit that they are living

in fresh water and *Cx. quinquefasciatus* mostly breeds and the larvae survive in the polluted water.

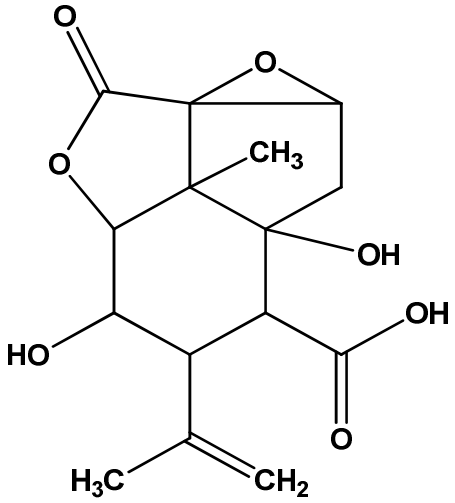
Figure 8: LC_{50} (ppm) of column fractions of *A. cocculus* against III instar of *Ae. albopictus* and *Cx. quinquefasciatus*



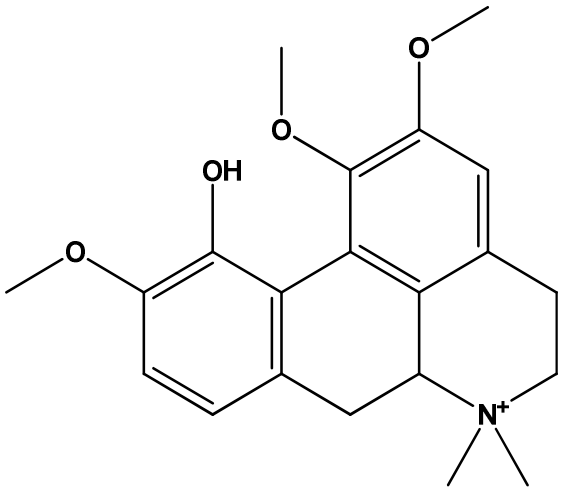
The activity of column fractions of *A. cocculus* was in the order Hexane (H) > Acetone (A) > H:EA (3:1) >H:EA (2:1)> H:EA (1:1): Ethyl acetate (EA): H:EA (1:2) >H:EA (1:3). And the LC_{50} values against *Ae. albopictus* and *Cx. quinquefasciatus* were 9.81> 12.36> 14.44> 17.96> 18.64> 19.4> 22.22> 24.22 and 10.1> 15.12> 16.84 > 19.96> 20.44> 22.4> 24.98> 28.86 respectively.

The most active fraction of *A. cocculus* was further purified using 'Thin layer chromatography' and the fractions were again tested for their larvicidal activity and the most active fractions were analyzed by NMR and LCMS to elucidate the structure of compound present in the fraction.

Chromatographic techniques are used to identify fractions and help to isolate chemical constituents from the selected extract. The fractions isolated from the hexane fraction of *A. cocculus* were **I** and **III**. The selected fractions which showed potent activities were further analyzed using Liquid Chromatography-Mass Spectrometry. The fraction I produced the molecular ion signal (m/z) of 309.13 and the identified compound may be 4, 6a-dihydroxy-1a1-methyl-2-oxo-5-(prop-1-en-2-yl)octahydro-1a1H-oxireno[2',3':1,2]indeno[7,1-bc]furan-6-carboxylic acid and its molecular formula is $\mathbf{C_{15}H_{18}O_7}$(Picrotin).

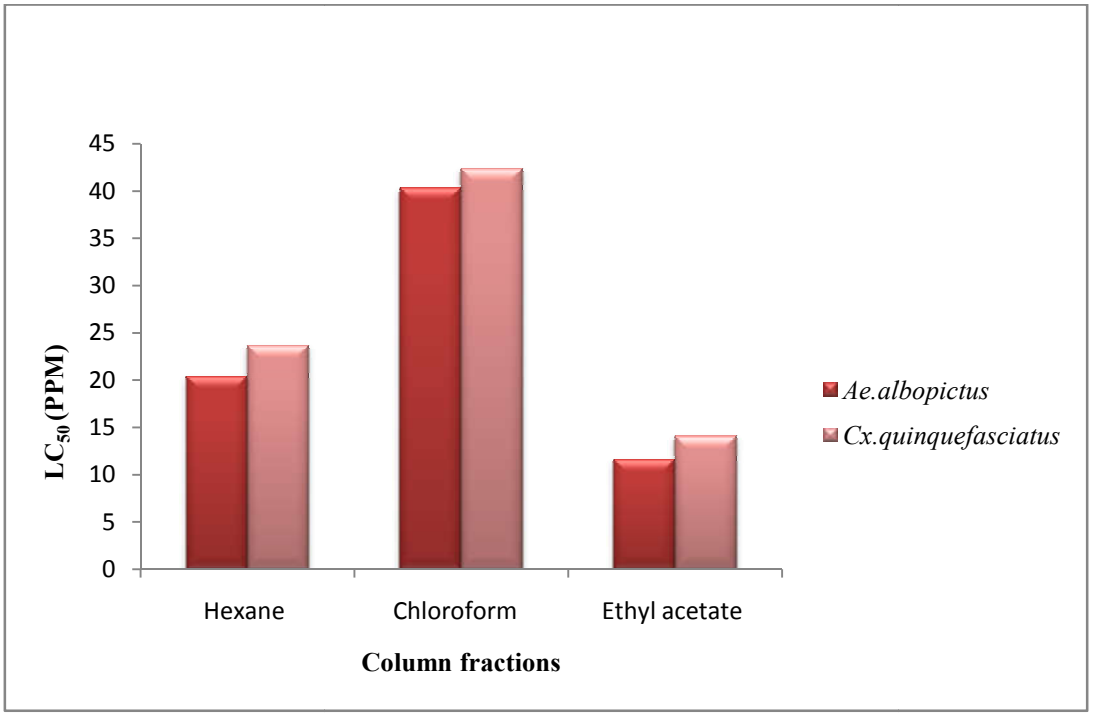


The fractions III produced the molecular ion signal (m/z) of 355.21 and the identified compound may be 11-hydroxy-1, 2, 10-trimethoxy-6, 6-dimethyl-5, 6, 6a, 7-tetrahydro-4H-dibenzo [de, g] quinolin-6-ium and its molecular formula are$\mathbf{C_{21}H_{26}NO_4^+}$ (Menispermine).

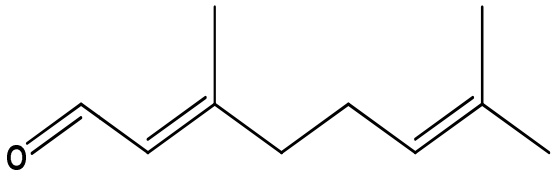


Three fractions of *M. leucadendron* leaf extracts viz, hexane, ethyl acetate and chloroform were taken using the column chromatography and analysed their larvicidal activity against 3rd instar larvae of both *Cx. quinquefasciatus* and *Ae. albopictus*. The mortality rates were recorded and the LC_{50} values were calculated. The chloroform fraction of *M. leucadendron* has more activity than hexane and ethyl acetate fractions. A slight difference is seen in the activity against *Cx. quinquefasciatus* and *Ae. albopictus*. In all the three fractions, *Ae. albopictus* shows less LC_{50} values as compared with *Cx. quinquefasciatus,* as in the case of *A*. *cocculus* extracts.

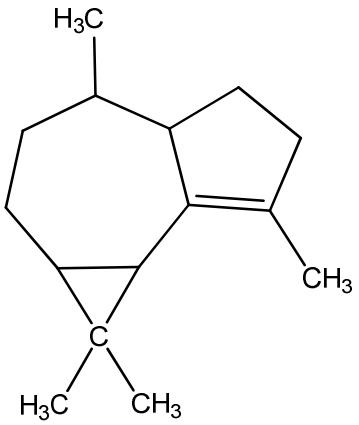
Figure 9:LC_{50} (ppm) of column fractions of *M. leucadedondron* against III instar of *Ae. albopictus* and *Cx. quinquefasciatus*



The fractions isolated from the chloroform fraction of *M. leucadendron* were **II and VII**. The fractions **II** produced the molecular ion signal (m/z) of 151.27 and the identified compound may be (E)-3, 7-dimethylocta-2, 6-dienal and its molecular formula is **$C_{10}H_{16}O$** (Citral).



The fractions VII produced the molecular ion signal (m/z) of 203.37 and the identified compound may be 1,1,4,7-tetramethyl-1a,2,3,4,4a,5,6,7b-octahydro-1H-cyclopropa[e]azulene and its molecular formula is **$C_{15}H_{24}$** (α-Gurjunene).



In conclusion, the ^{1}H and ^{13}C NMR as well as LCMS spectrum confirms two chemical constituents obtained from the fractions of the plant extracts of *A. cocculus* and two chemical constituents from *M. leucadendron.* The present study identified 4 compounds using the chromatographic fractions obtained from the extracts of the two plants, *A. cocculus* and *M. leucadendron*. The identified 4 compounds were namelypicrotin, menispermine, citral, α-Gurjunene. The identified compounds may lead the way to define its potential biological activity. These molecules can be further theoretically tested for its biological activity using *in silico* methods. In future, based on its theoretical activity the compounds can be further redesigned for its potent biological activity.

The results on GC-MS analyses of *M. leucadendron* essential oil is summarized in the table (51). Thirteen compounds have been identified in *M. leucadendron* leaf oil samples. The compounds were mostly monoterpene hydrocarbons, oxygenated monoterpes, sesquiterpene hydrocarbons, and oxygenated sesquiterpenes. The result showed γ –Selinene (39.66%) was the major compound in the oil followed by α- Pinene (9.59), Carophylline (9.07), Terpineol (5.97), Limonene (3.2), α- Gurjunene (3.11), Ledol (2.63), Terpene-4-ol (1.86), P- Cymene (1.73), β- Pinene (1.35), β- Eudesmol (1.21), Linalool (1.15), Spathulenol (1.08) respectively.

