Larvicidal Efficacy of *Croton bonplandianus* Baill. Extract and its Synergistic Effects with Different Conventional Insecticides against *Aedes aegypti* (Linnaeus, 1762) , a Dengue Fever Vector

Thesis submitted in partial fulfilment of the requirements for the Degree of

DOCTOR OF PHILOSOPHY IN ZOOLOGY

Under the Faculty of Science

University of Calicut

by

LAKSHMI K.V.

Under the supervision of

Dr. ANEESH E.M.
(Guide)
&

Dr. SUDHIKUMAR A.V.
(Co-Guide)

CHRIST COLLEGE (AUTONOMOUS)

IRINJALAKUDA - 680125, KERALA

DECEMBER 2023

DECLARATION

I, LAKSHMI K.V., hereby declare that the work embodied in the thesis **"Larvicidal Efficacy of *Croton bonplandianus* Baill. Extract and its Synergistic Effects with Different Conventional Insecticides against *Aedes aegypti* (Linnaeus, 1762) , a Dengue Fever Vector"**, submitted to the University of Calicut in partial fulfilment of the requirements for the Degree of Doctor of Philosophy in Zoology is a bonafide record of the research work carried out by me under the supervision of Dr. Aneesh E.M., (Guide), Assistant Professor, Department of Zoology, University of Calicut, and Dr. Sudhikumar A.V., (Co-Guide), Assistant Professor, Head of the Department of Zoology, Christ College (Autonomous), Irinjalakuda. No part of the thesis has formed the basis for the award of any degree, diploma or other similar titles of any university.

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This is to certify that the thesis entitled **"Larvicidal Efficacy of *Croton bonplandianus* Baill. Extract and its Synergistic Effects with Different Conventional Insecticides against *Aedes aegypti* (Linnaeus, 1762) , a Dengue Fever Vector"**, submitted to the University of Calicut for the award of the degree of Doctor of Philosophy in Zoology, is an authentic record of the work done by **Ms. LAKSHMI K.V.,** under my supervision and guidance and no part of the thesis has formed the basis for the award of any degree/diploma or other similar titles of any University.

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CERTIFICATE

This is to certify that **Ms. LAKSHMI K.V.,** has completed the research work for the full period prescribed under the Ph.D. ordinance of the University of Calicut. This thesis **"Larvicidal Efficacy of *Croton bonplandianus* Baill. Extract and its Synergistic Effects with Different Conventional Insecticides against *Aedes aegypti* (Linnaeus, 1762) , a Dengue Fever Vector"**, embodies the results of her investigations conducted during the period at which she worked as a research scholar. I recommend the thesis to be submitted for the evaluation for the award of the degree of Doctor of Philosophy in Zoology of the University of Calicut.

PRINCIPAL

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3.	Name of the supervisor	Dr. ANEESH E.M. (Guide) Dr. SUDHIKUMAR A.V. (Co-guide)		
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LAKSHMI K.V.

Dedication

This thesis is dedicated to my mother. Your altruism has become the catalyst for every single triumph of my life, and I sense satisfaction in achieving my dreams, realizing that they are an extension of yours. Thank you for being the cornerstone of my accomplishments.

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ABBREVIATIONS

Ae. - Aedes

An.- Anopheles

Ar.- Armigeres

Cx.- Culex

Lt.- Lutzia

Ma. – Mansonia

E. coli- Escherichia coli

S. aureus- Staphylococcus aureus

K. pneumoniae-Klebsiella pneumoniae

P. vulgaris-Proteus vulgaris

°E- Degree East Longitude

°N- Degree North Latitude

AChE- Acetylcholine Esterase

ANOVA- Analysis of Variance

ArcGIS- Aeronautical Reconnaissance Coverage Geographic Information System

BLAST- Basic Local Alignment Search Tool

BOLD- Barcode of Life Data System

bp- Base pair

BSA- Bovine Serum Albumin

CB1- Plant isolated Eicosane

CDNB-GSH -1-chloro-2,4-dinitrobenzene

CDRL- Communicable Disease Research Laboratory

CO1- Cytochrome Oxidase Subunit1

CTC- Co-toxicity Coefficient

DDT- Dichlorodiphenyltrichloroethane

DHF- Dengue hemorrhagic fever

dhs.kerala.gov- Directorate of Health Services Kerala Government

Dionex ASE 150- Dionex Accelerated Solvent Extractor

DMSO- Dimethyl sulfoxide

DNA- Deoxyribonucleic Acid

dNTP- Deoxynucleoside triphosphate

DSS- Dengue Shock Syndrome

DTNB- 5,5′-Dithiobis (2-nitrobenzoic acid)

DTT- Dithiothreitol

DV- Dengue Virus

EDTA- Ethylenediaminetetraacetic acid

EtBr- Ethidium Bromide

GABA- Gamma- Aminobutyric Acid

GCMS- Gas Chromatography-Mass Spectrometry

GIS- Geographical Information System

GPS- Global Positioning System

GST- Glutahione -s- transferase

HTML- Hypertext Markup language

IGR- Insect Growth Regulator

JE- Japanese Encephalitis

JEV- Japanese Encephalitis Virus

KH_2PO_4- Potassium phosphate

L/D- Light/Dark

LC- Lethal Concentration

MEGA- Molecular Evolutionary Genetics Analysis

MFO- Mixed- function oxidases

$MgCl_2$- Magnesium Chloride

mtDNA- Mitochondrial DNA

NADPH- Nicotinamide adenine dinucleotide phosphate

NaOH- Sodium hydroxide

NCBI- National Center for Biotechnology Information

NJ- Neighbor joining

NMR- Nuclear Magnetic Resonance

OC- Organo Chlorine

OD- Optical Density

OP-Organophosphate

p.adj- p adjusted value

PAST- Paleontological Statistics Software Package for Education and Data Analysis

PBO- Piperonyl butoxide

PCR- Polymerase Chain Reaction

pH- Potential of Hydrogen

PMSF- Phenylmethylsulfonyl fluoride

Py-Pyrrethroids

RNA- Ribonucleic Acid

rpm- Revolutions per minute

RR- Resistance Ratio

SC1- Synergistic Compound of Malathion and Eicosane

SC2- Synergistic Compound of Temephos and Eicosane

SC3- Synergistic Compound of Cypermethrin and Eicosane

SC4- Synergistic Compound of Lambda-cyhalothrin and Eicosane

SDS- Sodium dodecyl sulphate

SF- Synergistic Factor

SPSS- Statistical Package for the Social Sciences

TAE- Tris-acetate EDTA

TCA- Trichloroacetic acid

TLC- Thin Layer Chromatography

USA- United States of America

UV- Ultraviolet

WHO- World Health Organization

WNV- West Nile Virus

YF- Yellow fever

Ppm- Parts per million

mg- Milligram

min- Minute

ml - Millilitre

µl – Microliter

ABSTRACT

Mosquitoes, found ubiquitously across various regions of the world, play a pivotal role in transmitting many devastating diseases. Their significance as vectors has stimulated the alarming rise in mosquito-borne diseases as they are responsible for carrying and transmitting pathogens to humans and animals. The most significant health risks associated with mosquito vectors include dengue, malaria, chikungunya, yellow fever, zika virus infection, filariasis, and West Nile virus infection. These diseases collectively contribute to extensive global disease and mortality rates, with millions of cases reported annually. Accurate identification of mosquito vectors in specific regions is crucial for strategizing effective disease management and resource allocation as it enables early detection and targeted response measures, reducing disease impact.

For decades, Kerala has faced persistent intimidation of mosquito-borne diseases. Therefore, it becomes imperative to precisely identify, map, and document the vector mosquito population and the factors that drive their proliferation. The first objective of this study was to identify the important vector mosquito species within selected areas that represented urban and semi-urban regions of the Thrissur district in Kerala, India, using molecular identification techniques. According to the molecular data, a phylogenetic tree was constructed, comparing the genetic relations between the collected vector species with the other NCBI-deposited species from various regions around the globe. This objective presented detailed information on local vector populations, facilitating more focused and effective vector control strategies. This research combined traditional and molecular techniques to recognize mosquito species and utilised GIS technology for species mapping according to their habitat geographic region.

The study also evaluated different diversity indices, including alpha, beta and gamma diversity indices of the collected vector populations. These diversity indices provided an overall awareness of the diversity of selected vector mosquito species within the study area. The molecular identification of mosquito vectors confirmed the existence of 11 vector species of primary and crucial mosquito-borne diseases. The collected vector mosquito species were identified and documented under four different genera, *Anopheles, Aedes, Armigeres* and *Culex,* within the study area. The selection

of *Ae. aegypti*, as the experimental species for larvicidal activity studies was influenced by understanding the burden of disease associated with this vector mosquito and its role in disease transmission.

The next objective of the study involved screening various locally available plant extracts against *Ae. aegypti* vector using organic solvents with increasing polarity. It comprised the identification and isolation of bioactive compound within effective phyto-extract, and the evaluation of the susceptibility of fourth-instar larvae of *Ae. aegypti* to the plant extract. This assessment utilised the standard larval bioassay procedure outlined by the World Health Organization (WHO).

The following part of the study involved the larval susceptibility assay, where different conventional insecticides and the plant isolate were tested against *Ae. aegypti* larvae. The bioactive compound, identified as eicosane and termed CB1, and four conventional insecticides, lambda-cyhalothrin, cypermethrin, temephos, and malathion, were chosen for examination. The WHO protocol was followed for assessing larval susceptibility, and plant isolate bioassay, with modifications as needed to meet the specific requirements of the study. The results revealed the susceptibility status of *Ae. aegypti* towards all the tested compounds.

Antimicrobial activities were also considered to determine the efficiency of CB1 to limit the growth of microorganisms. Of the four different bacterial strains tested, the growth of all except one was limited by the plant isolate. This result indicated an add-on advantage of CB1 as it could inhibit certain microbial growth when released to the environment as a larvicide.

The fundamental objective of this study was to evaluate the synergistic impact, as synergy can enhance the efficacy of insecticides when combined with natural compounds like the plant isolate, thus improving the prospects of successful vector control. This cooperative approach also holds the potential to reduce the dependence on chemical insecticides alone, thereby promoting more sustainable vector management practices. Two distinct experimental approaches were established to examine the interplay between the insecticide and plant extract, yielding the Co-toxicity coefficient, CTC and the Synergistic factor, SF. These experiments aimed to gain a deeper understanding of the combined effects of each chemical insecticide with the plant's bioactive compound on *Ae. aegypti* larvae. The CTC analysis assessed the

combined mixture- influenced mortality rates in comparison to expected outcomes, while the evaluation of the SF aimed to explain the degree of synergism or antagonism observed between the insecticide and the plant isolate. These investigations provided a better perceptive of the interactions among these substances and their aptitude for successful vector control tactics. The results demonstrated that all combinations exhibited a synergistic effect on the test species, with the eicosane-lambda cyhalothrin combination, SC4, displaying the most pronounced impact.

SC4 was chosen for further analysis to investigate the possibility of resistance development in the laboratory reared *Ae. aegypti*. This involved quantitative assay of the detoxifying enzymes of the selected generations of *Ae. aegypti*, which had been raised through exposure to this compound for five consecutive generations and comparing their detoxifying enzyme activity with that of the susceptible strain. Bioassay experiments adhered to the standard WHO method and the Resistance Ratio (RR) assessment was also conducted following the WHO protocol. The derived lethal concentration (LC_{50}) values indicated that although there was a minor rise in LC_{50} values with the progression of generations, it did not reach a level indicative of resistance development against SC4, and the *Ae. aegypti* strain remained susceptible even after five generations.

Quantitative enzyme assays were also performed to analyse the mode of action of crucial detoxifying enzymes in a laboratory-reared susceptible strain of *Ae. aegypti* when exposed to the synergistic compound, SC4 and the plant isolate CB1 over different time intervals of 24, 48, and 72 hours. Specific enzyme activities of Acetylcholinesterase, Carboxylesterase, Glutathione S-transferase and Cytochrome P450 were evaluated along with the total protein concentration. The results consistently demonstrated a reduction in the activity of the tested detoxifying enzymes throughout all treated generations, implying a potential barrier to the rapid development of resistance to these compounds.

Keywords: Mosquito, *Ae. aegypti*, Barcoding, Larvicide, Synergy, Insecticides, Phyto- extract.

സംഗ്രഹം

ലോകത്തിന്റെ വിവിധ ഭാഗങ്ങളിൽ സർവ്വവ്യാപിയായി കാണുന്ന രോഗാണുവാഹകരായ കൊതുകുകൾ, ഡങ്കി, ചിക്കുൻഗുനിയ, മലേറിയ, സികവൈറസ് അണുബാധ, മന്ത് തുടങ്ങിയ മാരകമായ സാംക്രമിക രോഗങ്ങൾ മനുഷ്യരിലേക്കും മൃഗങ്ങളിലേക്കും പകർത്തുന്നതിൽ പ്രധാന പങ്ക് വഹിക്കുന്നു. ആഗോളതലത്തിൽ ഈ രോഗങ്ങൾ വിപുലമായ മരണനിരക്കിനു കാരണമാകുന്നത് നിമിത്തം ഇവയുടെ ഫലപ്രദമായ നിയന്ത്രണം ആരോഗ്യമേഖലക്ക് അനിവാര്യമാണ്.

കേരളത്തിലെ തൃശ്ശൂർ ജില്ലയിലെ തിരഞ്ഞെടുത്ത നഗര, അർദ്ധനഗര പ്രദേശങ്ങളിലെ പ്രധാന രോഗ വാഹക കൊതുകുകളുടെ തന്മാത്രാതലത്തിലുള്ള വർഗ്ഗീകരണവും അതിലൂടെ സാന്നിധ്യം, സാന്ദ്രത, വൈവിധ്യം എന്നിവയുടെ രേഖപ്പെടുത്തലും ആയിരുന്നു ഈ പഠനത്തിന്റെ ആദ്യലക്ഷ്യം. ഈ മേഖലയിൽ നിന്നും 11 വ്യത്യസ്ത സ്പീഷീസുകളിൽ പെട്ട പ്രധാന രോഗവാഹകകൊതുകുകളെ തിരിച്ചറിയാനും വിവരങ്ങൾ രേഖപ്പെടുത്താനും സാധിച്ചു. ഇത് ഏഡീസ് ഈജിപ്തി ഡെങ്കുരോഗാണുവാഹക കൊതുകിനെ അതുമായി ബന്ധപ്പെട്ട രോഗത്തിന്റെ നാശകശേഷിയുടെ അടിസ്ഥാനത്തിൽ ലാർവിസൈഡൽ പരീക്ഷണത്തിനായി തെരഞ്ഞെടുക്കാൻ സഹായകമായി.