The present study elucidate that the two plants, *A. cocculus* an *M. leucadendron* contain bio active components that offer comparatively better and promising biocontrol agents which can be used effectively against mosquitoes. The larvicidal, adulticidal and repellent properties exhibited by both the plants offer venues for utilization of the active components from these plants to be incorporated in mosquito control programmes which are more ecofriendly and environmentally sustainable, in addition to ensuring substantial reduction in mosquito populations.

3. SUMMARY

Economic losses to insect related infections has been a comparatively serious concern even in the modern high-tech world and a lion share of this is contributed by vector borne diseases especially mosquito borne diseases. Mosquitoes are dreadful insect vectors which transmit bacterial, viral and parasitic diseases including Malaria, Filariasis, Yellow fever, Chikungunya, Dengue fever, Japanese encephalitis etc. without being affected themselves. A careful and prolonged control of the vector can help to control these diseases out breaks; nevertheless, it is not an easy task due to its natural tolerance and early development of resistance to available insecticides. The early detection of resistance in vector mosquitoes can help the local government to plan and select appropriate alternative control measures or insecticides or effective control mechanisms. The present study investigated the resistance status with regard to synthetic insecticide usage to control *Culex quinquefasciatus* in different locations in Kerala and also analyzed the potential of selected plant derived botanicals from *Anamirta cocculus* and *Melaleuca leucadendron* as an alternative to synthetic insecticides against *Cx. quinquefasciatus* and *Aedes albopictus.*

The study area included five municipalities/corporations viz, Kozhikode corporation, Palakkad Municipality, Thrissur Municipal Corporation, Cochin Municipal Corporation, Ponnani Municipality in Kerala State. The selection of sampling sites was determined according to criteria based on the frequency of insecticide application to control mosquitoes. *Cx. quinquefasciatus* Say was the test orgranism in the study. Bioassays were conducted using the commonly used organophosphorus larvicide, temephos. Quantitative metabolic enzymes assay have been commonly used in the detection of insecticide resistance because it is a highly sensitive technique

and gives results rapidly even at low frequencies. Carboxylesterase, Glutathione -S- transferase (GST), Monooxygenase or mixed function oxidase (MFO) and Acetylcholinesterase activities in *Cx. quinquefasciatus* collected from the selected sites of Kerala were studied. Enzyme activities were analysed to estimate the detoxification status of *Cx. quinquefasciatus* from these selected sites. The susceptibility status of *Cx. quinquefasciatus* using WHO Susceptibility kit was done employing three insecticide impregnated papers viz, organophoasphate Malathion 5%, synthetic pyrethroids, Cyfluthrin 0.15% and Deltamethrin 0.05%. The survived mosquitoes from the WHO kit assay were taken; DNA was isolated and amplified partial *ace*1 gene and *kdr* gene. Site specific mutation in *ace*1 gene detected using restriction enzyme Alu1. Allele specific PCR assay were conducted to identify the site specific mutation in the *kdr* gene.

The results of biochemical assay conducted using 0.01ppm temephos and that of the field populations were elevated when compared to the laboratory population and the scarcely treated populations. The resistance ratio for the years 2014, 2015 and 2016 for all the five populations was more than one indicating the occurrence of insecticide resistance.There was a significant increase in the resistance ratio over the three years of investigations from 2014to 2016. Of the five areas the Cochin populations is having the highest value of LT_{50} and resistance ratio.

There is a significant difference in carboxylesterase, GST, MFO activities of field populations of *Cx. quinquefasciatus* with that of laboratory populations. The detoxification enzyme levels of *Cx. quinquefasciatus* were highin the areas where insecticides sprayed regularlywhen compared to the areaswhere insecticides are sprayed scarcely. The level of enzymes increased from year to year and it reinforces the need for constant surveillance of mosquito populations susceptibility against the insecticides used in control

programs as well as their effectiveness in the field. The presence of elevated enzyme levels indicated the multiple resistance mechanism in the field populations of *Cx. quinquefasciatus*.

The acetylcholine esterase assay was conducted to identify the resistance towards organophosphates and carbamates. The % remaining activity in propoxur inhibited fraction in the regularly insecticide treating field populations of *Cx. quinquefasciatus* were higher than 30% and scarcely treating area showed a value less than 30% in all the samples. The value greater than 30% indicates the chance to develop resistance in field populations.

The present investigation also revealed the development of resistance to pyrethroid insecticides in *Cx. quinquefasciatus* subjected to susceptibility tests in mosquito populations collected from all the five areas. The findings of the study has critical implications for vector control regimes followed in all the five areas as the mosquito populations has already developed resistance to commonly used insecticides. As the field population of Cochin showed comparatively high resistance to organophosphates, it implies that, the strategy used for vector control in Cochin Municipal Corporation may have to be reconsidered. In general, the current strategy for mosquito control in all these areas of investigation is based primarily on the dependence of temephos by using it as a larvicidal agent by spraying in ditches, which are the main breeding sites of *Cx. quinquefasciatus*. The emergence of resistance to organophosphates in the field strains indicates the need for the usage of an alternative strategy for effective control of mosquito populations of the area in order to avoid the adverse consequences of development of insecticide resistance and cross resistance.

The observation made on the incidence of *kdr* mutation may also be considered seriously as pyrethroids are used for IRS and ISS for the

immediate control of mosquito populations in areas reporting high incidence of lymphatic filariasis cases. The *kdr* mutation renders the *Cx. quinquefasciatus* populations resistant to the common household control measures used, as pyrethroids are the common constituent of mosquito mats, coils and repellents. The RFLP using Alu1 on the *ace* gene 1 also showed the presence of heterozygous genotype in all the five field populations except in Cochin where the C*x. quinquefasciatus* population possessed homozygous resistant genotype, which is an indication to the excessive dependence of usage of Temephos as a larvicide for extensive field applications.

The most suitable strategy for controlling disease vectors especially mosquitoes, the rotational use of insecticides of different modes of action altogether, rather than merely alternating members of any one chemical class or different chemical classes that address the same target site. For example, the presence of *kdr* resistance renders DDT and pyrethroids less effective, whereas carbamates, such as bendiocarb, or organophosphates can still be used, but the presence of modified AChE warns the regular usage of organophosphates in the field.

The evidence of development of resistance to synthetic insecticides in mosquitoes observed in the present study points to the need of employing new phytochemicals in the field as an alternative to synthetic chemical pesticides which would be easily degradable and have less harmful effect on other organisms. The present study evaluated the activity of two botanicals from *A. cocculus* and *M. leucadendron,* against *Cx. quinquefasciatus* and *Ae. albopictus* and isolated the active of compounds from these plants. As far as the percentage yield is considered, the plant materials offered a good source of bioactive agent with high larvicidal efficacy irrespective of the plant materials. Present study revealed that the acetone and methanol extracts of *A. cocculus* are found to be the best yielded one among the selected plants.

Among them acetone extract of *A. cocculus* elicited more yield (30%) than methanol extract (28%). In addition to that the yield of acetone and methanol extracts of *M. leucadendron* showed a bit lower than *A. cocculus*.

Critical lethal concentration of the selected plant extracts pays much attention to the effective usage of the plant based extracts against mosquitoes. In this part the selected extracts, either acetone or methanol elicit significant larvicidal activity particularly in the immature larval stages. Among the different extracts of both *A. cocculus* and *M. leucadendron* acetone extracts of the selected plants are found to be more active than methanol extracts. The extended exposure of the plant extracts (in 72 hrs) exhibited maximum mortality and the critical lethal concentration (both LC_{50} and LC_{90}) tend to decrease when the larval development increases. The decreased LC_{50} and LC_{90} values in the larval instars may be because of the differential response elicited by the larval instars.

The larvicidal activity of the two selected plants, *A. cocculus* and *M. leucadendron* acetone extracts against *Cx. quinquefasciatus* and *Ae. albopictus* found to be similar. But the activity of methanol extracts of the selected plants exhibited decreased larvicidal activity compared to the acetone extracts. Among the different extracts of *A. cocculus* it is observed that *Ae. albopictus* found to be more susceptible compared to *Cx. quinquefasciatus*. Usage of different type of extractions using the same solvent exhibited a small difference in the LC_{50} as the removal of fat elicited a negligible amount of decrease in the LC_{50} value in *A. cocculus*-*Cx. quinquefasciatus* system.

The column chromatography fractions shown more activity than the crude extracts. The most active fractions were subjected to NMR spectroscopy and LCMS analysis. Menispermine and Picrotin were present in the hexane fraction of *A. cocculus* and α-Gurjunene and Citral were present in the Chloroform fractions of *M. leucadendron.*

M. leucadendron showed adulticidal and repellent activity against mosquitoes. The GCMS data showed the presence of γ –Selinene, α- Pinene, Carophylline, Terpineol, Limonene, α- Gurjunene, Ledol, Terpene-4-ol, P-Cymene, β- Pinene, β- Eudesmol, Linalool and Spathulenol as major compounds.

In a nutshell the investigations and the observations made in the present study throws light on the emergence of development of resistance to temephos used as a mosquito control agent in selected centers of Kerala. The studies made on plant based mosquitocidal agents revealed the potentiality of *A. cocculus* and *M. leucadendron* as high yielding source of mosquito control agents. The investigations provide clear understanding of the status of insecticide resistance in some of the major cities in Kerala that can yield to revitalizing the mosquito control programmes in these areas and at the same time the outstanding knowledge gained from the bioassays and studies on mosquitocidal properties of plant based components can pave ways to develop better strategies by employing plant based, cost effective and ecofriendly means of mosquito control programmes.