പ്രാദേശികമായി ലഭ്യമായ വിവിധ സസ്യങ്ങളുടെ എക്സ്ട്രാക്ടിന്റെ ലാർവിസൈഡൽ സാധ്യത ഏഡീസ് ഈജിപ്തി കൊതുകിനെതിരെ പരീക്ഷിക്കുന്നതായിരുന്നു ഈ പഠനത്തിന്റെ അടുത്തലക്ഷ്യം. ഇതിനായി വിവിധ സ്പീഷിസിൽ പെടുന്ന മുപ്പതോളം സസ്യങ്ങളുടെ ഭാഗങ്ങൾ ശേഖരിക്കുകയും അവയുടെ എക്സ്ട്രാക്ട് വേർതിരിച്ചെടുക്കുകയും അവയിൽ ക്രോട്ടൺ ബോൺപ്ലാൻഡിയാനസ് ചെടിയുടെ ഇലകളുടെ എക്സ്ട്രാക്ടിന്റെ മികച്ച ആകൃവിറ്റി മനസിലാക്കുകയും ചെയ്തു. ഇതിൽ തന്നെ ഇകോസൈൻ എന്ന സംയുക്തമാണ് ഈ ചെടിയുടെ ലാർവിസൈഡൽ ആകൃവിറ്റിക്ക് പ്രധാന കാരണം എന്നു മനസിലാക്കി വേർതിരിച്ചെടുത്തു.

നിലവിലെ പഠനത്തിന്റെ അടുത്ത ഘട്ടത്തിൽ ഈ വേർതിരിച്ചെടുത്ത സംയുക്തത്തിന്റെയും മറ്റു നാലു രാസകീട നാശിനികളായ മാലത്തിയോൺ, ടെമഫോസ്, സൈപർമെത്രിൻ, ലാംഡസൈഹാലോത്രിൻ എന്നിവയുടെയും ലാർവിസൈഡൽ ആകൃവിറ്റി ഏഡീസ് ഈജിപ്റ്റി കൊതുകുകൾക്കെതിരെ പരീക്ഷിക്കുകയും അവയുടെ ലീതൽ കോൺസെൻട്രേഷൻ നിരക്ക് രേഖപ്പെടുത്തുകയും ചെയ്തു.

പ്ലാന്റ് ഐസോലേറ്റ് പോലുള്ള പ്രകൃതിദത്ത സംയുക്തങ്ങളുമായി സംയോജിപ്പിക്കുമ്പോൾ കീടനാശിനികളുടെ ഫലപ്രാപ്തി വർദ്ധിപ്പിക്കാൻ സാധിക്കും എന്ന സിനർജി എന്ന ആശയത്തിന്റെ പരീക്ഷണമായിരുന്നു ഈ പഠനത്തിന്റെ അടിസ്ഥാനലക്ഷ്യം. ഇതിനായി, ഇകോസെയിൻ, മറ്റു നാലു രാസകീടനാശിനികളുമായി പ്രത്യേക അനുപാതത്തിൽ സംയോജിപ്പിക്കുകയും അവയുടെ ലാർവിസൈഡൽ ആകൃവിറ്റി പരിശോധിക്കുകയും ചെയ്തു. അതിൽ നിന്നും ലഭിച്ച കോ-ടോക്സിസിറ്റി കോ-എഫിഷ്യന്റെയും സിനർജിസ്റ്റിക് ഫാക്ടറിന്റെയും ഫലങ്ങൾ താരതമ്യപഠനത്തിന് വിധേയമാക്കിയപ്പോൾ അതിൽ സിനർജിക് മിശ്രിതങ്ങൾ കൊതുകിന്റെ മരണനിരക്കിനെ കൂടിയ നിരക്കിൽ സ്വാധീനിച്ചതായി കാണപ്പെട്ടു. ഇതിൽ തന്നെ ലാംഡസൈഹാലോത്രിൻ ആയുള്ള മിശ്രിതമാണ് കൂടുതൽ കാര്യക്ഷമത കാഴ്ചവെച്ചത് എന്നതിനാൽ ആ സംയുക്തത്തെ ലബോറട്ടറി കൾച്ചർ ചെയ്ത ഏഡീസ് ഈജിപ്റ്റിയുടെ രാസനാശിനിക്കെതിരായുള്ള പ്രതിരോധ വികസനത്തിന്റെ സാധ്യതകൾ വിലയിരുത്താനായി തെരഞ്ഞെടുത്തു. ഇതിനായി ഡീടോക്സിഫിക്കേഷൻ എൻസൈമുകളുടെ ക്വാന്റിറ്റേറ്റീവ് അസെ ആണ് പരീക്ഷിച്ചത്. എന്നാൽ തുടർച്ചയായ ഈ മിശ്രിതം ഉപയോഗിച്ച അഞ്ചാമത്തെ തലമുറയിൽ പോലും പ്രതിരോധ വികസനത്തിന്റെ അസാന്നിധ്യം രേഖപ്പെടുത്തുകയും അതിലൂടെ ഈ മിശ്രിതം പ്രതിരോധത്തിന്റെ ദ്രുതഗതിയുള്ള വികസനത്തെ തടയുന്നതായും മനസ്സിലാക്കാൻ സാധിച്ചു.

GENERAL INTRODUCTION

Mosquitoes are a humongous group of insects observed throughout the world's temperate and tropical regions and beyond the Arctic Circle. They belong to the Culicidae family of the order Diptera (Harbach and Kitching 2016). They are recognized as one of the most pertinent groups of arthropods in the public health field, as they can act as vectors for many lethal disease parasites. A total of 3,719 species of mosquitoes, divided into 113 genera and two subfamilies, are currently recognized, with many more species still in need of confirmation (Harbach, 2023). More findings and nomenclature of sibling species, primarily in *Anopheles*, as a result of the use of DNA-based methods, can be expected to result in a three to fivefold increase in current numbers (Harbach and Besansky, 2014). After Brazil, Indonesia, Malaysia, and Thailand, India stands fifth in terms of mosquito biodiversity (Foley et al., 2007).

Many species of mosquitoes are crucial to tropical medicine and public health because they carry pathogens of infectious diseases, such as nematodes, protozoans, and arboviruses, which can cause serious harm to humans. These diseases are particularly concerning tropical and temperate countries and are considered as a significant public health risk (Rocklöv and Dubrow, 2020). According to WHO reports, vector-borne diseases, including those transmitted by mosquitoes, make up over seventeen percent of all infectious diseases in humans and lead to approximately 700,000 deaths each year (WHO, 2014; Chaiphongpachara et al., 2022).

Biology

Based on their characteristics, general morphology, and lifecycle, mosquitoes are organised into two subfamilies: Anophelinae, and Culicinae. Both Culicine and Anophelinae subfamilies comprise blood-feeding female mosquitoes. Unlike these, the Toxorhynchitinae females do not need blood to complete their life cycle, and their general morphology excludes bloodsucking mouthparts (Cauich-Kumul et al., 2018). The general distinguishing morphological characteristics of the Culicinae subfamily include long antennae and a proboscis with shorter maxillary palpi, which are adapted for piercing and sucking fluids from plants and animals. In comparison, the palpi and

proboscis of the Anophelinae subfamily are approximately equal in length (Resh and Carde, 2009).

The scutellum is the posterior portion of either the mesonotum or the metanotum of the thorax region that can be considered a distinguishing characteristic among different subfamilies of mosquitoes. In mosquitoes, it is seen as a small, plate-like structure on the dorsal side of the thorax. The scutellum of the Anophelinae subfamily has a rounded or semi-lunar shape and is comparatively flat without any lobes, whereas the Culicinae subfamily has a distinct triangular shape due to its trilobed structure. The scales on the wings of mosquitoes are another important feature used in the conventional morphologic classification. The Anophelinae mosquitoes has ornamental wings with pale and dark scales arranged in a distinctive pattern. However, such scales are usually absent in Culicinae subfamily. Even though some members of the Culicinae have ornamental scales on the wings, the scale pattern is only prominent on the abdominal area where the wings are connected to the body part and less on the main wing region. This pattern can also be used to identify different species and subfamilies of mosquitoes (Resh and Carde, 2009; Wilkerson et al., 2021).

Life Cycle

Mosquito is a holometabolous dipteran with four distinct life stages, Egg, larva, pupa and adult. Except for the Adult, which is a free-flying phase, all the other forms are entirely aquatic and require water for their survival. Usually, mosquito species can complete their life cycle and become adult in a relatively short period of 8 to 10 days, depending on several factors that influence their survival and growth, like availability of food, climatic conditions, mosquito species and other environmental factors (Kalman, 2004).

Egg: The female mosquitoes lay eggs, which is the first stage of the mosquito life cycle. The eggs are laid as single eggs or in clusters. Each cluster may contain up to hundreds of eggs. The species coming under *Aedes* and *Anopheles* lay single eggs, while *Culex* and *Culiseta* lay eggs in cluster form. The selection of oviposition sites also shows a wide range of habitats within the subfamilies, such as stagnant water, floating plants, artificial containers, tree holes, or damp areas which are prone to flooding (Rueda et al., 2008). After a few hours of laying, the color of the mosquito

egg shifts from white to dark brown to black. Mosquito eggs vary in shape depending on the species. While some mosquito eggs are boat-shaped, others are ovoid in shape. Typically, their length ranges from 0.5 to 1 mm. In two to three days after being laid, mosquito eggs will hatch into larvae under typical, favourable conditions. Certain species, on the other hand, can endure for several months in hostile, arid conditions before hatching. Several factors, including temperature, humidity, and the availability of nutrients, influence the timing of egg hatching. Once hatched, the larvae undergo several developmental stages before becoming pupae and adult mosquitoes (Hall and Tamïr, 2022).

Larvae: The mosquito larvae undergo four stages of development known as instars before reaching the pupal stage. The body of larvae is divided into three distinct parts: a small head, an elongated thorax, and a tubular abdomen. After hatching, the mosquito larvae start to feed on tiny aquatic plants and animals, such as phytoplankton and zooplankton, as well as organic waste. Certain mosquito larvae have a predatory behaviour and consume other mosquito larvae as food. The larvae can take four to ten days to go through the four instar stages before they moult into the pupae. Mosquito larvae are usually found near the water surface to inhale air. They can draw air in by penetrating the surface film with a siphon that is attached to the end of their body. However, the larvae belonging to the species of *Mansonia* and *Coquilletidida* complex have a unique way of breathing. They have a modified piercing siphon that enables them to attach themselves to plant stems below the water surface to obtain oxygen from the plant tissues (Jackman and Olson, 2002; Becker et al., 2010a; Hall and Tamïr, 2022).

Pupae: Pupae, often referred to as "tumblers," are the stage of development that follows the fourth moult of the larval stage in mosquitoes. During the pupal stage, mosquitoes are almost completely still and only move when disturbed. In this stage, some larval organs disappear, and the development of adult body structure occurs. Unlike larvae, pupae do not consume any food, and they typically survive for a period of 1 to 3 days before emerging as adult mosquitoes. (Jackman and Olson, 2002; Becker et al., 2010a).

Adult: The last stage of metamorphosis is finished, and adult mosquitoes typically emerge 1 to 2 days following the appearance of pupae. Male mosquitoes do not reach sexual maturity upon emergence, which distinguishes them from female mosquitoes,

and consequently, the male population generally emerges a day or two prior to females. Adult mosquitoes typically obtain nourishment from sources such as nectar, plant sap, and other types of carbohydrates. Upon emergence, adult mosquitoes are prepared to commence their life cycle, which involves mating, feeding, and oviposition. In most species, female mosquitoes need to consume a blood meal as a source of protein before they can lay eggs (Jackman and Olson, 2002; Becker et al., 2010a).

Breeding Habitats

Mosquitoes breed in a wide range of aquatic habitats such as standing water, temporary rain pools, marshes, swamps, and other similar environments, with different species preferring different environments. Except for marine ecosystems that have significant levels of salt, mosquitoes can survive in a variety of habitats, including freshwater, brackish water, or any other sort of water (clear, turbid, or contaminated). The breeding sites may be natural or artificial and can vary in size from small containers, like discarded tires, cans, and bottles, to extensive water bodies, for instance, rivers, lakes, and ponds. Several factors, including the availability of water, temperature, humidity, and the presence of suitable organic matter for larval nutrition, determine the breeding habitat of mosquitoes. Mosquitoes usually opt for stagnant water that is rich in organic matter, containing decaying leaves and algae to lay their eggs. The diversity of mosquito species varies depending on the geographical region, and several researchers have investigated mosquito biodiversity (Adeleke et al., 2008; SNR et al., 2011; Dejenie et al., 2011; Gopalakrishnan et al., 2013; Chatterjee et al., 2015).

Vector Status

Despite their small size, mosquitoes play a significant role in the ecosystem as pollinators and vectors of numerous diseases. According to Naddaf et al. (2012), mosquito complexes are responsible for causing severe discomfort and transmitting life-threatening pathogens and parasites like arboviruses, protozoans and nematodes to humans and animals. Due to their capacity to spread mosquito-borne illnesses that affect humans, the *Anopheles*, *Aedes*, *Armigeres* and *Culex* genera of mosquitoes are medically and economically significant (Gubler, 2010). *Culex* mosquitoes are majorly known to spread filariasis, West Nile virus and Japanese encephalitis. *Aedes*

mosquitoes are particularly well known for transmitting deadly diseases like dengue, chikungunya and zika. *Anopheles* mosquitoes, the members of the Anopheline group are primarily responsible for passing on one of the most fatal strains of malaria-causing pathogens, along with several arboviruses and parasites (Reisen, 2010; WHO, 2014).

The rate at which a virus spreads depends on a multitude of factors that interact in a given landscape, including susceptibility, abundance and rate of contact. Differentially distributed patterns of disease transmission may arise because of changes in the number, range, and variety of disease-carrying vectors and hosts. Such patterns might spread the disease repeatedly, which could have catastrophic consequences. The consequences of global changes, including changes in temperature, agricultural management, population expansion, and urbanization, can drastically affect the diversity and complexity of the mosquito population and the frequency of transmission of mosquito-borne illnesses (Sutherst, 2004). The environment and climate substantially impact how vectors, particularly mosquitoes, are distributed both temporally and spatially (Wanji et al., 2009). A productive and effective sustainable mosquito control program by the local authorities depends on their knowledge of the variety and faunal richness in the region. The underlying ecological and environmental changes directly impact the variety and abundance of mosquito species (Radhakrishnan, 2019). It is crucial to enumerate mosquito species diversity to understand the epidemiology of various illnesses. Such methodical investigations based on the spatial distribution of vector mosquitoes will support a more accurate assessment of the risk of transmission of vector-borne illnesses (Sajith et al., 2015).

The Oriental region, which includes India, is said to be one of the world's richest biogeographic regions for mosquitoes, along with the Neotropics. The expansive and abundant foliage topography of India has provided an inclusive array of determinant variables that nourished mosquito breeding and vector-borne disease outbreaks throughout the country. The rich diversity of mosquito species in the Oriental Region can be attributed to several factors, including its tropical climate, which provides ideal conditions for mosquito breeding and proliferation, as well as its diverse range of habitats like forests, wetlands, and urban areas, which offer a variety of niches for different mosquito species. Moreover, the extended history of human habitation and

trade in the area has contributed to the dispersal of mosquito species over various regions. (Gaston and Hudson, 1994).

With its diverse geography and climate, India has consistently been a focal point for transmitting mosquito-borne diseases such as dengue, chikungunya, malaria, Japanese encephalitis, and filariasis (Sabesan et al., 2000; Williams et al., 2000; Mackenzie et al., 2004). Mosquito-related difficulties and outbreaks of diseases transmitted by mosquitoes cause significant and demanding challenges that have profoundly impacted public health, particularly in developing urban areas. These difficulties have proven to be highly detrimental, necessitating immediate attention and practical strategies to alleviate their adverse effects on the well-being of the population (Dale et al., 1998; Palaniyandi, 2012; Dev et al., 2015).

In Kerala, the predominant mosquito species, including *Anopheles*, *Aedes*, *Culex*, and *Armigeres*, serve as carriers for transmitting various vector-borne diseases. The conducive temperature prevailing throughout the year in Kerala provides an optimal environment for the proliferation and survival of disease-carrying vectors, particularly mosquitoes. Additionally, the significant annual rainfall experienced in the state further creates suitable breeding habitats for mosquitoes, amplifying their population and potential for disease transmission. The abundance of breeding sources, such as stagnant water bodies and vegetation, further exacerbates the vulnerability to vector-borne diseases in Kerala (Sumodan, 2012; Balasubramanian and Nikhil, 2013). There has been a marked increase in reported outbreaks related to mosquito-borne diseases in both rural and urban areas of the state, and this trend stances a substantial challenge to the public health sector in Kerala, as highlighted by Vanaja and Sumodan (2019).

Aedes aegypti

Among the thirteen genera within the Culicidae family, the genus *Aedes* poses the most significant public health concern worldwide (Rajesh et al., 2013). The proliferation of *Ae. aegypti* populations have coincided with an apparent rise in the frequency of dengue virus outbreaks worldwide. As rainfall contributes to the proliferation of *Aedes* mosquito populations, the risk of dengue infections is exceptionally high during or after these periods, thereby amplifying the potential for disease transmission (Pandya, 1982; Mackenzie et al., 2004).

Aedes species are frequently spotted in natural and artificial containers that contain uncontaminated and tidy water. The females of *Ae. aegypti* predominantly prefer artificial containers filled with water for oviposition, and it has been observed that the number of eggs laid increases significantly when these containers are dark in colour and contain standing water with a low concentration of decomposing organic matter (Harrington et al., 2008). Ant traps, clay jars, flowerpots, drums, concrete tanks, coconut shells, and abandoned tires are some favoured hatching places (Paupy et al., 2009., Paupy et al., 2010; Dom et al., 2013). Unlike other mosquito genera, *Aedes* lay eggs just above the water surface, and they can withstand desiccation for a long time before hatching into larvae once they encounter water (Fay and Perry, 1965; Reiter 2007). The females enrich the development and persistence of their juvenile forms by choosing oviposition sites of low to moderate encounter with parasites (Zahiri et al., 1997), predators (Pamplona et al., 2009) and competition (Ponnusamy et al., 2008).

The biology of the species explains that one of the key distinguishing features of *Ae. aegypti* is the pattern and placement of silvery-white scales on different body sections. The tori, clypeus, and palpi tips have white patterning that is similar to the pale scales that developed on the central vertical region and stretched into the space between the eyes. Scutellum also has flat, whitish scale patterns. On its frontal aspect, the mid-femur is nearly entirely covered by a long, pale band without extending all the way to the knee. A band of white scales that almost completely covers the front part of the hind femur stretches to the knee area. On segments 1-2 or 1-3 of the mid and hind leg tarsi, there is a thin white band; on segments 1-4, there is a totally white fifth segment. Segments of the abdomen range from brown to black. II-IV or V tergites have light, pale scales at the base, occasionally with vivid spots. I-VII tergites' dorsal views reveal lateral white spots. The larvae have lateral teeth that are substantially distinct and carry 8–12 comb scales. Near the base of the shaft, a single antennal hair appears. In the mesothoracic and metathoracic segments, undivided pleural hairs are moderately developed. A long syphon (longer than the length of the basal diameter) has 3-5 sets of hair that extend over the centre of the syphon from the base. Chitin completely covers the anal segment; the papillae have rounded borders and a double-length fan (Barraud, 1934; Nagpal and Sharma, 1995).

Dengue Fever

Dengue fever is a severe viral illness associated with life-threatening complications. The origins of dengue fever can be traced back to ancient times, as it was described in a Chinese medical encyclopaedia from the Jin Dynasty in AD 992, referred to as "water poison" associated with flying insects. The term "dengue" itself originated from the Swahili phrase Ka-dinga pepo, which translates to "cramp-like seizure." In the 1780s, the first recorded dengue outbreaks occurred simultaneously in Asia, Africa, and North America. Benjamin Rush, in 1789, reported the first clinical case of Dengue during the 1780 Philadelphia outbreak, naming it "break-bone fever" due to the prevalent symptoms of muscle and joint pain (Morens et al., 2013).

Dengue viruses (DV) belong to the Flaviviridae family and have four known serotypes: DV-1, DV-2, DV-3, and DV-4. DV is a positive-stranded RNA virus with three genes encoding structural and seven genes encoding non-structural proteins. Dengue viruses are predominantly transmitted through the bites of mosquitoes, particularly by the species *Ae. aegypti* and *Ae. albopictus*. These mosquitoes act as the primary vectors for the spread of dengue viruses to humans (Halstead, 2008a; Halstead, 2008b).

The WHO classified dengue infection in 1997 into undifferentiated fever, known as Dengue Fever (DF), and Dengue Hemorrhagic Fever (DHF), which are commonly used worldwide, although it was revised in 2009 to uncomplicated and severe fever (Deen et al., 2006). In India, dengue outbreaks have increased since the mid-1990s, particularly in urban areas. After that, it quickly spread to areas that were previously dengue-free, such as Orissa, Arunachal Pradesh, and Mizoram. As per the available data, dengue was initially documented in Madras (Chennai) in 1780, and its first epidemic occurred in Calcutta, Kolkata, in 1963. Since then, the disease has spread extensively throughout India. Large-scale outbreaks of dengue shock syndrome (DSS) and dengue haemorrhagic fever (DHF) started in 1996, first affecting the areas around Delhi and Lucknow before moving across the country (Dar et al., 1999). Since 1956, four serotypes (types one through four) of the dengue virus have been isolated from various parts of India, with DENV2 and DENV3 having the most severe impacts on the Indian subcontinent (Raheel et al., 2011). The overall incidence of dengue cases in the nation has significantly increased since 2001. A few southern states (Maharashtra, Karnataka, Tamil Nadu, and Kerala) and some northern states witnessed dengue

outbreaks in the early 2000s. The disease's geographic distribution has noticeably changed with the increased quantity and severity of cases. The spread of the dengue virus and its carriers has been accelerated by insufficient efforts to control vector mosquitoes (Mutheneni et al., 2017).

Dengue cases, with some resulting in deaths, were initially reported in Kerala in 1997. However, previous records revealed that even before this, the strains DEN-1, DEN-2, and DEN-4 had already been identified in human blood samples. Since 1998, Kerala has experienced a high incidence of dengue (Mutheneni et al., 2017; Karunakaran et al., 2014). Dengue has become a growing concern in Kerala, with increasing reported cases, high co-infection rates, a high mortality rate, and the presence of all four virus serotypes, making it a hyper-endemic region for the disease. In 2003, Kerala emerged as the Indian state with the highest dengue-related mortality, and since then, the reported cases of dengue in this region have been steadily rising (Thenmozhi et al., 2007; Karunakaran et al., 2014; Suresh et al., 2021).

Barcoding

The precise identification and thorough documentation of regional vector species are of utmost importance in implementing effective vector management strategies. With a thorough understanding of the presence and distribution of vector species, it becomes possible to formulate insecticidal interventions specifically targeted at controlling these vectors. This approach not only helps mitigate the risk of pathogen transmission but also ensures the use of appropriate insecticidal combinations that could optimize vector control efforts. Traditionally, the identification of most mosquito species has relied on morphological taxonomy, which involves using physical characteristics as a means of classification, as proposed by Linnaeus. While morphology continues to be the most commonly employed technique for distinguishing adult mosquitoes, it does have certain limitations. The fragility of mosquito scales and their probability of rubbing off or damage during collection is a significant challenge for morphology-based identification. Furthermore, mosquitoes frequently belong to closely related sibling species that exhibit morphological similarities, resulting in the formation of species "complexes." Additionally, larger groups of related species with overlapping morphology further complicate the task of morphological identification (Bortolus, 2008; Wang et al., 2012). The presence of these challenges has led to a shift towards

DNA sequencing, also known as "barcoding," as a reliable method for mosquito identification. This approach involves using concise, standardized gene regions as internal tags for species, enabling quick, efficient, and precise automated identification. The cytochrome oxidase I gene (COI or COX1) in mitochondrial DNA has become a valuable resource for evaluating mosquito biodiversity. The mitochondrial (mt) genome is currently the most studied genomic area in the insect world, outdoing nuclear genomes, including representatives from all insect orders, in contrast to only a few nuclear genomes (Caravas and Friedrich, 2013; Cameron, 2014). This gene provides a universally comparable DNA sequence that can be employed for comparative analysis, making it an essential tool in assessing and studying the diversity of mosquito species (Avise et al., 1987; Naddaf et al., 2012; Paramasivan et al., 2013). The COI barcode region is situated at the gene's 5' end. This region is typically 648 base pairs long and surrounded by conserved regions, which are used as a basis for designing PCR primers. The COI barcode region is characterized by significant differences in genetic distance within species (usually less than 3%) compared to that between species (usually 10-25%) (Hebert et al., 2003a; Hebert et al., 2003b; Ratnasingham and Hebert, 2007). For this reason, it acts as a logical initial step for the identification of mosquitoes (Beebe, 2018).

Diversity

Gaining insights into the diversity and distribution of mosquito vectors of different species within a specific area is crucial for establishing effective methods of vector monitoring and control, as highlighted by Rueda (2008). Utilizing the concepts of mosquito diversity (alpha diversity) and community structure in which they coexist (beta diversity) can provide valuable information for developing more efficient population control programs. Alpha diversity (α) represents the diversity within a single community in a homogeneous area under investigation. Beta diversity (β) refers to the variation in composition between different communities across a landscape. The combined diversity of these community clusters, resulting from the interaction between alpha and beta diversity, is known as gamma diversity (γ), as defined by Whittaker (1972) and Magurran (1988). Utilizing diversity indices, including alpha, beta, and gamma diversity, allows the overall analysis of the influence of climate, physical and biological factors, and human activities on biodiversity. These indices provide a quantitative framework for assessing how these

factors shape mosquito species composition and distribution within a particular ecosystem. The exploration of these relationships aids in understanding the intricate dynamics between environmental variables and mosquito diversity, thus facilitating the development of targeted strategies for effective mosquito population control and management (Piovezan et al., 2013; Nikookar et al., 2015)

Vector Control

Mosquito control strategies can be categorized into three primary methods: physical, chemical, and biological. Physical methods include actions that reduce mosquito breeding habitats or make them less hospitable for mosquitoes to lay eggs, such as draining stagnant water and removing objects that collect water. Chemical methods involve using insecticides to kill adult mosquitoes or their larvae. On the other hand, biological methods use natural predators or bacteria to control the mosquito population (Pates and Curtis, 2005; Golding et al., 2015).

Chemical Insecticides

The World Health Organization classified chemical pesticides into four different categories: I. Organochlorines (OC), II. Organophosphates (OP), III. Carbamates, and IV. Pyrethroids (Leng et al., 1997). Pesticides manufactured from organic molecules containing chlorine atoms are classified as OCs. Pesticides classified as OPs belong to the second group and have phosphorus as a primary component. The third class of chemical insecticides are carbamates. These substances, made from carbamic acid, are frequently applied to manage nematodes, mites, and insects. Pyrethroids, artificial pesticides made from natural substances present in chrysanthemum flowers, comprise the fourth category. Because pyrethroids work well against a variety of pests, they are frequently used in both household and agricultural settings (Casida and Quistad, 1998).

In 1892, an inventive milestone was achieved with the discovery of dinitro-o-cresol, the first synthetic organic insecticide. Over the following decades, a diverse array of comparable compounds emerged through synthesis, although with limited application in mosquito control initiatives. Subsequent advancements in insecticide research led to the development of more sophisticated and efficacious products, exemplified by dichlorodiphenyltrichloroethane (DDT) and malathion. Nevertheless, the unrestrained and inappropriate utilisation of these chemical agents has propelled the evolution of

insecticide resistance among mosquitoes, creating a formidable challenge to their efficacy (Cremlyn, 1978). From the 1920s onward, insecticides were the preferred method of controlling insect pests because of their greater efficacy regarding pest management. Mosquito control has been primarily connected with the use of synthetic insecticides ever since organic insecticides were pioneered. These synthetic insecticides, such as DDT and malathion, were widely used for mosquito control due to their efficacy in killing both adult mosquitoes and their larvae (Lee and Yap, 2003). DDT was also utilised in agriculture to manage pests like potato beetles and boll weevils. However, worries about how it would affect the environment surfaced. DDT is difficult to decompose and remains persistent in the environment. Its ability to travel over long distances through the air and water, accumulate in the tissues of animals and humans, and affect human health and non-target species like fish and birds raised concern. Research revealed that DDT may be carcinogenic to humans and was connected to issues with development and reproduction in birds and other animals. As worries about DDT increased, substitute pesticides were created. The development of OP in Germany quickly followed, starting in 1932 when Lange and von Krueger created organofluorophosphate esters for the first time, which quickly took the place of DDT in many applications. Although OPs degrade more quickly in the environment and do not accumulate as much in the food chain as DDT, they are more toxic to insects. OPs are also harmful to humans and can have significant negative health effects, especially for those who work with them. As an alternative to DDT and OPs, carbamates were created in the 1950s and gained popularity in the 1960s (Cremlyn, 1978).