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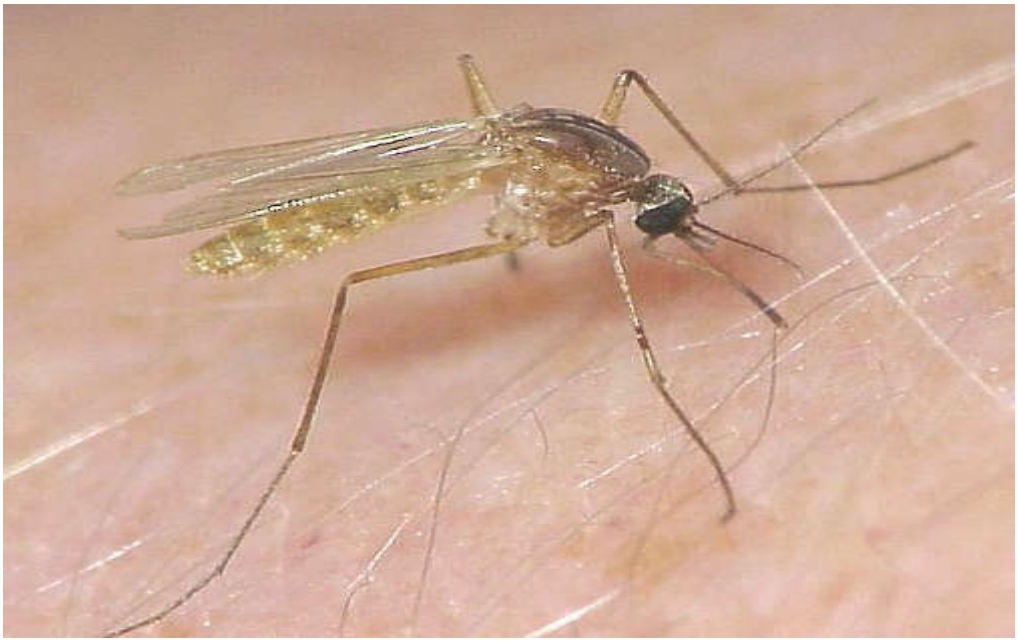
PLATE 2.1

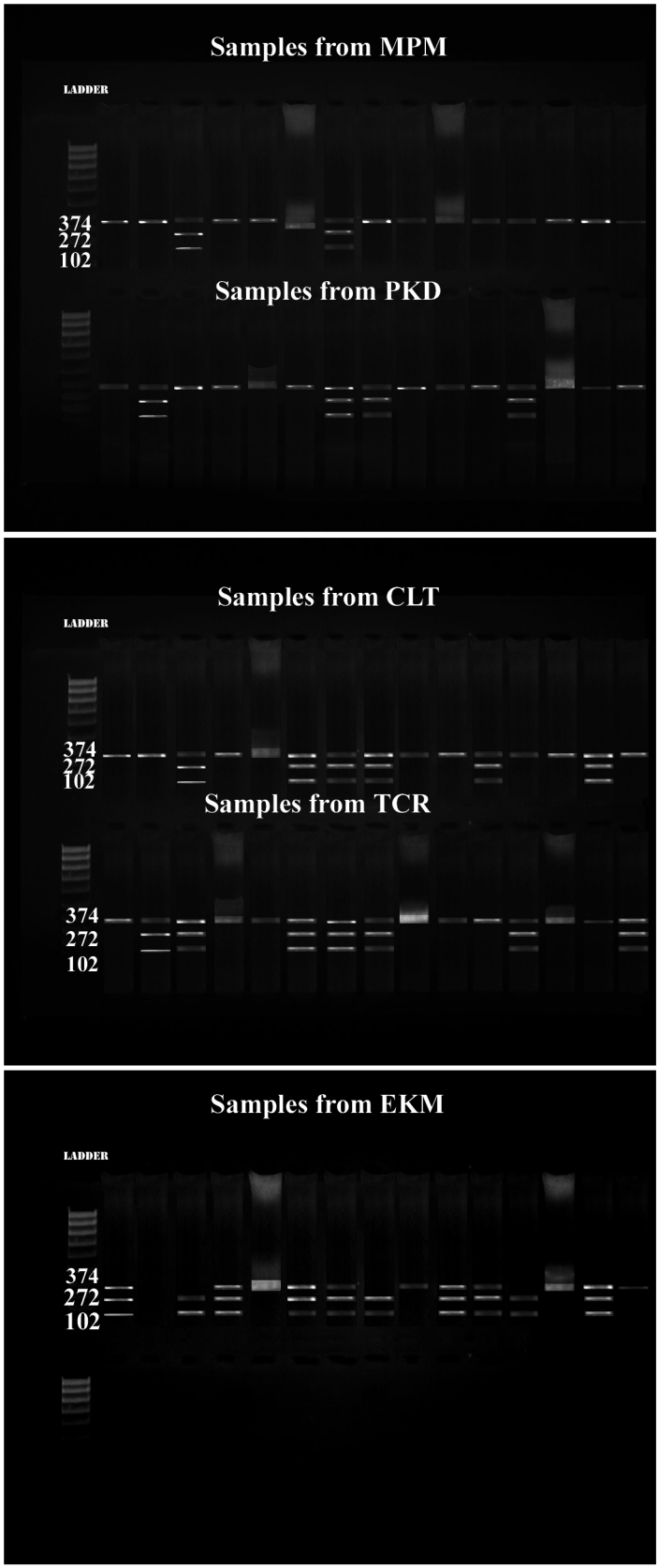
TEST ORGANISM

***Aedes albopictus* Skuse**



***Culex quinquefasciatus* Say**

PLATE 1.4



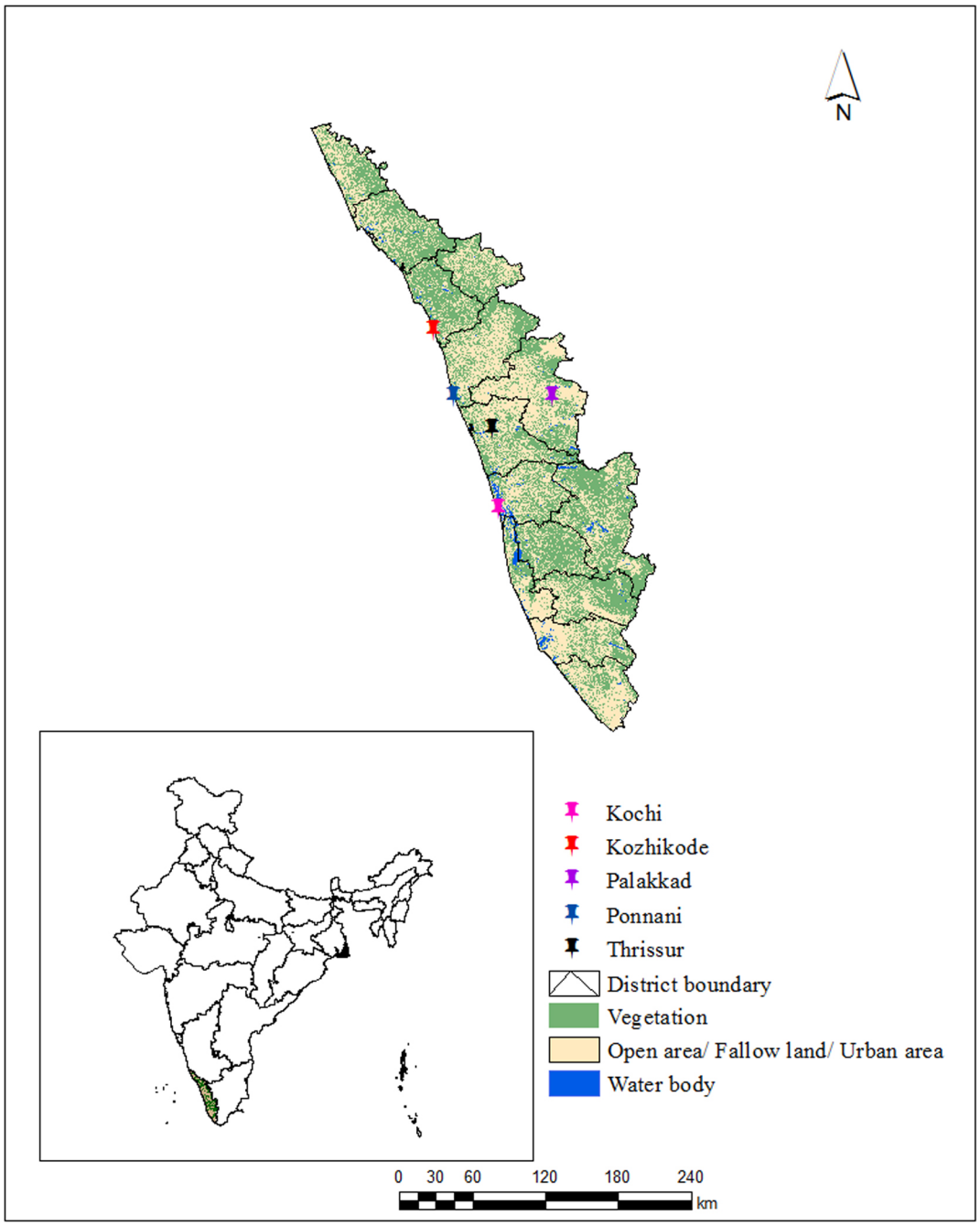
B

Agarose gel showimg *ace1* genotypes in the field collected *Cx. quinquefasciatus* obtained after RFLP using AluI enzyme

PLATE 1.3

Collection of Sample

PLATE 1.2



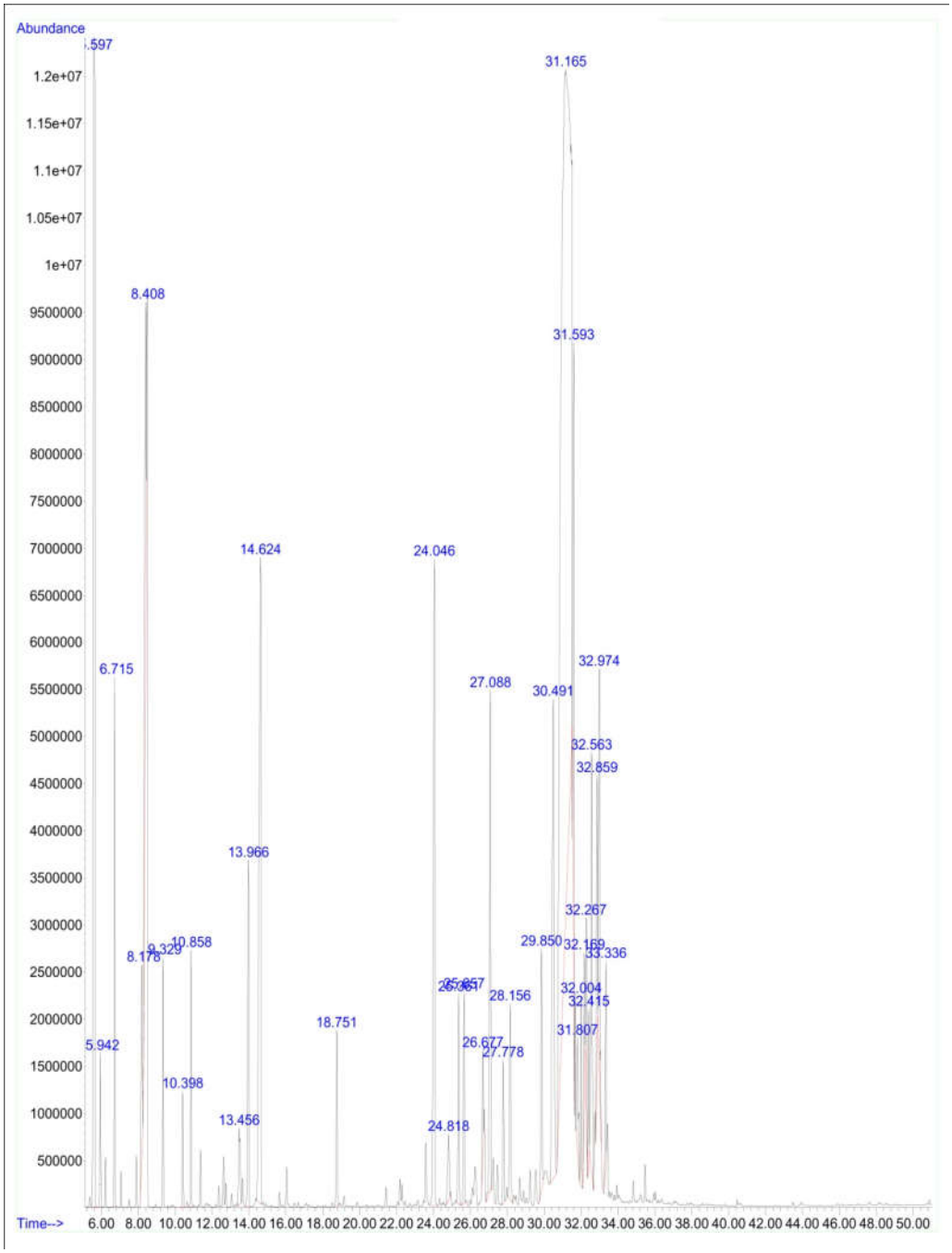
Area of Sample Collection

PLATE 1.4

A. *Anamirta cocculus* (L.) Wight abd Arn; B. Seed

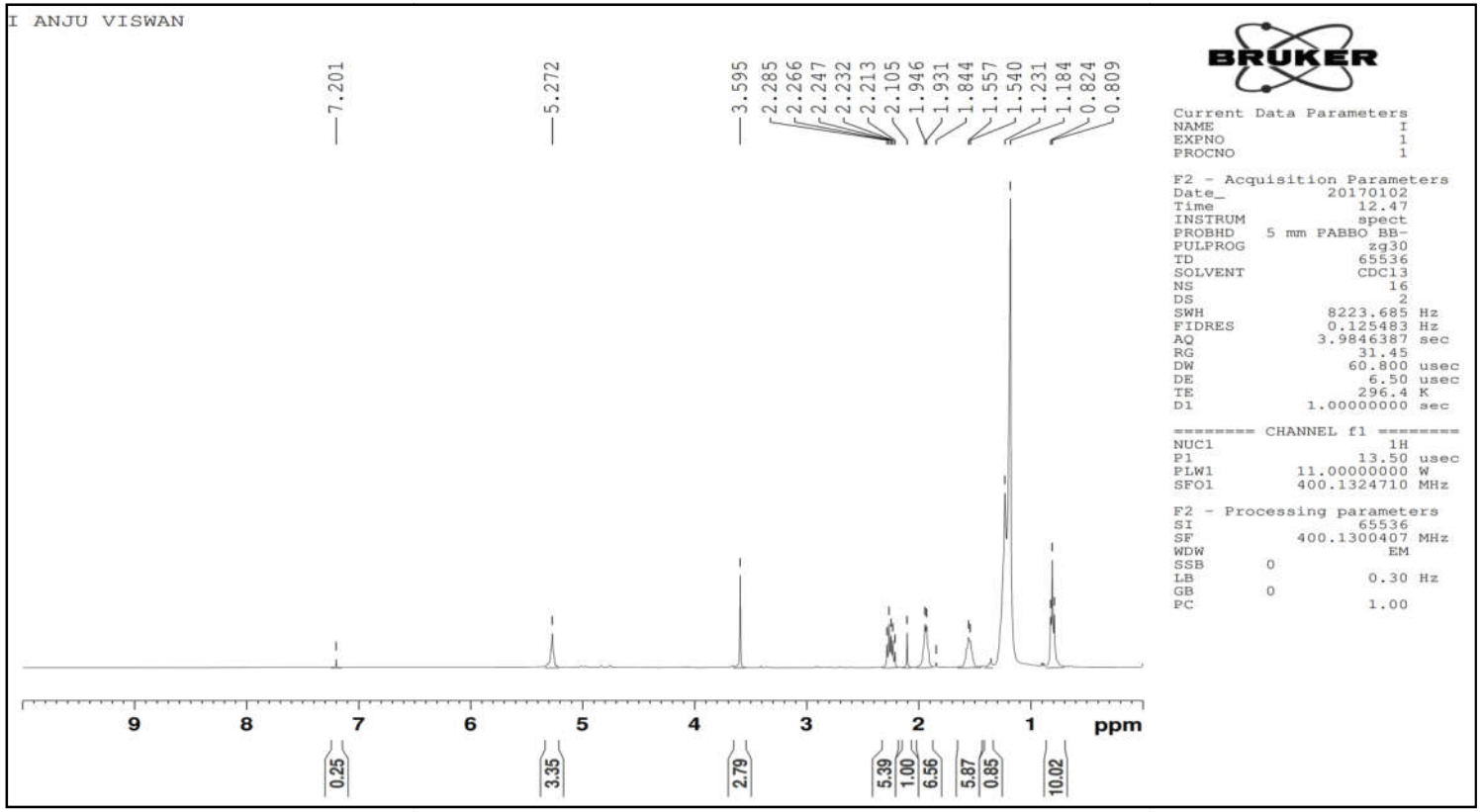
C. *Melaleuca leucadendron* (L.); D. Leaves

PLATE 2.3



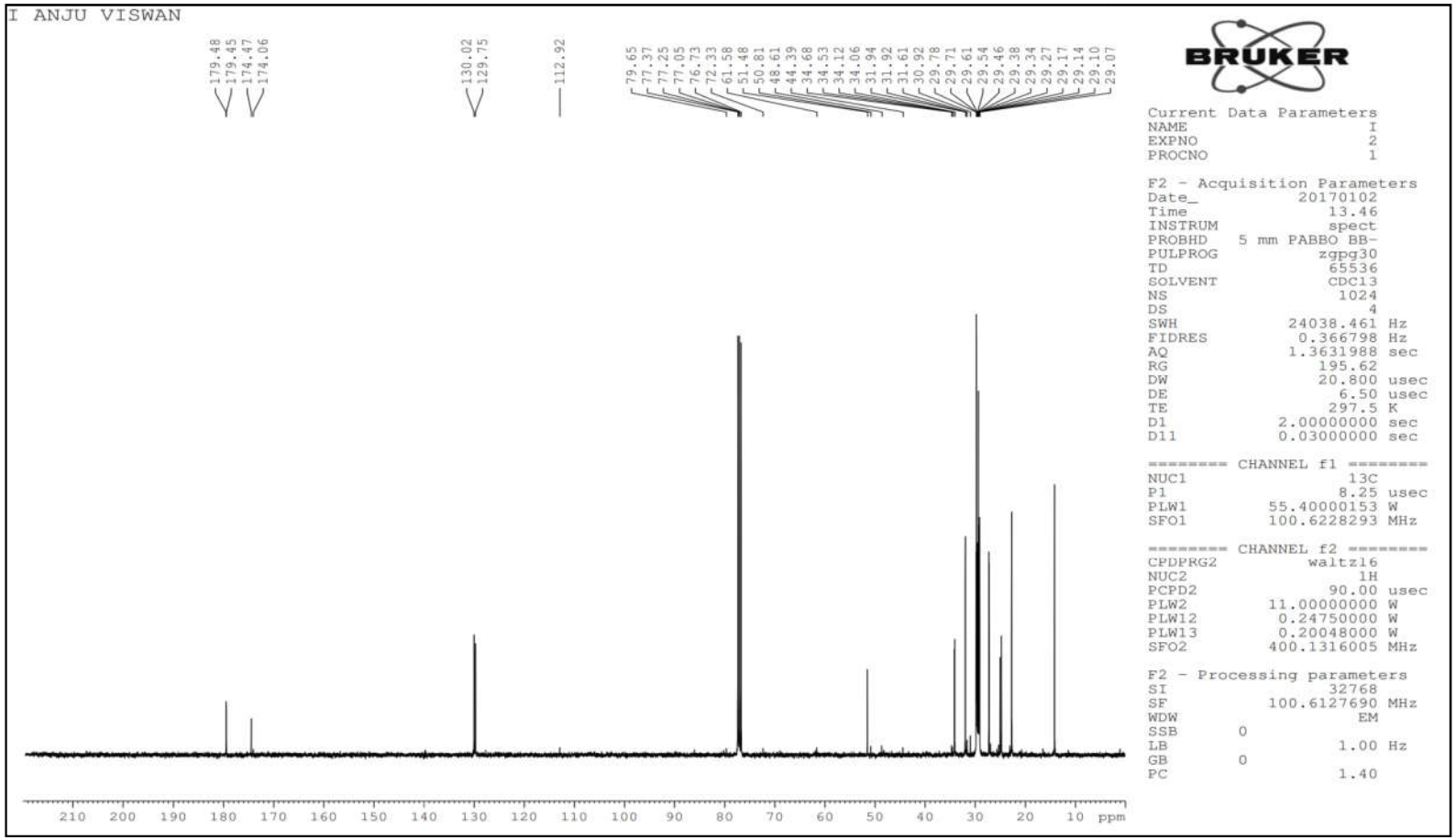
Total Ion chromatogram of *M.leucadendron* essential oil with retention time