The 1960s and 1970s observed a significant change in the way insecticides were developed and governed. During this time, the discovery and development of photostable pyrethroids had a significant impact on insecticide administration. Photostable pyrethroids represent a group of synthetic insecticides that are structurally modelled after the chemical composition of natural pyrethrins, originating from the blossoms of chrysanthemum flowers. These compounds are known for their fast-acting and potent insecticidal properties and are highly effective against a wide range of insects. For over two hundred years, it has been known that pyrethrum flowers and their extracts contain insecticidal properties in the form of pyrethrins and have been documented in various sources (McLaughlin, 1973; Casida,1980; RUIGT, 1985;

Proudfoot, 2005; Ensley, 2018). Over the past several decades, pyrethroids and pyrethrins have risen to prominence as a primary group of insecticides employed to combat pests in agricultural fields, gardens, and homes. They currently represent a substantial portion, making up 25 per cent, of the worldwide insecticide market. Pyrethroids have been adopted as an alternative to extremely toxic and persistent organochlorine and organophosphorus pesticides, as noted in the study conducted by Katsuda in 1999. From 1940 to 1970, several synthetic compounds, known as pyrethroids, were created and subjected to testing as analogues of natural pyrethrins. During this timeframe, the research and development of pyrethroids were predominantly led by Japan and the United Kingdom (Elliott et al., 1973; Elliott, 1980). The discovery of these compounds revolutionized the field of insecticide administration and paved the path for the formulation of safer and more efficient insecticides. Notably, specific synthetic pyrethroid compounds like allethrin, tetramethrin, and resmethrin exhibited remarkable insecticidal characteristics, leading to their successful commercial production as insecticides (Gajendiran and Abraham, 2018).

The global utilisation of synthetic insecticides for vector control, which involves controlling and managing disease vectors, including mosquitoes, has been well-documented in research conducted by Becker et al. (2010b). Reportedly, the annual usage of 547 tonnes of active OCs, 437 tonnes of OPs, 24 tonnes of carbamates, and 162 tonnes of pyrethroids were recorded for vector control purposes. These formulations have been widely used to prevent vector species successfully for several decades. Their primary method of action is to interfere with the nervous system of insects, which is analogous to the nervous system of mammals. Insects are extremely susceptible to even minute concentrations of pesticides, which can lead to their mortality because of their small size and quick metabolism. Even though these dosages might not be fatal to humans, risks could still be involved. Because non-pest insects, humans, animals, and pets share similar nervous system components, designing insecticides that only target pest insects is difficult. However, the goal of the most recent pesticide generation, according to Prato et al. (2012), is to minimize environmental persistence while exhibiting improved specificity.

Nevertheless, the development of insecticide resistance in mosquito vectors has made the reappearance of mosquito-borne diseases a concern yet again. Insecticide

resistance is a genetic adaptation that allows mosquitoes to survive exposure to insecticides, rendering them ineffective towards the interventions aimed at controlling mosquito-borne diseases. Insecticides with high fatality rates have been used all over the world to control pest insects, including mosquitoes. Although these insecticides have successfully managed mosquito populations for a long time, resistance to these compounds has emerged due to their widespread use and dependence on a few active ingredients. Developing resistance in vector mosquitoes towards synthetic insecticides is an extreme menace to public health worldwide because it reduces the effectiveness of fundamental measures against diseases spread by mosquitoes (Brown, 1986). Investigations into the development of resistance have revealed that, *Ae. aegypti* has triggered resistance to almost all insecticides, including carbamates, OCs, OPs and pyrethroids (Ranson et al., 2010).

Statistics from the WHO indicate that over 250,000 individuals succumb to pesticide poisoning each year. These fatalities are primarily attributed to factors such as incorrect administration, including inaccurate dosage or application methods and improper handling of pesticides (Stoytcheva, 2011). The concerns regarding the detrimental effects of pesticide residues on human and animal health stem from numerous scientific investigations that have demonstrated the harmful effects of specific synthetic active components found in pesticides. These studies provide a foundation for understanding the potential risks associated with high pesticide exposure. Furthermore, several community-based research studies have explored the correlation between pesticide exposure and cancer development, shedding light on the potential health implications of prolonged pesticide exposure (Wolff et al., 1993; Settimi et al., 2003). Studies suggest pesticides can impact cancer development through various non-genotoxic mechanisms, such as peroxisome proliferation and hormonal imbalance. Additionally, pesticides can influence the carcinogenic process through multiple pathways, potentially altering the genome and providing neoplastic cells with a growth advantage. These findings highlight the complex nature of pesticide-induced carcinogenesis, and the various ways pesticides can contribute to the development and progression of cancer (Hodgson and Levi, 1996). Most pesticides are known to have harmful effects on various systems of the human body, including the nervous, renal, respiratory, and reproductive systems. These chemicals can cause detrimental impacts and disruptions to the normal functioning of these vital

physiological systems (Stoytcheva, 2011). Insecticides, while intended to disrupt the nervous system of insects and their normal functions, can also harm mammals, causing immediate and long-lasting neurotoxic effects due to the fundamental similarities in the neurological systems of mammals and insects (Osman, 2011). Intensive or improper application of insecticides has been associated with numerous negative consequences, such as the development of pesticide resistance in certain pests, the pollution of water, soil, and chemicals with residues that can accumulate in the food chain, a decline in biodiversity and nitrogen fixation, the destruction of marine and avian life, and the potential for genetic abnormalities in future generations (Stoytcheva, 2011; Naqqash et al., 2016).

Studies also indicate that when mosquitoes become resistant to a particular insecticide, they frequently exhibit resistance to other insecticides as well, a phenomenon referred to as cross-resistance. Consequently, the utilisation of alternative insecticides may not produce the desired outcomes when combating resistant mosquito populations. Moreover, the rapid development of insecticide resistance implies that the regular development of new insecticides is imperative to adapt to the ever-changing resistance patterns exhibited by mosquito populations (Liu et al., 2006). Insufficient knowledge regarding the development of resistance and cross-resistance has led to the widespread use of multiple chemical insecticides, resulting in detrimental effects on non-target populations and the environment.

Botanicals

The emergence of resistance and the growing environmental catastrophe have prompted researchers to shift their focus towards phytochemicals to achieve successful vector control devoid of such tribulations. The use of plants for pest control purposes can be traced back to ancient times, with historical evidence supporting its practice for an extensive duration. Previous studies have indicated that several aromatic plants or their derived extracts, obtained from specific plant components, were employed as effective measures to combat various insects (Isman, 2006; Benelli, 2015). This traditional approach to pest control relied on recognizing that certain plants possessed insecticidal properties. In mosquito vector management, phytochemicals played a crucial role until the 1920s, when synthetic chemicals gradually replenished them following the introduction of DDT. One notable study

conducted in 1933 by Campbell et al. demonstrated the potential of plant alkaloids as a larvicidal agent against mosquitoes. Researchers isolated several alkaloids, including anabasine, methyl anabasine, nicotine, and lupinine, from *Anabasis aphylla*, a common weed in the Middle East. The results showed that these substances had strong larvicidal activity against mosquitoes, suggesting that they could be useful as natural pesticides for minimizing mosquito populations. Following that study, other phytochemicals with the potential to control mosquito populations were explored. Notable examples include eucalyptus oil, citronella oil, and neem oil. These substances may prevent adult mosquitoes from flying or impede the growth and development of mosquito larvae. Plants are a rich source of insecticidal compounds, and traditional medical practices around the world are well acquainted with this fact. Plants have been used for medical purposes for many ages. Scientists started methodical research into the insecticidal qualities of many plant species in the early 1900s, and by the 1990s, over 1,200 species had been identified as potentially insecticidal. Many of these plants are effective natural insecticides because they contain phytochemicals that disrupt insects' biological processes (Roark and Mclndoo, 1945). But, with the onset of synthetic pesticides like OPs, OCs, carbamates, DDT, and pyrethroids, their influence in the field significantly declined (Ghosh et al., 2012).

Most compounds classified as botanicals are secondary metabolites, which are the chief principal defence mechanism of plants against constant herbivore and environmental selection pressure. Additionally, some plant-based compounds have inherent insecticidal properties as well. An inclusive review of previous research provides compelling evidence supporting the use of plant-derived products as effective insecticides against mosquito populations. Numerous phytochemicals from diverse classes, such as phenolics, alkaloids, steroids, terpenoids, and essential oils derived from various plants, have been extensively studied for their insecticidal properties. Several variables, including the extraction technique, polarity of the solvents used, specific plant species, mosquito species, regional variations, and the specific plant parts employed in the extraction process, can affect the degree of insecticidal activity displayed by these plant extracts (Shaalan et al., 2005b).

A diverse range of plant species, including herbs, shrubs, and towering trees, have been utilised to extract compounds with mosquito-controlling properties. It has been discovered that some herbal extracts function as insect growth regulators (IGRs),

having a significant impact on different stages of insect development. These effects include hindering growth, influencing adult emergence, delaying egg hatching, and affecting fecundity and fertility. As a result, these herbal extracts offer practical solutions for controlling mosquito populations. More than a thousand plant species have been found to be possible sources of bioactive compounds that act as IGRs, including Phyto-ecdysones, Phyto-juvenoids, and antijuvenile hormones (Tiwari et al., 1998). Phytochemicals found in plants represent a vast and relatively unexplored resource that could be usefully employed in mosquito control programs rather than heavily depending on synthetic pesticides. Several studies have examined the chemical structure and composition of phytochemicals to ascertain the extent to which they inhibit the development of mosquito larvae. The potential benefits of various secondary plant-derived compounds, such as lignans, alkaloids, steroids, isoflavonoids, and terpenes, have been documented by this analysis. Additionally, numerous studies have identified and isolated biologically active components from diverse plant sources and investigated their lethal effects on various mosquito species (Kishore et al., 2011; Isman, 2020).

Synergy

Although plant extracts appear to be promising substitutes for mosquito control, they have certain drawbacks. Their wide variations in efficacy can be attributed to a multitude of factors, such as the type of plant utilised, the area of origin, and the extraction technique. Because of this variance, standardizing plant extracts in terms of potency and consistency for the purpose of controlling mosquitoes becomes more difficult. Another disadvantage of plant extracts as adulticides or larvicides is their shorter shelf-life when compared to synthetic pesticides. More frequent applications are needed to ensure long-term efficacy. The potency of plant extracts may also vary amongst mosquito species, with some exhibiting a greater tolerance or resistance to these substances. Furthermore, the expense and demand of the resources associated with the extensive production of plant extracts make them hard to use in large-scale mosquito control initiatives. To maximize the effectiveness, consistency, and usefulness of plant extracts in mosquito control initiatives, these limitations must be addressed through additional research and development (Pavela, 2014).

In the quest for efficient and environmentally sustainable mosquito control strategies, exploring synergistic interactions between synthetic insecticides and botanical compounds has gained considerable attention. In the field of toxicology, synergism pertains to the occurrence where the combined toxicity of a mixture surpasses the expected level when considering the individual toxic effects of its components (Sarup et al., 1980; Mohan et al., 2006). The combination of synthetic insecticides with specific botanical compounds has shown the potential to enhance their collective efficacy in vector control. By harnessing the complementary properties of these two components, synergistic effects can be achieved, resulting in improved mosquito control outcomes. This approach offers a promising ability to reduce the reliance on high concentrations of synthetic insecticides while maximizing their impact on mosquito populations. Furthermore, utilizing botanical compounds alongside synthetic insecticides may help alleviate the development of insecticide resistance, offering a more resilient and long-term solution for mosquito management (Morales-Rodriguez and Peck, 2009; Bhan et al., 2015).

Research endeavours that use plant extracts as a means of vector control and their synergistic effects with conventional pesticides hold significant potential for enhancing the effectiveness of vector control measures while minimizing environmental risks. Such studies can provide valuable perceptions for developing integrated pest management approaches against vector mosquitoes.

Objectives

- To Screen a few locally available plant extracts against *Aedes aegypti*, using organic solvents of increasing polarity.
- To identify and isolate the bioactive compound in effective Phyto-extract.
- To evaluate the susceptibility status of 4^{th} instar larvae of *Ae. aegypti* towards the plant isolate by employing the standard larval bioassay procedure prescribed by WHO.
- To evaluate the synergistic effects of selected plant-isolate with different conventional insecticides on *Ae. aegypti*.
- To recognize different mosquito vector species from selected areas of the Thrissur district, Kerala, India, by molecular identification and the preparation of a phylogenetic tree

Organization of the Thesis

Chapter I: Molecular Identification of Important Vector Mosquito Species from Selected Sites of Thrissur District, Kerala, India.

Chapter II: Screening of Locally Available Plants for Their Larvicidal Effect on Fourth Instar Larvae of *Ae. aegypti* and Bioactive Compound Isolation from the Selected Plant.

Chapter III: Susceptibility Assessment of Four Different Conventional Insecticides and Isolated Plant Bioactive Compound on Fourth Instar Larvae of *Ae. aegypti*.

Chapter IV: The Effect of Synergistic Interaction between Plant Isolate and Four Conventional Insecticides on Fourth Instar Larvae of *Ae. aegypti*.