PLATE 2.4A



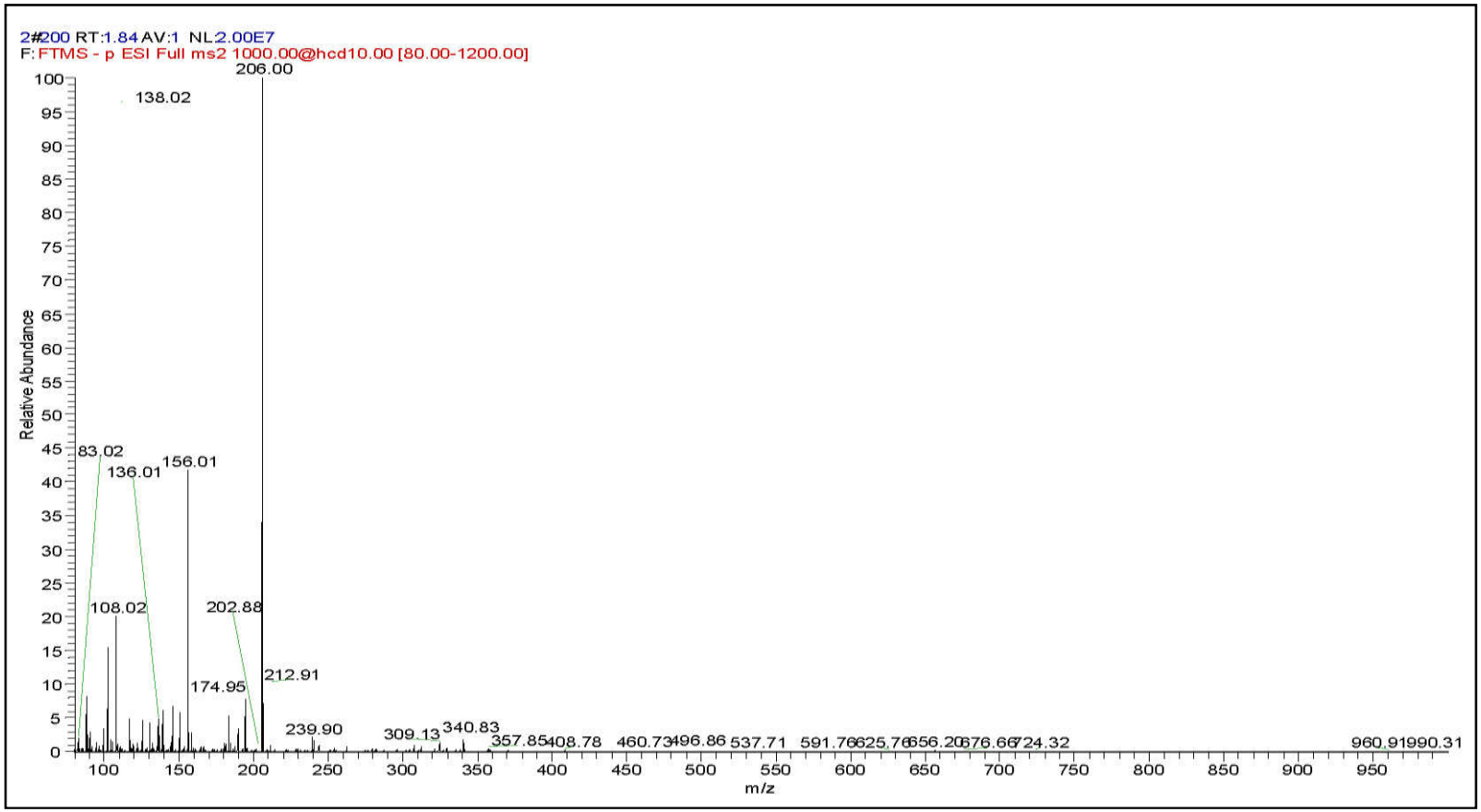
^{1}H NMR spectrum of Fraction 1 (Compound 1)

PLATE 2.4B



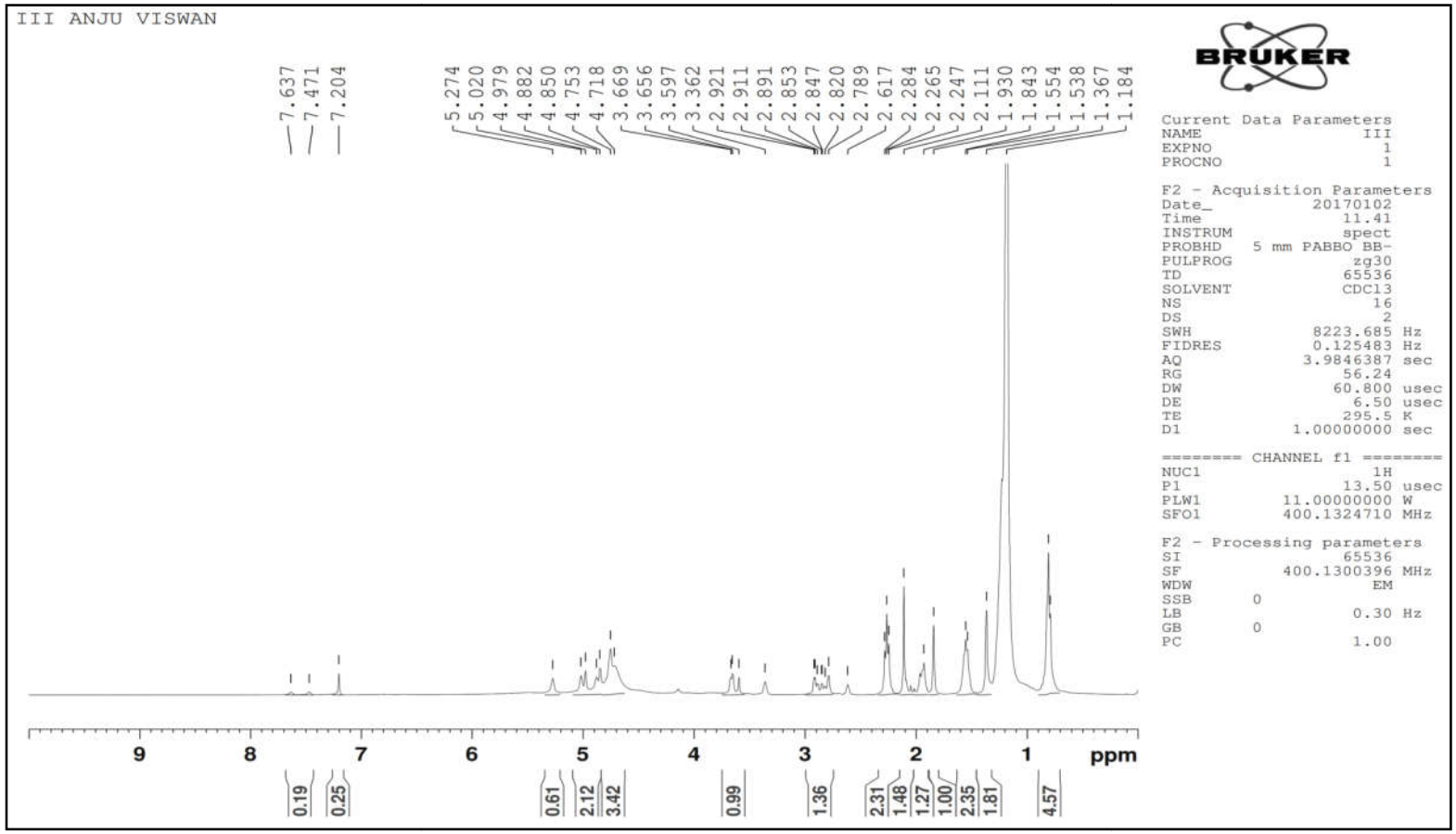
^{13}C NMR spectrum of Fraction 1 (Compound 1)

PLATE 2.4C



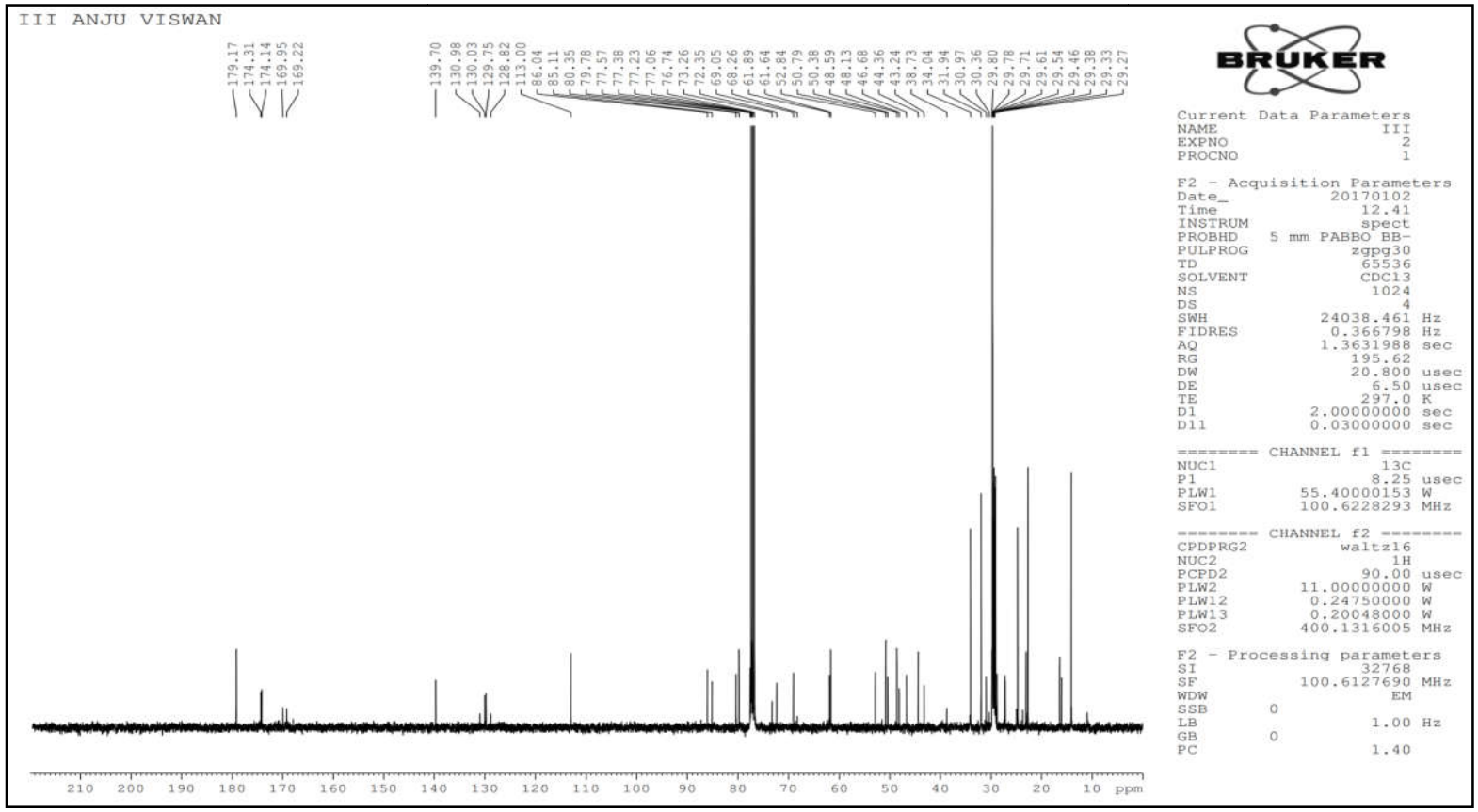
LCMS spectrum of Fraction 1 (Compound 1)

PLATE 2.5A



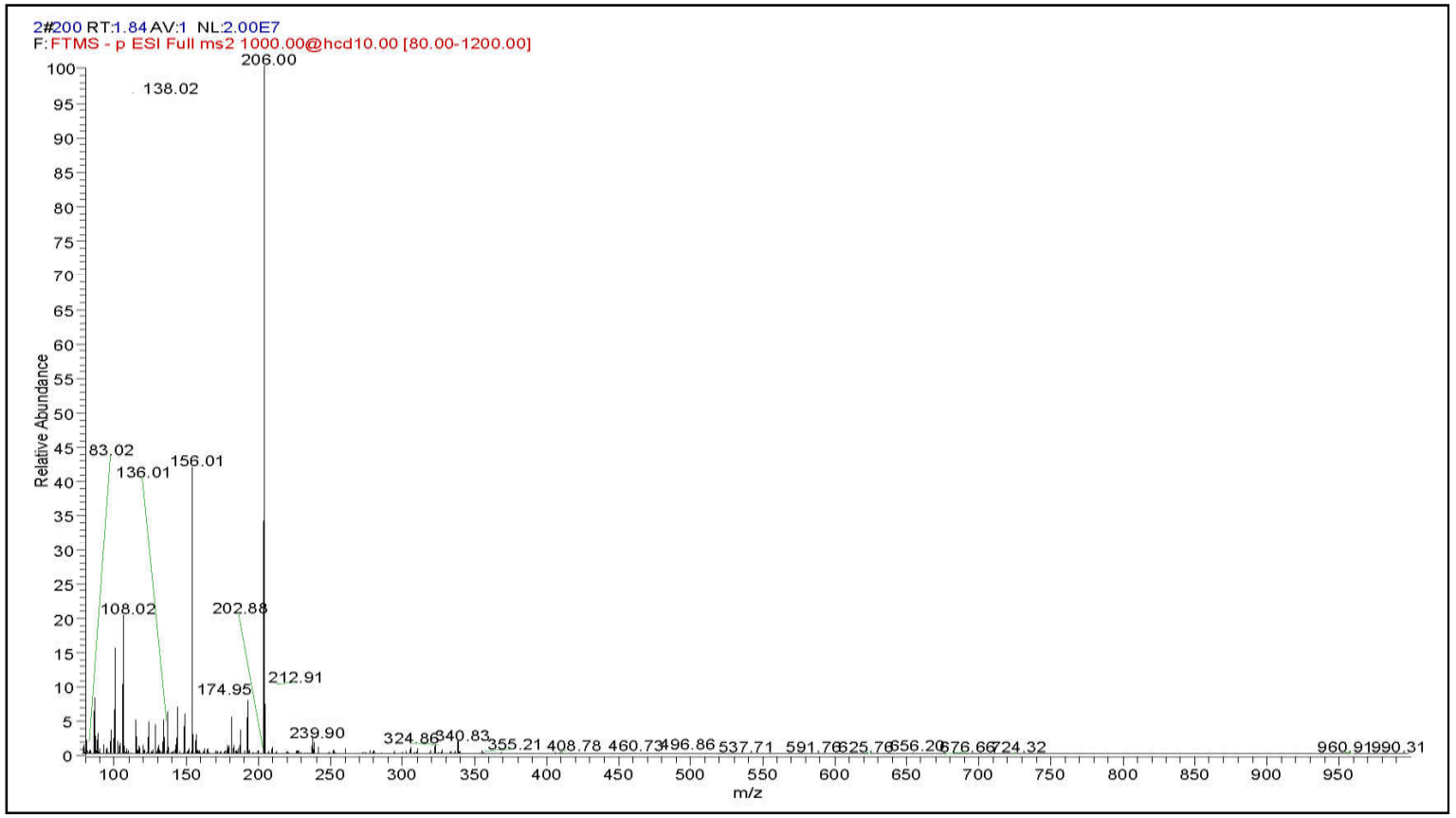
^{1}H NMR spectrum of Fraction III (Compound 2)

PLATE 2.5B



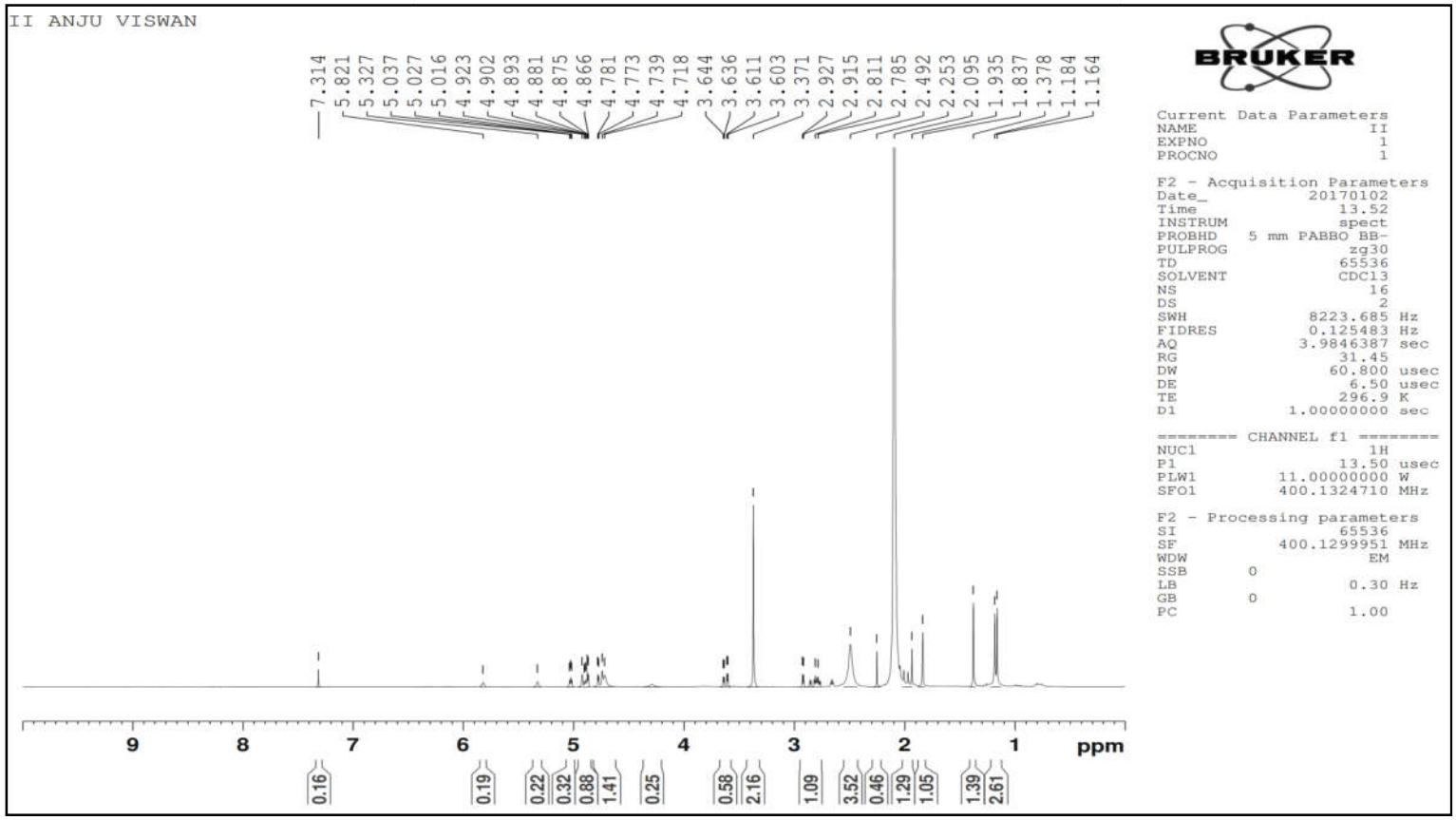
^{13}C NMR spectrum of Fraction III (Compound 2)