REVIEW OF LITERATURE

Mosquito Diversity

Since the earliest days of human civilization, mosquitoes have been a persistent source of annoyance and the carriers of devastating diseases, causing frequent suffering and significant economic burdens. These tiny insects transmit some of the most lethal illnesses known to humanity, including malaria, yellow fever, dengue, encephalitis, filariasis, and several other infections, causing a significant loss of human lives (Gubler, 1991). Despite extensive research efforts and decades of mosquito control measures implemented worldwide, mosquitoes remain a global public health challenge. The eradication of traditional mosquito-borne diseases remains a distant goal for many developing nations, while some developed countries have witnessed the introduction and establishment of exotic mosquito species, leading to the emergence of previously unknown mosquito-borne diseases in these regions. Mosquitoes exhibit exceptional adaptability, thriving in diverse habitats across the globe, except for permanently frozen areas. Their larvae breed in a wide range of water bodies, whether temporary or permanent, highly polluted, or clean, large or small, stagnant or overflowing, and even the tiniest accumulations, such as water-filled buckets, flower vases, old tires, hoof prints, or leaf axils (Becker et al., 2010a).

Mosquito Vectors

After developing into the adult phase, the capacity of a mosquito species to transmit diseases to humans is determined by a range of factors, which are commonly assessed using the concept of vectorial capacity. Vectorial capacity provides a quantitative measure of the transmission risk caused by a mosquito species, explicitly indicating the daily rate at which new human infections are likely to arise from a current infected human case. With over 350 mosquito species capable of spreading infections, mosquito vectors are one of the leading causes of disease and death worldwide (Gubler, 1996; Reiter, 2001).

Chikungunya: Chikungunya (CHIK), a viral disease transmitted by mosquitoes, is caused by the CHIK virus, an alphavirus belonging to the Togaviridae family. The term refers to the arched posture of individuals experiencing severe joint pain. The CHIK virus was first identified in Tanzania, East Africa, in the 1950s. The first documented occurrence of chikungunya in India was in Kolkata city, West Bengal, in 1963, resulting in numerous fatalities, especially among children, followed by the outbreak in Barsi in 1973. However, the virus re-emerged in December 2005 and has been persistently spreading ever since (Shah et al., 1964; Mourya et al., 2001; Naresh and Sai, 2010). *Ae. aegypti* and *Ae. albopictus* are the primary vectors of chikungunya in Asia and the Indian Ocean region, although other *Aedes* species, along with *Cx. annulirostris* and various *Anopheles* species contribute to the transmission of the virus worldwide (Jupp et al., 1981; Lam et al., 2001; Pialoux et al., 2007).

Dengue Fever: Dengue poses a current threat to more than 2.5 billion individuals, which accounts for over 40% of the global population. The dengue virus consists of four serotypes (DV-1, DV-2, DV-3, and DV-4) belonging to the Flaviviridae family. Based on the information provided by WHO, approximately 100 million people are affected by dengue each year, leading to hospitalization for around 500,000 severe cases, predominantly among minors. The fatality rate for dengue infections is estimated to be nearly 5%. The initial significant outbreak of DHF occurred during 1953-1954 in the Philippines, which was soon followed by the rapid global spread of DF and DHF epidemics (Rigau-Pérez et al., 1998). Subsequently, in 1996, India experienced its first major widespread epidemic of DHF, starting in areas near Delhi and Lucknow before spreading throughout the entire country (Dar et al., 1999; Agarwal et al., 1999; Shah et al., 2004; Gupta et al., 2012). The incidence of dengue fever in Kerala has steadily increased since its first reported case in the Kottayam District in 1997, with a notable surge observed from 2006 onwards (Lal and Prasittisuk, 2004; Kumar et al., 2013).

Filariasis: Lymphatic filariasis is caused by a thread-like filarial parasite, *Wuchereria bancrofti*, that is transmitted to people by mosquitoes. The primary causative agents of lymphatic filariasis are *W. bancrofti* (98%), followed by *Brugia malayi* and *B. timori* (2%), which are responsible for Bancroftian and Brugian filariasis, respectively (Simonsen and Mwakitalu, 2013). The Indian

subcontinent is significantly affected by this disease, making it a prevalent health issue in the region. Lymphatic filariasis continues to be a substantial socio-economic challenge in many tropical countries, including India (Udonsi, 1986). The presence of microscopic parasitic worms in the lymphatic system impairs the immune system and contributes to the severity of the illness. Lymphatic filariasis affects over 120 million people globally, with approximately permanent disability in 40 million individuals, including genital illness in over 25 million males and lymphedema in more than 15 million patients, and the majority of cases, around 65%, are found in Southeast Asia. In India, it is estimated that approximately 23 million individuals experience symptoms of filarial disease, and more than 31 million people are carriers, with Kerala being identified as the state with the second-highest prevalence of lymphatic filariasis in the country (Rajasekariah et al.,1991; Regu et al., 2005; WHO, 2005b). In the Indian context, *W. bancrofti* is the primary causative agent of lymphatic filariasis, and it is primarily transmitted by the widely prevalent mosquito vector, *Cx. quinquefasciatus* (Agrawal and Sashindran, 2006).

Japanese Encephalitis: JEV (Japanese Encephalitis Virus), a member of the Flaviviridae family, is closely related to the West Nile, Murray Valley, St Louis, and encephalitis viruses, as well as the dengue virus (Heinz and Stiasny, 2012). JEV primarily circulates in a zoonotic cycle involving mosquitoes, pigs, and water birds, with humans being considered dead-end hosts for the virus. JEV has been isolated from various mosquito species through field research. While the principal mosquito vectors may vary across regions, *Cx. tritaeniorhynchus* has been identified as the most significant mosquito vector. In specific locations such as Malaysia and Australia, *Cx. gelidus* has been recognized as the primary JEV vector, and it is now emerging as a secondary vector in several other countries (Heathcote, 1970; Gould et al., 1974; Reuben and Gajanana, 1997; Bhattacharya and Basu, 2014). Japanese encephalitis is estimated to cause 50,000 cases and 10,000 deaths annually, primarily affecting children under five. Reports indicate that the virus has established itself in India. *Anopheles* species, including *An. subpictus*, are strongly implicated in the transmission of JE in the Indian states, in addition to the primary vector, *Cx. tritaeniorhynchus*. During an outbreak in Cuddalore, Tamil Nadu, India, *An. subpictus* was identified as a vector for JEV. Another species, *Ar. subalbatus* is also considered a potential vector of JE in India (Pearce et al., 2018).

Malaria: *Plasmodium falciparum* and *P. vivax* are the primary parasite species responsible for human malaria infections, posing a significant global health concern, with transmission occurring in 97 countries and risking the lives of approximately 3.4 billion individuals. Annually, approximately 2 million confirmed cases of malaria and 1,000 deaths are reported, although the estimated figures by the WHO Southeast Asia Regional Office indicate around 15 million cases and 20,000 deaths. India accounts for 77% of the total malaria cases in Southeast Asia (Peters et al., 2002; Kumar et al., 2007). In India, several mosquito species have been identified as the principal malaria vectors, along with *An. stephensi* (Balasubramanian et al., 1984). Except for *An. stephensi*, all the primary vectors belong to species complexes (Sharma, 2002). *An. vagus*, a prevalent species in Southeast Asian countries, including India, carries important malarial pathogens that contribute to their transmission among humans (Alam et al., 2010; Verhaeghen et al., 2010). In recent years, *An. vagus* has been identified as an increasingly important vector for malaria transmission in various global regions, highlighting its growing role in carrying human pathogens (Maheswary et al., 1994; WHO, 2016b; Alam et al., 2017).

West Nile Virus: West Nile virus (WNV) is a significant flavivirus transmitted by arthropods, typically resulting in a mild infection known as West Nile fever (WNF) in humans. Mosquitoes serve as the primary carriers of WNV. Different species of *Culex* mosquitoes have been identified as vectors in various geographical areas, including India. The detection of West Nile antibodies in humans was initially documented in Mumbai in 1952, indicating the presence of the virus in India (Smithburn et al., 1954). Experimental investigations have demonstrated that mosquitoes, counting *Cx. pipiens*, *Cx. tritaeniorhynchus*, *Cx. bitaeniorhynchus*, *Cx. quinquefasciatus*, and *Ae. albopictus*, have the capability to act as potential vectors of WNV (Varma, 1960; Ilkal et al., 1997; Paramasivan et al., 2003).

Yellow Fever: The primary causative agent of YF is the prototype member of the Flavivirus genus, which falls under the Flaviviridae family (Barrett and Higgs, 2007). Productive infection of the virus is limited to a relatively small range of hosts, and its natural maintenance occurs through transmission between non-human primates and blood-feeding mosquitoes, primarily from the *Aedes* genera. From the 18th Century to the early 20th Century, yellow fever posed a significant risk to human health, leading to repeated epidemics in coastal towns and cities located far

away from endemic regions in North America, the Caribbean, and Europe. YF sporadically infects humans when they are bitten by mosquitoes that have previously fed on virus-carrying monkeys. However, humans can also become hosts for inter-human transmission, primarily facilitated by the *Ae. aegypti* mosquito (Monath and Vasconcelos, 2015). Other *Aedes* species, *Ae. albopictus* (Reiter, 2007) and *Ae. vittatus* (Sudeep and Shil, 2017) has also been identified as a potential vector for transmitting YF.

Zika Virus: ZIKV, a type of virus that falls under the Flaviviridae family, was initially detected in 1947 following its isolation from *Rhesus macaque* monkey found in the Zika Forest of Uganda (Dick et al., 1952). During the period from the 1960s to the 1980s, isolated cases of ZIKV infections were sporadically identified in various regions of Africa and Asia. However, since 2007, significant outbreaks of Zika virus disease have been documented in Africa, the Americas, Asia, and the Pacific (Puntasecca et al., 2021). It belongs to the positive-sense single-stranded RNA virus group. Similar to other flaviviruses, the ZIKV is primarily transmitted by mosquitoes, with the *Aedes* genus being the primary carrier. Different *Aedes* species, including *Ae. aegypti*, *Ae. albopictus* and *Ae. vittatus* have been implicated in the transmission. In Asia, the *Ae. aegypti* mosquito is considered the primary vector for ZIKV (Lanciotti et al., 2008; Plourde and Bloch, 2016; Sudeep and Shil, 2017). India reported the initial four instances of ZIKV in 2007, originating in Gujarat and Tamil Nadu (Bhardwaj et al., 2017). In 2021, the first confirmed case of ZIKV was reported in Thiruvananthapuram, Kerala, followed by 83 additional cases reported in the region (Sasi et al., 2021).

Molecular Barcoding

Scientists have successfully categorized approximately 1.7 million species based on their morphology, which refers to the shape and structure of plants and animals, and this method continues to be fundamental in taxonomic diagnosis following the Linnaean system. However, relying solely on morphology to describe the diversity of life has limitations. Firstly, variations in the appearance of species and genetic variability in the traits used for identification can lead to incorrect classifications. Secondly, many groups often have hidden or cryptic taxa that cannot be distinguished based on morphology alone. Thirdly, morphological keys are often

limited to specific life stages or genders, making it challenging to identify many individuals. Additionally, traditional identification methods based on morphology can be time-consuming and may not always provide accurate species-level identification (Paramasivan et al.,2013). Lastly, while modern interactive versions of keys have improved the process, their practical use still requires a high level of expertise, increasing the likelihood of errors (Knowlton, 1993; Jarman and Elliott, 2000). The limitations associated with morphology-based identification systems and the shortage of taxonomists highlight the necessity for a new approach to recognize taxonomic groups. Micro-genomic identification systems, which utilise the analysis of a small portion of the genome, offer a highly promising method for identifying biological diversity. While this approach has gained significant hold in studying complex groups such as viruses, bacteria, and protists, the challenges posed by morphological taxonomy demand its expansion to incorporate all life forms (Pace, 1997; Brown et al., 1999; Vincent et al., 2000; Allander et al., 2001; Hamels et al., 2001; Hebert et al., 2003a)

The DNA barcode technique has emerged as a promising method for rapid characterization of biodiversity. This approach involves amplifying a specific segment of mitochondrial DNA from animals. Genomic techniques for taxon identification utilise the diversity found within DNA sequences to identify and distinguish organisms. These sequences can be considered genetic "barcodes" that are present in every cell (Kurtzman,1994; Wilson, 1995). Notably, the DNA barcoding technique has proven effective in distinguishing sibling species of mosquitoes. The concept of DNA barcoding is not novel and was initially introduced in 1993, although it needed to attain significant attention from the scientific community at that time. However, the field of DNA barcoding experienced a resurgence in 2003, marking its golden age (Hebert et al., 2003a; Naddaf et al., 2012).

The analysis of the mitochondrial genome of animals is considered superior to the nuclear genome due to several factors, including the absence of introns, minimal exposure to recombination, the maternal lineage, and its haploid mode of inheritance, as highlighted by Saccone et al. in 1999. Among mitochondrial genes, Cytochrome c oxidase subunit 1 is widely recognized as the most conserved gene in terms of amino acid sequences, providing a clear advantage for taxonomic studies

(Knowlton and Weigt, 1998; Hebert et al., 2003b; Kumar et al., 2007). This characteristic renders it highly suitable for investigating evolutionary timeframes in scientific studies. Using reliable primers facilitates the regular retrieval of targeted regions within the mitochondrial genome (Folmer et al., 1994).

The precision and adaptability of DNA barcoding make it an essential tool for species identification in vector surveillance (Ashfaq and Hebert, 2016). The existing morphological identification keys for mosquitoes have limitations as they primarily cover only certain developmental stages like imaginal and fourth instar. This restricts their effectiveness in identifying other field-collected developmental stages without laboratory rearing. Moreover, adult specimens obtained during routine disease surveillance programs are prone to damage, resulting in the loss of important identifying features like bristles and scales, rendering them unidentifiable. Identifying sibling species with identical morphological characteristics adds further complexity to the conventional method (Kumar et al., 2007).

To examine significant mosquito species for their impact on public health in rural South India, Paramasivan et al. (2013) conducted a study analysing the genetic sequences of four species: *Ae. aegypti*, *Ae. albopictus*, *Cx. tritaeniorhynchus*, and *Cx. quinquefasciatus*. The study concluded that DNA barcoding is a rapid and efficient methodology for identifying and classifying mosquito species. This technique enables differentiation between mosquito species and provides important insights into their phylogenetic relationships. Furthermore, incorporating DNA-based species identification methodologies can significantly enhance the traditional morphology-based taxonomical techniques used in mosquito classification. In a study aiming to generate DNA barcodes for various mosquito species in India, particularly those of significance as vector species, samples were collected from multiple regions, including eight Indian states and union territories. Through molecular identification, they successfully documented 111 mosquito specimens from 15 taxa, highlighting the effectiveness of DNA barcoding as a complementary approach to traditional morphology-based taxonomical methods in identifying and classifying mosquito species (Kumar et al., 2007). Bindu and Sabastian (2014) investigated the genetic structure of COI gene in the mosquito species *Ar. subalbatus* obtained from Kerala. The gene sequences from these specimens exhibited a 100% similarity to those obtained from the same species collected in

Pakistan. This study emphasised that one of the essential advantages of DNA barcoding is its capacity to evaluate the global geographic and spatial distribution of species. Using a specific fragment of mitochondrial DNA from the COI region enabled the identification of Canadian mosquito species. This approach successfully generated barcodes for 37 mosquito species in Ontario and New Brunswick provinces. The study revealed a notable difference in the sequence divergence of the COI region, with significantly higher variations observed among species within the same genus compared to variations within a species (Cywinska et al., 2006). These findings were further supported by Wang et al. (2012), who utilised the COI gene to generate DNA barcodes for common mosquito species in China, including the primary disease vectors. Their research confirmed a significant disparity in the sequence divergence of the COI gene, with species within the same genus exhibiting approximately 30 times higher divergence compared to individuals within the same species.

In the research conducted by Chan et al. (2014), an analysis was carried out on 128 adult mosquito specimens belonging to 45 species from 13 genera. The researchers constructed phylogenetic relationship trees for different mosquito genera such as *Aedes*, *Anopheles*, *Culex*, and others. The study focused on comparing the distinct clustering patterns of different species with their taxonomic identities. The results showed that using DNA barcoding based on the CO1 gene achieved a 100% success rate in accurately identifying mosquito species. Additionally, the study contributed to the existing knowledge by providing COI-based barcode sequences for 16 mosquito species that were not formerly presented in sequence databases. In the process of molecularly identifying mosquitoes in southeastern Australia, researchers obtained the COI sequences from 113 specimens that had been morphologically identified. These specimens represented 29 distinct species, six tribes, and 12 genera. The study involved all developmental stages of the collected species, including eggs, to conduct a comprehensive molecular analysis (Batovska et al., 2016).

The molecular identification of mosquito species has been the focus of several studies investigating the utility of the COI gene sequences. Research focused on the use of mitochondrial markers for molecular identification of West African *Aedes* mosquitoes, which are known vectors of arboviral diseases, revealed a

significant molecular divergence between *Ae. furcifer*, and *Ae. taylori*, despite their similar morphological characteristics. These findings underscored the effectiveness of molecular identification techniques, particularly utilizing the mitochondrial gene COI, in accurately documenting interspecific species (Cook et al., 2005). In an analysis conducted in Japan, the nucleotide sequence of the COI gene was utilised to evaluate 512 mosquito specimens collected from multiple sites spanning from Hokkaido to Kagoshima. The findings revealed that 240 mosquitoes, representing 45 species and 11 genera, had their gene sequences deposited in GenBank. Traditional taxonomy methods faced significant challenges in northern Japan when distinguishing morphologically similar *Aedes* mosquitoes with black legs, specifically those belonging to the punctor-subgroup like *Ae. punctor* and *Ae. communis*. However, the specimens could be categorized into two genetically distinct populations through COI gene sequence analysis (Maekawa et al., 2016).

Díez-Fernández et al. (2018) conducted a study in southern Spain where they employed a molecular method to amplify and sequence the COI gene of *Ae. vittatus* mosquito larvae and adults. The sequenced species exhibited a similarity of only 94% with other *Aedes* species. When comparing the mosquito sequences isolated from Spain with those published in public databases, a 99% similarity was found with sequences of two *Aedes* mosquitoes, *Ae. vittatus* and *Ae. cogilli*. Notably, at that time, there were no previous records of the mosquito species *Ae. cogilli* in Europe and was exclusively seen in India. A study conducted by Hernández-Triana et al., (2019) focused on using DNA barcoding techniques to aid in identifying British mosquitoes, documented cryptic genetic diversity, and examined invasive species. The study analysed a total of 42 mosquito species from different genera, including *Aedes, Culex, Anopheles, Coquillettidia,* and *Orthopodomyia*. Based on this analysis, the authors proposed that combining morphological characteristics with DNA barcoding provided an efficient approach for accurately identifying mosquitoes, particularly those invasive species that were a threat to public health. Furthermore, this method also helped to uncover hidden genetic diversity within species groups, enhancing the understanding of mosquito populations. In a survey conducted in Mananthavady Taluk of Wayanad district, Kerala, mosquito vectors of JE were identified using molecular barcoding techniques. The study revealed the presence of 12 mosquito species belonging to three genera, *Anopheles, Culex*,

and *Mansonia*, in the region. *Cx. tritaeniorhynchus*, *Cx. quenquefasciatus*, *Cx. gelidus*, *Cx. bitaeniorhyncus*, *Cx. vishnui*, *Cx. pseudovishnui*, *Cx. fuscocephala*, *Ma. indiana*, *Ma. uniformis*, *An. barbirostris*, and *An. Peditaeniatus* were the identified species. The findings supported the recommendation of employing molecular barcoding to identify mosquito vectors effectively (Thankachan et al., 2021).

The collective findings of these studies underscore the effectiveness and promise of employing COI gene sequences and DNA barcoding for the molecular identification and classification of mosquitoes. Utilizing molecular approaches enhances the understanding of genetic diversity, population dynamics, and interspecific relationships and complements traditional morphological identification methods in mosquito research and surveillance. Furthermore, these studies emphasized one of the significant advantages of DNA barcoding in assessing the spatial and geographic distribution of mosquito species on a global scale. Integrating molecular techniques with traditional methods could hold great potential for improving the knowledge of mosquitoes and their impact on public health.

Chemical Control

Historically, the utilisation of insecticides dates back to ancient times, with arsenic as insect bait in the 9th century AD. Subsequently, compounds such as lead arsenate ($PbHAsO_4$), cryolite (Na_3AlF_6), and borax ($Na_2B_4O_7$) were employed as cellular poisons and dehydrators (Popov et al., 2021). The chemical industry has witnessed significant advancements, leading to crucial breakthroughs in managing pathogens, vectors, and pests. Chemical insecticides gained widespread usage in the mid-19th century, with Paris green, a mixture of copper acetoarsenite, proving effective in combating the Colorado potato beetle in 1871 (Alyokhin, 2009). Paris green remained a prevalent choice until the mid-20th century in various countries, particularly for controlling the malaria vector, *Anopheles* mosquito (Symes, 1952; Majori, 2012).

DDT, perhaps the most renowned chemical pesticide, was synthesized in 1874 by Austrian chemist Othmar Tseidler (Bate, 2007) and its insecticidal properties were discovered by Swiss scientist Paul Müller, employed at J. R. Geigy Ltd., in 1939 (Davies et al., 2007). In recognition of his seminal research on the effectiveness of

DDT as a contact toxin, Paul Müller was honoured with the Nobel Prize in Medicine in 1948 (Mischke, 1985). Subsequent research revealed the detrimental impact of DDT on various non-target organisms, including mammals, birds, and reptiles, in addition to pest insects.

Organophosphates

The use of DDT in plant protection prevailed until the latter half of the 20th century, after which it was succeeded by a widespread adoption of organophosphates (e.g., dichlorvos, cyanophos, fonofos, malathion, temephos) and carbamates (such as carbaryl, carbofuran, aldicarb). OPs represent a group of insecticides, some possessing high toxicity levels. They were extensively employed as insecticides and were considered among the most prevalent options until the beginning of the 21st century (O'Brien, 1967). OPs consist of two components, namely a phosphate (or thio- or dithio-phosphate) group and an organic moiety. Typically, the phosphate group is O, O-dialkyl substituted. These pesticides exhibit strong inhibitory effects on cholinesterase enzymes through reversible or irreversible covalent binding with the serine residue positioned in the active site of acetylcholinesterase. As a result, the natural breakdown of neurotransmitters is hindered, causing similar adverse effects in insects, wildlife, and humans (Barr and Buckley, 2011).

Classified as an Op insecticide, malathion was first made available by the American Cyanamid Company in 1950. Malathion is well-known for its broad range of effectiveness and has been used extensively to control a variety of insect species (Flessel et al., 1993). The lipid-soluble nature of malathion makes it easier to absorb by the skin, respiratory system, or digestive tract (Gunther et al., 1968). Since 1956, public health officials have used malathion as a tool to control mosquito populations when needed. Malathion proved to be an effective mosquito repellent with negligible hazards to human health and the environment when used in accordance with the recommended application rate and safety precautions (Penner, 2005). The study on insecticide susceptibility of *Ae. aegypti* in various regions of Thailand indicated that malathion remained effective in control programs, as all larval populations exhibited minimal resistance to this insecticide (Ponlawat et al., 2005).The assessment of insecticide resistance in *Ae. aegypti* across different localities in Colombia indicated that the species displayed susceptibility to malathion and did not exhibit resistance or cross-resistance (Ocampo et al., 2011).

The extensive utilisation of malathion in vector control programs has been well-documented in the literature. Nevertheless, the emergence of resistance to these compounds has raised concerns, as it has been observed in diverse mosquito vectors across different geographical regions (Bisset et al., 1991; Gopalan et al., 1996; Poopathi et al., 2000). A study assessed the development of resistance to malathion in *Ae. albopictus*. The larvae of selected colonies, subjected to 10 generations of malathion-induced selection pressure, exhibited reduced susceptibility to malathion compared to the susceptible strains. Susceptibility tests using a diagnostic dosage of 5 % malathion-impregnated paper showed varying degrees of resistance among adult mosquitoes (Selvi et al., 2010). Likewise, in the research carried out by Hidayati and colleagues in 2011, it was demonstrated that subjecting *Ae. aegypti* mosquitoes to continuous malathion exposure over 45 generations of selective treatment led to a progressive escalation in their resistance to the insecticide. This discovery lent acceptance to the idea that extended and recurrent malathion usage could lead to the development of resistance within mosquito populations.

Temephos, classified as a non-systemic organophosphorus insecticide, is primarily utilised as a larvicide for mosquito control, particularly in domestic water containers and drinking water storage facilities (WHO, 2009). Temephos continued to be the predominant chemical used for controlling the immature stages of *Ae. aegypti* in Southeast Asia and became a crucial component in mosquito control efforts. The repeated utilisation of insecticides has led to the development of resistance in vector populations, thereby abating the efficacy of operational interventions (Saeung et al., 2020). Resistance to temephos has been extensively observed in *Ae. aegypti* populations in the Americas, as evidenced by studies conducted by Rodríguez et al. (2007) and Ocampo et al. (2011). However, the Asian region has relatively fewer reports on temephos resistance, as described by Chaiphongpachara and Moolrat (2017). Several studies, such as those conducted by Liew et al. (1994) in Singapore, Polson et al. (2001) in Cambodia and Paeporn et al. (2004b) in Thailand, employed a diagnostic dosage of 0.02 mg/L of temephos. These studies have consistently reported the presence of temephos resistance in *Aedes* mosquitoes. *Ae. aegypti*'s resistance to temephos has been the subject of several studies, with Paeporn et al. reporting a substantial rise in resistance through

selection, Wirth and Georghiou observing elevated resistance in a laboratory-reared colony, and Tikar et al. documenting a significant increase in resistance in a particular strain of *Ae. aegypti* (Wirth and Georghiou,1999; Paeporn et al., 2003; Tikar et al., 2009). In a scientific investigation that sought to assess the susceptibility or resistance status of *Ae. aegypti* to temephos in three districts of Tamil Nadu, the populations showed a considerable degree of resistance, ranging from high to moderate, and the authors concluded that the widespread use of temephos to control *Ae. aegypti* populations in the corresponding areas were the cause of this elevation in resistance (Muthusamy and Shivakumar, 2015a).

Pyrethroids

Pyrethrum is a plant-based insecticide derived from the flower heads of a white-flowered plant in the chrysanthemum genus. It has a long history as a botanical insecticide. Its insecticidal properties have been recognized for over 150 years, with references to the chrysanthemum flowers originating from ancient Chinese history and potentially being introduced to Europe through the Silk Roads. The efficacy of pyrethrum as an insecticide was first observed in the 19th century when it was used by Caucuses tribes for controlling body lice, as noted by Jumticoff, an American researcher (Glynne-Jones, 2001). Since the 1840s, it has been well-established that pyrethrins are highly susceptible to degradation by light, with a short half-life of less than five hours when exposed to direct sunlight. This inherent photosensitivity has significantly restricted their practical application in commercial settings. To overcome this limitation, the initial development of synthetic pyrethroids involved modifying specific structural elements of pyrethrin I with isosteric substitutes to enhance their metabolic and photochemical stability. Although the synthesis of pyrethrin analogues commenced shortly after identifying the active constituents, it was not until 1949 that the first commercially successful pyrethroid, allethrin, was introduced (Casida, 1980). However, their usage declined after World War II due to the introduction of more cost-effective organochlorines, organophosphorus compounds, and carbamate insecticides. Nevertheless, concerns about environmental contamination and vertebrate toxicity prompted a renewed interest in pyrethrins and the development of synthetic pyrethroids with improved stability and potency. These synthetic analogues, incorporating structural modifications, exhibit a wide range of insecticidal activity. These synthetic compounds can display structural

similarities to the original pyrethrins or have significantly different chemical structures (Bradbury and Coats, 1989; Valentine, 1990; Schleier and Peterson, 2011).

Cypermethrin, a potent synthetic pyrethroid insecticide, was initially synthesized in 1974 and introduced to the market in 1977. It is chemically composed of the alpha-cyano-3-phenoxybenzyl ester of the dichloro analogue of chrysanthemic acid, specifically 2,2-dimethyl-3-(2,2-dichloro vinyl) cyclopropane carboxylic acid (WHO, 1989). Evaluation of cypermethrin resistance in the dengue vector, *Ae. aegypti* population from Lahore, Pakistan, revealed that the tested population displayed a lower level of resistance to cypermethrin (Jahan and Shahid, 2013). *Ae. aegypti* in various regions of Vietnam demonstrated continued susceptibility to the pyrethroid insecticide cypermethrin, as revealed by studies conducted at different locations in eleven provinces and cities (Huong et al., 2004). Monitoring the resistance to the pyrethroid cypermethrin in *Ae. aegypti* populations collected in Brazil indicated the development of resistance in the selected species, potentially attributed to the repeated use of insecticides without available alternatives (Da-Cunha et al., 2005). The evaluation of *Ae. aegypti* populations from Pernambuco, Brazil, revealed resistance across all tested populations for cypermethrin, as documented by de Araújo et al. (2019). The resistance status of *Ae. aegypti* to cypermethrin in Bengkulu City, Indonesia, was examined, and it was found that *Ae. aegypti* populations from dengue endemic and sporadic areas in Bengkulu City remained susceptible to cypermethrin. However, there were indications of the emergence of resistance mechanisms in some populations, suggesting the potential development of resistance over time (Triana et al., 2019).