PLATE 2.5C



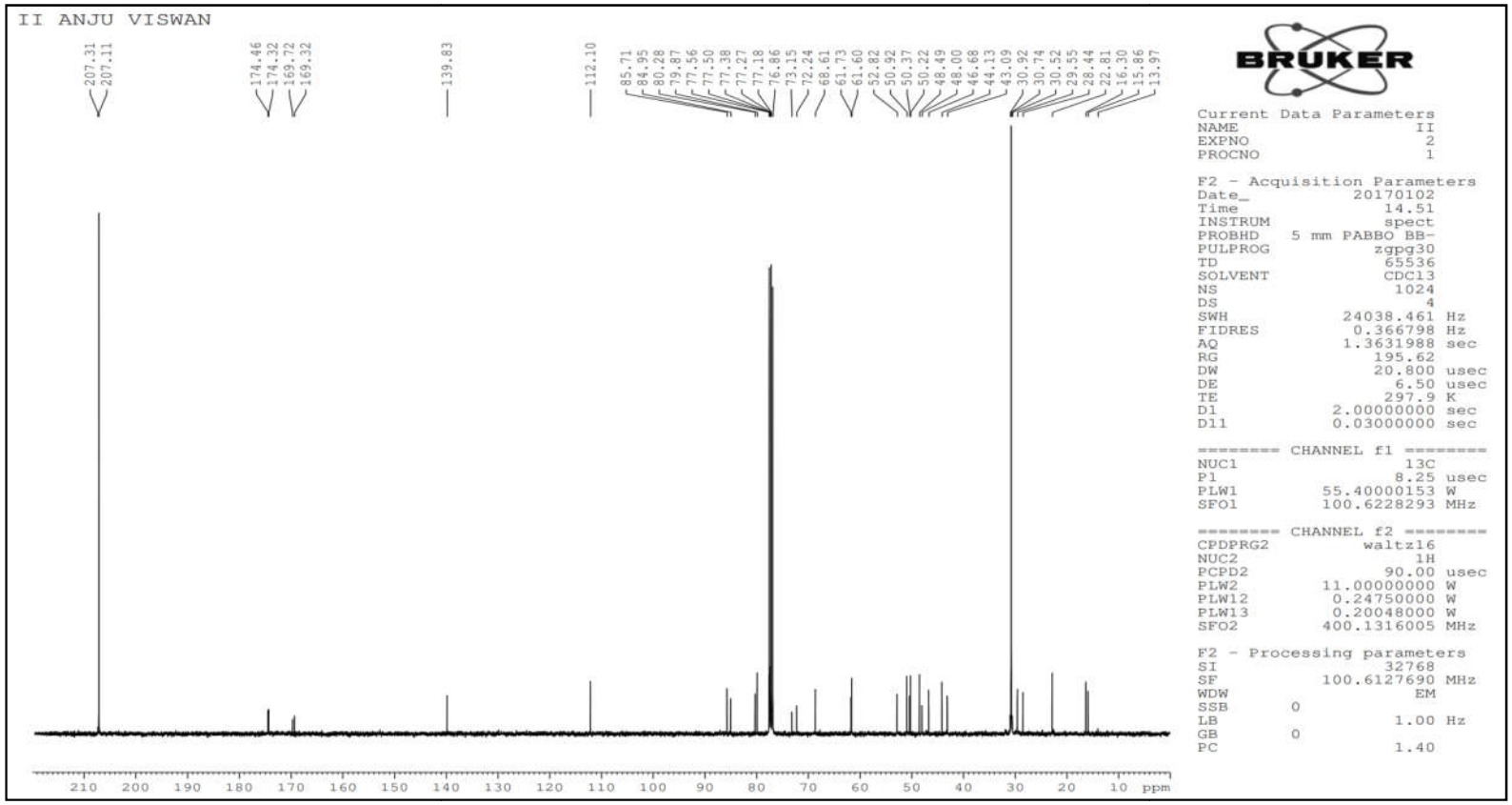
LCMS spectrum of Fraction III (Compound 2)

PLATE 2.6A



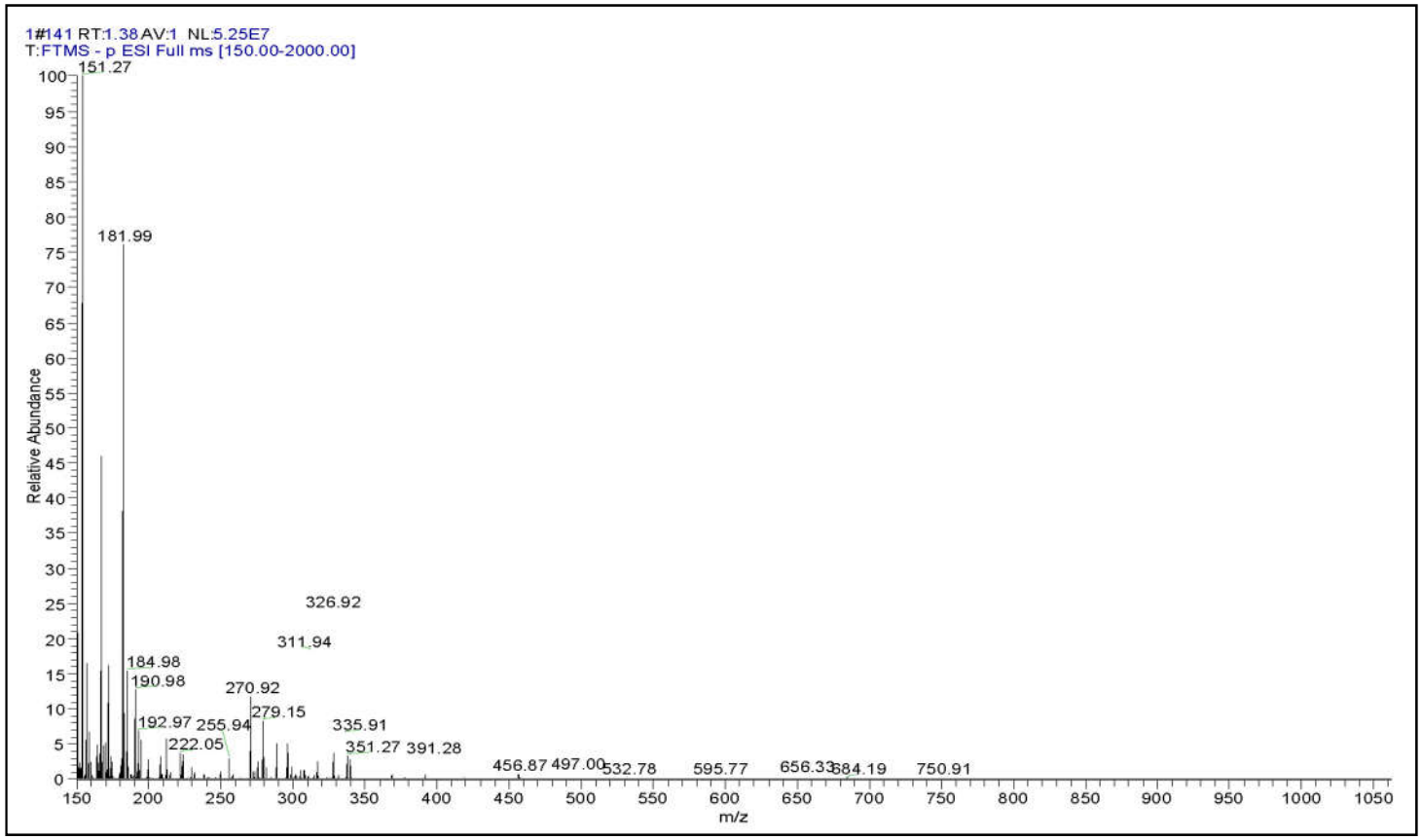
^{1}H NMR spectrum of Fraction II (Compound 3)