The results of the susceptibility assay conducted on *Ae. aegypti*, *Ae. albopictus*, *Cx. quinquefasciatus*, and *An. subpictus* mosquitoes exposed to cypermethrin revealed varying vulnerability among the mosquito species. While *Ae. aegypti* mosquitoes exhibited resistance, *Ae. albopictus* and *Cx. quinquefasciatus* displayed tolerance, with *An. subpictus* mosquitoes remained susceptible to the insecticide (Ramadhani et al., 2020).

Lambda-cyhalothrin was introduced in 1988, belongs to the pyrethroid class of insecticides and is widely utilised for effective pest control against a diverse range of pests (Fetoui et al., 2015). Lambda-cyhalothrin, identified explicitly as cyano-3-

phenoxybenzyl 3-(2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropane-carboxylate, emerged as an insecticide that striked a favourable balance between efficacy and toxicity. With widespread application in public health and animal health sectors, lambda-cyhalothrin had proven effective in controlling a broad spectrum of insects, including mosquitoes (Anadon et al., 2006). A research study was carried out at the International Airports of Thiruvananthapuram and Cochin in southern India to evaluate the susceptibility of *Ae. aegypti* and *Ae. albopictus* to lambda-cyhalothrin in their aquatic and adult stages. The findings revealed that both the larval and adult populations of *Ae. aegypti* and *Ae. albopictus* displayed susceptibility to lambda-cyhalothrin (Sharma et al., 2004). A research investigation by Husham et al. (2010) aimed to assess the susceptibility of the dengue vector *Ae. aegypti* to pesticides in Port Sudan City and samples of *Ae. aegypti* in their aquatic stage were collected from nine entomological stations for testing. The results of the study indicated that, *Ae. aegypti* demonstrated tolerance to lambda-cyhalothrin. During a survey analysis carried out in a malaria-endemic area of Southeastern Iran, Fathian et al. (2015) observed that both *An. stephensi* and *An. culicifacies* showed susceptibility to the insecticide lambda-cyhalothrin. The examination of the molecular mechanisms underlying resistance to lambda-cyhalothrin in a population of *An. funestus* from Senegal unveiled the development of resistance in the species to the insecticide (Samb et al., 2016).

Plant Extract

Current developments have led to limitations in using synthetic insecticides for mosquito control due to various factors. These include the absence of new insecticides, high costs associated with synthetic alternatives, and concerns about sustainability and their adverse effects on human health and non-target organisms. Synthetic insecticides are non-biodegradable and can lead to biological magnification in ecosystems, contributing to the emergence of global insecticide resistance. Researchers have actively pursued alternative strategies prioritising effective and transparent mosquito management approaches, including public education, monitoring, source reduction, and eco-conscious larval control methods with reduced toxicity. Consequently, there has been a shift in focus within control programs towards eco-friendly alternatives like plant-derived insecticides, reducing reliance on chemical insecticides (Ghosh et al., 2012).

In previous times, botanical insecticides like nicotine and pyrethrum held a prominent position in managing household pests until the late 1930s, when the insecticidal properties of DDT and methyl parathion were unveiled (Morgan, 2004). However, the subsequent emergence of cost-effective and highly efficient synthetic insecticides, such as organochlorines, organophosphates, and carbamates, led to the diminished significance of botanicals in the pest control industry starting from the 1970s. A resurgence of scientific interest in botanical insecticides was sparked by the discovery of the potent insecticidal properties of azadirachtin, a triterpenoid compound isolated from the seeds of the *Azadirachta indica*, commonly known as Indian neem tree, during the 1960s (Isman, 2006). This led to more research and the organization of international conferences dedicated to neem and neem-based insecticides throughout the 1980s and 1990s (Isman and Grieneisen, 2014). Sukumar et al. (1991) conducted a comprehensive review documenting 344 botanical agents with insecticidal properties, further highlighting the significance of botanical compounds in pest control.

In recent years, alternative approaches for effective pest and vector control have proliferated, leveraging advancements in genetic engineering, plant breeding, and a deeper understanding of plant-pest-predator interactions (Bakhsh et al., 2015). Among these options, one that shows promise and is effective is using secondary plant metabolites produced by certain plant species as part of their natural defence mechanisms against pests and diseases (Miresmailli and Isman, 2014). Numerous investigations in botanical studies have revealed the remarkable evolutionary adaptations of different plant species. Diverse chemical and physical defence mechanisms have emerged because of these adaptations, intending to overcome a variety of insect threats. These defence mechanisms exerted by plants include a wide range of compounds, including alkaloids, terpenoids, polyphenols, and phenols. These chemical compounds have unique properties and can be extracted from plants using a variety of techniques. Simple mixing in water, the use of organic solvents with varying polarities, supercritical fluid extraction, and various distillation techniques are examples of standard extraction techniques. The complex and frequently compelling chemicals plants have developed to lessen the harm that herbivorous insects inflict have been thoroughly examined and documented in the scientific literature (Mithöfer and Boland, 2012). A deeper understanding has been

gained regarding the precise mechanisms by which various plant defensive metabolites operate, emphasizing their capacity to deter insects (Nerio et al., 2010; Maia and Moore, 2011). Numerous plant species from diverse geographical regions have been found to have phytochemicals capable of exerting both acute and chronic toxic effects. Several botanical extracts have demonstrated notable detrimental effects on the fertility and viability of mosquito eggs. Additionally, these extracts have shown significant and promising larvicidal properties, highlighting their potential as effective mosquito control agents (Shaalan et al., 2005b).

In their research, Prempree and Sukhapanth (1990) observed that the crude extracts derived from various parts of the *Derris elliptica* bentham plant exhibited toxicity towards mosquito larvae. The extracts were found to disrupt the formation of both the cuticle and internal tissues of the larvae, ultimately resulting in total mortality. These findings indicate the potential of this plant as a natural larvicide against mosquitoes. Amusan et al. (2005) compared the toxicity effect of oil extracts from *Hyptis suaveolens* and *Citrus sinensis* and found that ethanolic extracts derived from the peel of *Citrus sinensis* demonstrated the highest mortality rate when tested against *Ae. aegypti*, in comparison to *Hyptis suaveolens*. These results of the study revealed the aptitude of extract taken from *Citrus sinensis* peel as a persuasive larvicide against *Ae. aegypti* mosquitoes, suggesting its possible application in mosquito control strategies. Chapagain and Wiesman (2005) conducted a study to examine the larvicidal effects of *Balanites aegyptica* (desert date) on *Cx. pipiens* mosquito larvae. They found that extracts from different plant parts, such as the seed kernel, fruit pulp, roots, bark, and leaves, displayed larvicidal activity. Among these extracts, the aqueous extract from the roots exhibited the highest larvicidal mortality, achieving significant results even at minute concentrations. In a study conducted by Chansang et al. (2005), it was observed that the aqueous extract derived from both unripe and ripe fruits of *Piper retrofractum* displayed varying degrees of larvicidal effectiveness against *Cx. quinquefasciatus* larvae. Furthermore, the extract exhibited comparable toxicity towards both resistant and susceptible strains of *Bacillus sphaericus* in Thailand. Interestingly, *Ae. aegypti* mosquitoes were more susceptible to the extract obtained from ripe fruits than *Cx. quinquefasciatus*. Komalamisra et al. (2005) performed a screening of larvicidal activity using various Thai plants against four species of mosquito vectors. They

conducted experiments on 96 ethanolic extracts obtained from 84 Thai plants. The results showed that extracts derived from *Rhinacanthus nasutus*, *Trigonostemon redioides*, *Acorus calamus*, *D. elliptica*, *Stemona tuberose*, and *Homalomena aromatic*, demonstrated significant larvicidal activity, suggesting their efficacy in this regard. Additionally, the petroleum ether extract of *R. nasutus* demonstrated larvicidal effects against all tested mosquito species. George and Vincent (2005) conducted a comparative investigation to assess the effectiveness of *Annona squamosa*, *Pongamia glabra*, and *Az. indica* against mosquitoes. They found that both *P. glabra* and *A. squamosa* extracts exhibited larvicidal properties, with *P. glabra* demonstrating a more pronounced effect. *Ae. aegypti* larvae were the most susceptible, followed by *An. stephensi* and *Cx. quinquefasciatus* for both extracts.

According to Obomanu et al. (2006), *Lepidagathis alopecuroides* exhibited potential as an affordable and efficient larvicide for mosquitoes. The study revealed its lethal effects on *An. gambiae* and *Cx. quinquefasciatus* mosquito larvae. Furthermore, *Cx. quinquefasciatus* demonstrated greater susceptibility to the extracts of *L. alopecuroides* and *Az. indica*. In a separate study, Pushpananthan et al. (2006) observed that steam-distilled oil derived from *Cymbopogan citrus* caused mortality in *Cx. quinquefasciatus* larvae after 24-hour treatment, and it displayed complete ovicidal activity. An examination was undertaken to analyse the larvicidal efficacy of the leaf extract derived from *Ageratina adenophora*, an extensively cultivated weed plant in the higher terrains of the Nilgiris district. The prime focus of this study involved the detailed analysis of the extract's impact on two species of mosquitoes, *Ae. aegypti* and *Cx. quinquefasciatus*. In comparison to neem, it was discerned that the leaf extract obtained from *A. adenophora* exhibited heightened toxicity towards both *Ae. aegypti* and *Cx. quinquefasciatus*. Consequently, the extract demonstrated significant potential for efficaciously restricting the proliferation of mosquito larvae (Mohan and Ramaswamy, 2007). Das et al. (2007) determined that the ethanol extract of *A. squamosa* exhibited the highest activity against *Cx. quinquefasciatus*, followed by ethanol extract of *Aristolochia saccate*, methanol extract of *A. squamosa*, and methanol extract of *Gymnopetelum cochinchinensis*. Tiwary et al. (2007) conducted a study on the larvicidal activities of the essential oil from the seed of *Zanthoxylum armatum* against three mosquito vectors, and they observed that *Cx. quinquefasciatus*, *Ae. aegypti*, and *An.*

stephensi displayed sensitivity to the oil. Matasyoh et al. (2008) discovered that the ethyl acetate extract of *Aloe turkanensis*, as well as the hexane, acetone, and methanol extracts of *Aloe fibrosa*, and all extracts of *A. andongensis*, demonstrated larvicidal effects against *An. gambiae*. Notably, the ethyl acetate extract of *A. turkanensis* demonstrated the most significant larvicidal activity, achieving 100% mortality.

An. culicifacies, *Ae. aegypti*, *Cx. quinquefasciatus*, and *An. stephensi*, were tested in a study assessing the efficacy of aqueous and hexane extracts from the dried fruit of *Solanum nigrum* against these species. It was found that the aqueous extracts showed lethal effects, causing 100% mortality in all tested species during the larval bioassay at a concentration of 1000 ppm (Raghavendra et al., 2009). Borah et al. (2010) proposed that *Toddalia asiatica* holds promise as a larvicide. Their research demonstrated that hexane, acetone, and methanol extracts from the leaves of *T. asiatica* displayed larvicidal activity against *Ae. aegypti* and *Cx. quinquefasciatus*. The hexane extract from the fruits of *T. asiatica* exhibited the highest larvicidal activity against both mosquito vectors. Dua et al. (2010) revealed that the essential oil derived from the leaves of *Lanthana camara* possessed adulticidal activity against *Cx. quinquefasciatus*, *Ae. aegypti*, and *An. stephensi* exhibited extended effectiveness at low storage temperatures. Jawale et al. (2010) investigated the larvicidal activity of *Oestrum nocturnum* against *Ae. aegypti* and found that the methanol extract of *O. nocturnum* displayed the highest larvicidal activity, resulting in 100% mortality of tested species.

Kamaraj et al. (2011) discovered that the ethyl and methanol extracts from the bark of *A. squamosa*, ethyl acetate and methanol extracts from the leaves of *Chrysanthemum indicum*, as well as acetone and ethyl acetate extracts from *Tridax procumbens*, showed potential as eco-friendly means to control *An. subpictus* and *Cx. tritaeniorhynchus*. In an examination of the larvicidal activity of *Eugenia jambolana* extracts against three mosquito species, *Ae. aegypti* exhibited the highest sensitivity, followed by *Cx. quinquefasciatus* and *An. stephensi*. Crude petroleum extract demonstrated greater larvicidal effectiveness (Raghavendra et al., 2011). The larvicidal activity of seaweed extracts, specifically *Enteromorpha intestinalis*, *Acanthopora spicifera*, and *Dictyota dichotoma*, dissolved in dimethylsulfoxide, was investigated against fourth instar *Ae. aegypti* larvae. The study reported that

ethanolic extracts from the seaweed *D. dichotoma* contained active compounds that exhibited larvicidal activity, indicating their potential for developing effective larvicidal agents (Beula et al., 2011).

The larvicidal activity of *P. pinnata* extract was evaluated against three mosquito vectors. The bark of *P. pinnata* was used to prepare methanol and hydro alcohol extracts, which were tested against fourth instar larvae of *An. Stephensi, Ae. aegypti*, and *Cx. quinquefasciatus*. The findings revealed that the methanol extract derived from *P. pinnata* presented the highest larval mortality compared to the hydro alcohol extract (Kolli and Sundararajan, 2013). Another study assessed the larvicidal potential of extracts from several Indian medicinal plants against *Ae. aegypti*. Among the extracts tested, the acetone extract of *Elaeagnus indica* showed the highest larval mortality, followed by the acetone extract of *Maesa indica* (Shivakumar et al., 2013). Kamiabi et al. (2013) observed that the crude extracts obtained from *Cyperus aromaticus* exhibited notable inhibitory effects on the growth of *Ae. aegypti* and *Ae. albopictus*. The wing length of treated adults from both mosquito species, particularly females of *Ae. albopictus*, showed a significant decrease. Additionally, the longevity of the mosquitoes was reduced, sterility indices were increased in the female parental generation, and pupal formation and adult emergence were delayed in both *Aedes* species.