PLATE 2.6B



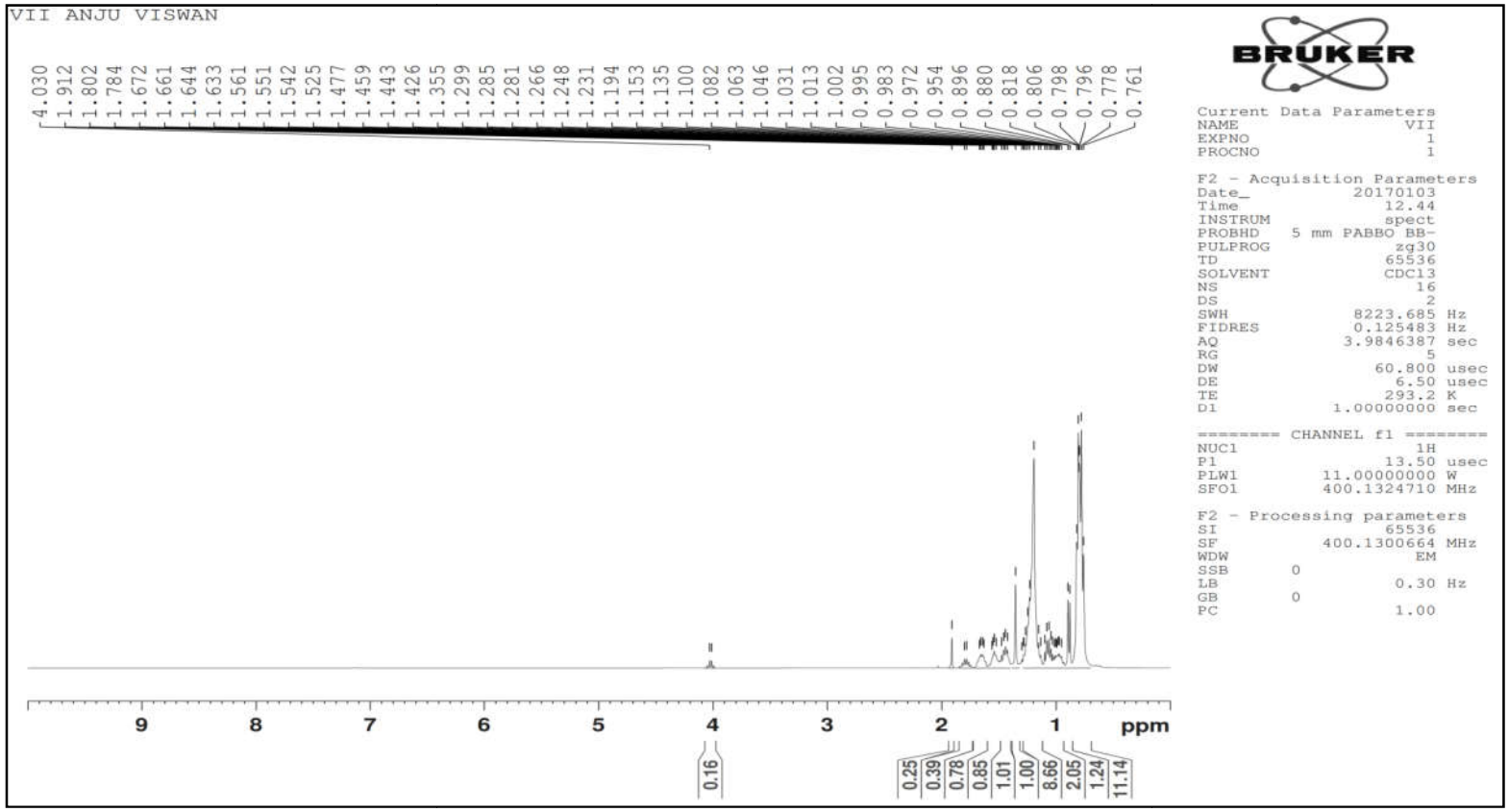
^{13}C NMR spectrum of Fraction II (Compound 3)

PLATE 2.6C



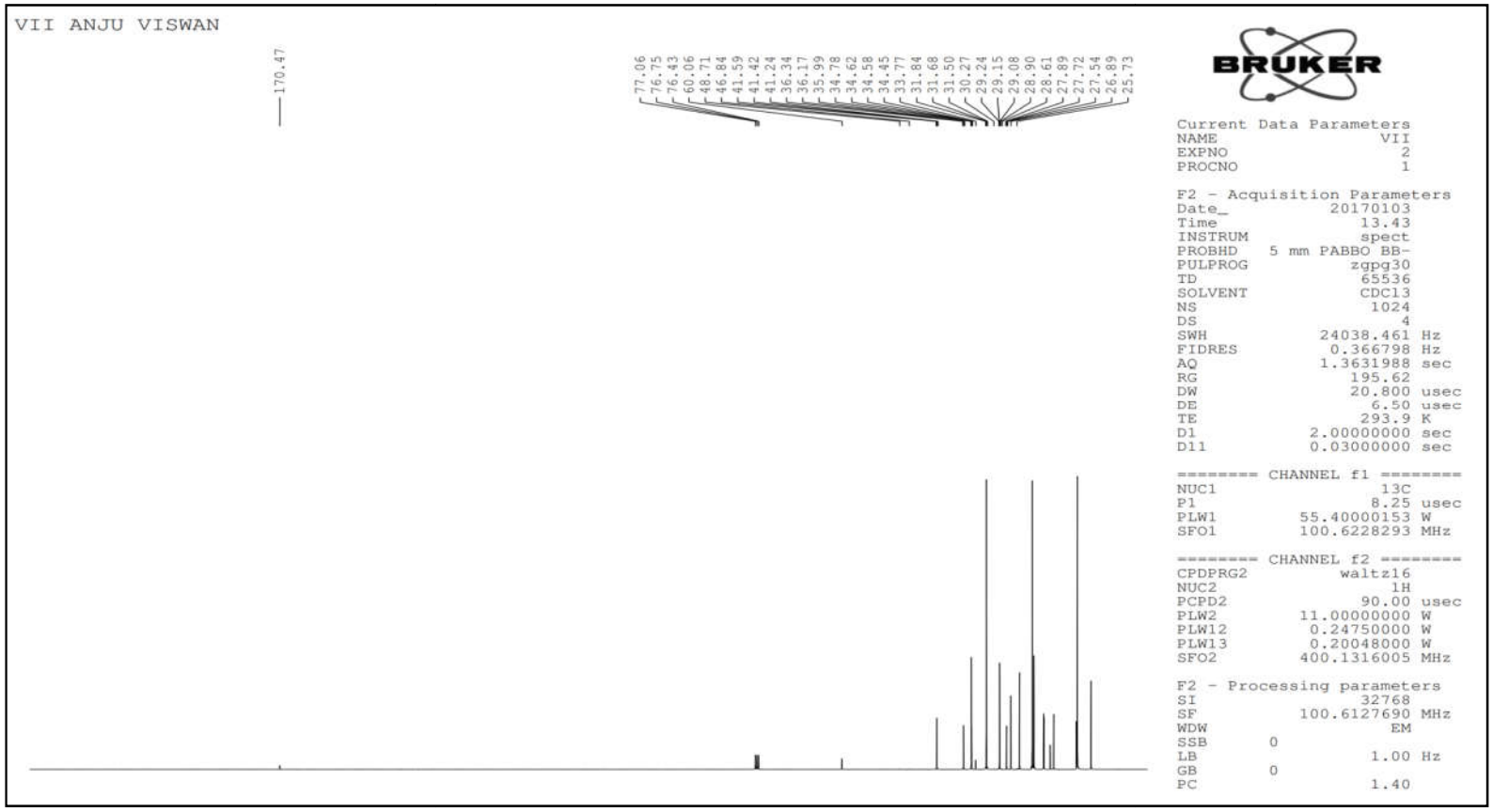
LCMS spectrum of Fraction II (Compound 3)

PLATE 2.7A



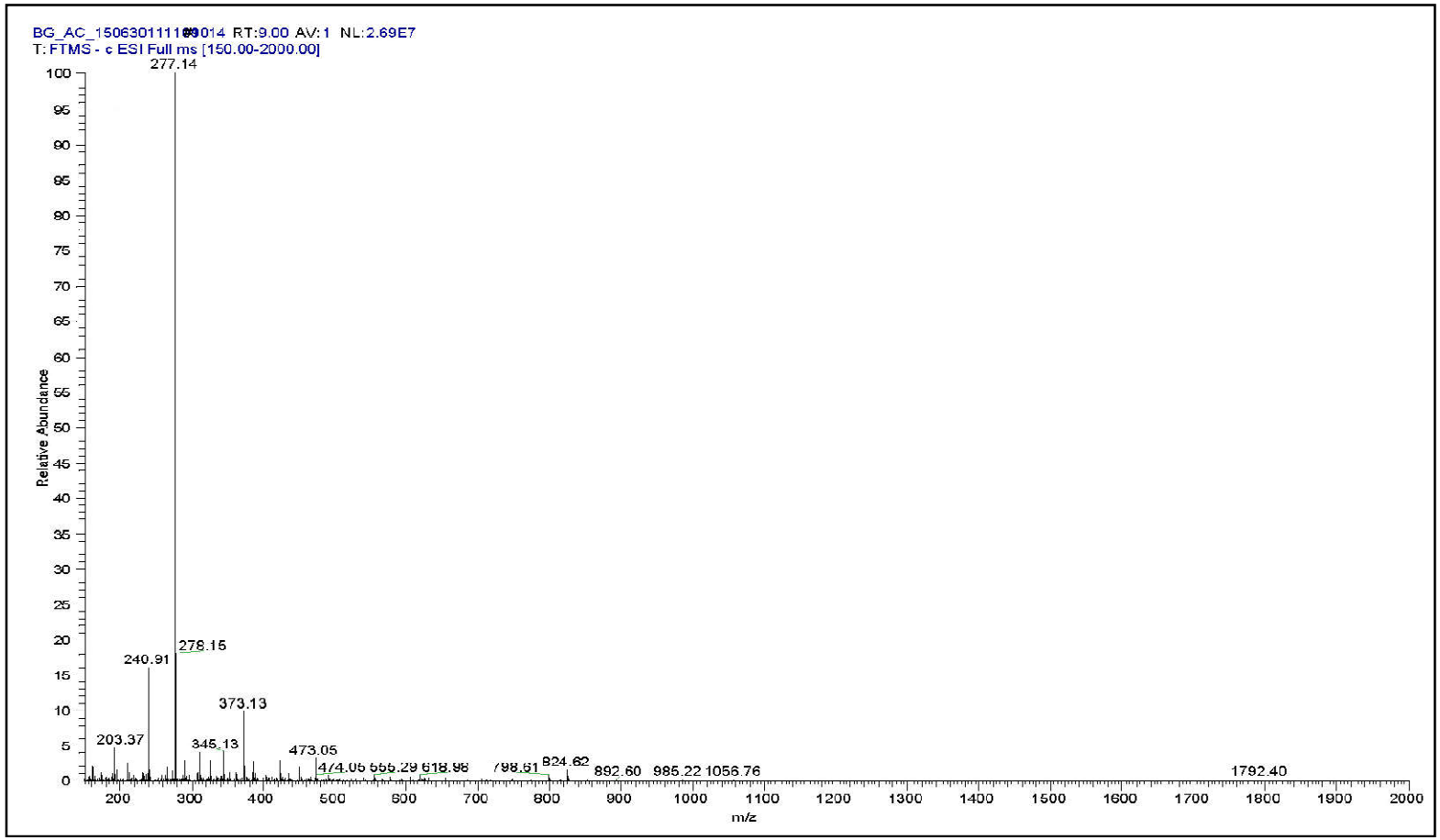
^{1}H NMR spectrum of Fraction VII (Compound 5)

PLATE 2.7B



^{13}C NMR spectrum of Fraction VII (Compound 5)

PLATE 2.7C



LCMS spectrum of Fraction VII (Compound 5)