The results of the study organised by Paul et al. (2020) indicated that the crude extracts, combined extracts, and bioactive fractions derived from two different plant species *Andrographis paniculata* and *Tinospora cordifolia* showed promise as larvicides against third-instar larvae of *Ae. aegypti*. In the research carried out by Aziz et al. (2021), an investigation was conducted to assess the larvicidal effect of a methanolic extract from *Vitex ovata* leaves against *Ae. aegypti*. The study demonstrated that the crude extract of *V. ovata* possessed bioactive compounds that could be utilised as bio-larvicides for controlling the *Aedes* mosquito vector.

Plant species that demonstrate high success rates are known to possess a wide variety of moderately toxic defence compounds or a small number of highly toxic substances. This enables them to effectively target a diverse range of molecular components, including proteins (enzymes, receptors, ion channels, structural proteins), nucleic acids, bio-membranes, and secondary metabolites. These specific or non-specific interactions contribute to the plant's ability to defend against threats.

Secondary metabolite analogues can disrupt essential components of cellular signalling systems by interfering with vital enzymes and processes involved in nervous system signalling. These analogues can affect neurotransmitter synthesis, storage, release, binding, re-uptake, receptor activation, and function, as well as enzymes responsible for signal transduction. Additionally, they can block metabolic pathways, leading to the disruption of crucial cellular processes. This indicates the diverse mechanisms through which secondary metabolite analogues exert their effects (Wink, 2000). It is evident that plant-based insecticides impact insect physiology through diverse mechanisms and at multiple receptor sites. For instance, terpenes can block the stimulating effects of glucose and inositol on chemosensory receptor cells (Gershenzon and Dudareva, 2007). Essential oils and their constituents disrupt biochemical processes, leading to imbalances in insect endocrinology and interfering with normal morphogenesis (Reynolds, 1987; Balandrin and Klocke, 1988). Neurotoxicity is observed in several monoterpenoids, causing hyperactivity and paralysis in insects (Coats et al., 1991). Insecticides like avermectin and milbemycin act on glutamate-gated chloride channels, while pyrethrins affect sodium channels (Bloomquist, 2003). Rotenone interferes with cellular energy metabolism in mitochondria by either inhibiting the electron transport system or uncoupling it from ATP production (Fukami, 1961; Yamamoto and Kurokawa, 1970). Other compounds like Sabadila, nicotine, and veratridine interfere with nerve function, energy metabolism, and cell division. Azadirachtin has been identified as an anti-mitotic insecticide, and specific mutations in GABA receptors confer resistance to certain insecticides (Richards and Cutkomp,1945; Höld et al., 2000, Rattan, 2010).

Synergy

Insecticide synergy has emerged as a valuable approach to enhance the efficacy of insecticides by amplifying their lethal effects. In this approach, single or multiple compounds are mixed with an insecticide. Synergistic compounds have successfully controlled the resistance development of targeted vector populations to conventional insecticides. The administration of synergists, even in sublethal doses, has presented escalation in the lethality of insecticides (Brindley and Selim, 1984). Synergists have been widely employed in commercial applications over the past five decades as they have proved to improve the competence of insecticides. The objective of

improving the stability of a pyrethroid insecticide permethrin, a potent insecticide for controlling houseflies, mosquitoes, and other household pests, can be considered the origin of insecticide synergists. The first practical and economically feasible synergist was piperonyl butoxide (PBO), which significantly decreased the production costs related to natural pyrethrum. However, a more promising approach was discovered when Haller and colleagues interpreted Eagleson's finding that adding sesame oil to pyrethrum extracts significantly enhanced their efficacy (Haller et al., 1942). They credited this enhanced effectiveness to the synergistic impact of sesamin, a component found in the oil. Later, it was identified that the presence of a methylene-dioxy-phenyl group in sesamin was a contributing factor. Subsequently, they synthesized the amides of 3,4-methylene-dioxy cinnamic acid and found them to exhibit synergistic activity with pyrethrins (Wachs, 1947).

Metcalf (1967) defined a synergist as a passive component in a mixture that enhances its toxicity when combined with an insecticide. Synergists are often metabolic inhibitors, implying a specific mode of action. Additionally, the term "potentiation" described the greater-than-additive effect observed in synergistic combinations of insecticides, where each insecticide is used at its toxic level and targets specific sites (Raffa and Priester, 1985). Moreover, the term "quasi-synergism," as proposed by Sun and Johnson in 1972, should be applied explicitly to chemicals that facilitate the penetration, transport, or accessibility of the insecticide rather than affecting its toxicity or mode of action. The use of synergists with increasing specificity continued to play a crucial role in managing resistant pests, helping to minimize selection pressure, primarily when they target a specific detoxification pathway of an insect. These synergistic agents served as valuable tools in maintaining effective pest control strategies while reducing the development of resistance (Wachs, 1947).

Thangam and Kathiresan (1991) investigated the synergistic interaction between marine plant extracts and insecticides (DDT, BHC and malathion) against *Ae.aegypti*. The study revealed that the stilt root of *Rhizophora apiculata* exhibited the highest level of synergistic activity when combined with BHC (Benzene Hexachloride). The larvicidal activity of plant extracts, both individually and in combination with synthetic larvicidal agents, was evaluated by Harve and Kamath (2004) against *Ae. aegypti* mosquitoes. The results showed that

extracts of *Murraya koenigii*, *Ferula asafetida*, and *Trigonella foenum graceum* exhibited potential synergistic activity when combined with the synthetic agents. At the same time, they showed poor larvicidal activity when tested individually. Shaalan et al. (2005b) evaluated the synergistic efficacy of *Khaya senegalensis*, *Daucus carota*, and *Callitris glaucophylla* in combination with and without synthetic insecticides against *Ae. aegypti* and *Cx. annulirostris* mosquitoes. The results indicated that all mixtures resulted in 100% mortality of *Cx. Annulirostris* larvae within 24 hours. Furthermore, the mixtures exhibited synergistic effects on *Ae. aegypti* larvae, except for one mixture that demonstrated an additive effect. In a distinct study conducted by Mohan et al. (2007), the synergistic efficiency of a combination of cypermethrin and petroleum ether extract of *Solanum xanthocarpum* at a 1:1 ratio was examined against *Cx. quinquefasciatus* mosquitoes. The results demonstrated that this combination exhibited the highest efficacy in controlling the target mosquito species. Chenniappan and Kadarkarai (2008) focused on the synergistic activity of *Andrographis paniculata* extracts against *An. stephensi* larvae. The study found that combining plant extract with deltamethrin resulted in the highest larvicidal activity, indicating synergistic effects. Furthermore, a 1:4 ratio of deltamethrin and ethanolic extract was identified as the most effective combination.

In a study by Mohan et al. (2010), the larvicidal activity of a combination of *Solanum xanthocarpum* extract and specific synthetic insecticides was investigated against *Cx. quinquefaciatus* mosquitoes. The results indicated that when fenthion and the plant extract were combined at a 1:1 ratio, they exhibited synergistic effects, resulting in the most favourable outcomes regarding larvicidal action against the target organism. Interestingly, oils derived from *Amyris balsamifera*, *Sesamum indicum*, *Helichrysum italicum*, *Santalum album*, *Juniperus virginiana*, and *Piper nigrum* exhibited significant synergistic effects when combined with carbaryl. However, paradoxically, all these oils reduced the toxicity of permethrin against *Ae. aegypti* larvae. None of the oils showed toxicity or synergistic effects on *Ae. aegypti* adult mosquitoes (Tong and Bloomquist, 2013). Lakshmi et al. (2021) undertook an experiment to investigate the synergistic effect of *C. bonplandianum* with two pyrethroid insecticides, cypermethrin and lambda-cyhalothrin, against *Ae. aegypti*. The study examined both the individual larvicidal

properties of these compounds and their synergistic effects. The findings revealed that synergism significantly enhanced the larvicidal activity of these compounds and was effective against *Ae. aegypti* larvae. In a study on the synergistic effect of bioactive monoterpenes against *Cx. pipiens* mosquitoes, researchers investigated the insecticidal impact of various binary monoterpene combinations utilizing a synergistic design approach. The findings indicated that all the individual monoterpenes, when tested within these binary mixtures, worked together to enhance the effectiveness of the insecticide (Ramzi et al., 2022). A study exploring the synergistic repellent and irritant effects of a mixture comprising β-caryophyllene oxide and vetiver oil against mosquito vectors demonstrated that this combination had a more substantial impact on mosquitoes than the individual compounds. The blend of β-caryophyllene oxide and vetiver oil exhibited an additive contact irritability effect, functioning as a noncontact repellent and led to knockdown activities at lower concentrations, indicating that utilizing these two repellent compounds in combination could result in a more effective mosquito repellent than using a single compound (Nararak et al., 2023).

Many research investigations have underscored that distinct mosquito species utilise a range of resistance mechanisms, emphasising the crucial role played by enhanced metabolic detoxification of insecticides via detoxifying enzymes (Hemingway and Ranson, 2000). Within the framework of mosquito insecticide metabolism, three enzyme families, namely, cytochrome P450 monooxygenases (P450), glutathione transferases (GST), and carboxy/cholinesterases (CCE) are intricately involved. These enzyme groups each catalyse a wide range of detoxification reactions and serve as the primary enzymatic defence against xenobiotics, which are foreign substances. Furthermore, they are responsible for clearing many by-products of metabolism, hold essential roles in numerous biosynthetic pathways, and actively participate in chemical communication processes (Feyereisen, 2005; Ranson and Hemingway, 2005; Strode et al., 2008; Oakeshott et al., 2010). The rapid increase in both numbers and variety of these enzymes, referred to as 'detoxification enzymes', in insects can be attributed to their evolutionary adaptation to a broad spectrum of natural xenobiotics found in their environment (Després et al., 2007; Marcombe et al., 2009).

Research findings have shown that changes in carboxylesterase activity are generally associated with resistance to organophosphate insecticides in many insects. The specific modifications in activity vary significantly depending on the sensitivity of the insects and differences between strains. Uplifted esterase activity is the underlying factor behind resistance to organophosphates, carbamates, and pyrethroid insecticides (Terriere, 1984; Oppenoorth, 1985). Esterases represent a category of enzymes linked to resistance in mosquitoes against organophosphates, carbamates, and, to a lesser extent, pyrethroids (Hemingway and Ranson, 2000; Sogorb and Vilanova, 2002). Glutathione S-transferases are actively involved in the detoxification process of xenobiotics, including insecticides (Hemingway et al., 2004; Gan et al., 2021). Cytochrome P450 monooxygenase is implicated in developing resistance to both organophosphates and pyrethroids. The heightened levels of these enzymes are accountable for the accelerated degradation of insecticides, ultimately leading to a modification in the susceptibility status of mosquitoes (Paeporn et al., 2004a; Adhikari et al., 2022). An accurate assessment of the susceptibility or resistance of a species to insecticide compounds could be achieved by conducting a quantitative enzymatic assay on these detoxifying enzymes (Jangir and Prasad, 2022).

GENERAL METHODOLOGY

Study Sites for Vector Collection

Four areas of Thrissur district, Kerala, India, were selected for the mosquito vector species sampling. The chosen regions include two urban areas, Thrissur Corporation Area (10.519682N latitude, 76.228148’ longitude) and Irinjalakuda Municipal Area (10.349122’ N latitude, 76.214167’E longitude), and two semi-urban areas, Muthuvara (10.553486’ N latitude, 76.177160E’ longitude) and Palakkal (10.474126’ N latitude, 76.215130’E longitude).

Mosquito Sampling

Sampling Methods

This study employed random sampling methods. Eggs were collected from both natural oviposition sites and artificial ovitraps. The larval collection was carried out using pipettes, dippers, and aquatic nets. Adult specimens were collected through attractant and non-attractant traps, sweeping insect nets, and an aspirator. The diversity study spanned from January 2016- December 2016. The identification of collected specimens was done using standard taxonomical keys. Various indices such as Dominance (D), Simpson's (1-D), Shannon (H), Evenness (H/S), Margalef, Biodiversity index, and Berger-Parker were utilised to demonstrate the alpha diversity of the study area. Jaccard and Sorenson diversity indices were used to compare two individual sites, and gamma diversity analysis was also conducted. The diversity analysis was performed using the 'PAST' software.

GIS Data Preparation

GPS coordinates were documented for every sampling location of mosquito species data collection throughout the diversity study timeframe. These records were then utilised in preparing the geographical information system (GIS). The ArcGIS software was employed for GIS preparation.

DNA Barcoding

Collection and Preservation

Mosquitoes were collected, morphologically identified, and preserved in 70% ethanol at -20ºC. Each specimen was assigned with a unique voucher number for future reference.

Extraction of Genomic DNA and PCR Amplification

Thoracic legs of mosquitoes were used to extract genomic DNA with the ORIGIN Genomic DNA Isolation Kit. Agarose gel electrophoresis was carried out to confirm DNA present in the reaction mixture. The process of amplification was conducted using a DNA thermal cycler (Takara). The amplification was achieved through 30 cycles, each comprising an initial denaturation at a temperature of 95°C (10 seconds), followed by annealing at 50°C (1 minute), and extension at 72°C (45 seconds), completed by a final extension at 72°C (3 minutes).

The forward and reverse primers were (5'-GGTCAACAAATCATAAAGA TATTGG-3') and (5'-TAAACTTCAGGGGGACCAAAAAAAAAAAATCA-3'), respectively. The initial step of the process involved denaturation at 95°C for 5 minutes.

Agarose Gel Electrophoresis

PCR-obtained products were subjected to separation by electrophoretic technique using a 2% TAE agarose gel stained with EtBr. The size of the product was determined using a Gene Ruler (Thermo Scientific; GeneRuler 100bp DNA Ladder, #SM0242). EtBr functioned as an intercalating agent within the DNA bases, emitting an orange colour by DNA under ultraviolet light.

PCR Product Purification

After completing the PCR amplification of the COI fragment of the specimen, the remaining PCR-obtained product was purified using the Fermentas GeneJET PCR purification kit. The GenElute TM PCR Clean-up Kit was specifically developed for the rapid PCR amplified product purification from surplus components present in the reaction mixture, including excessive DNA polymerase, primers, nucleotides, salts and oils. Subsequently, the purified product was once again subjected to examination by being separated on a 2% agarose gel to verify the existence of DNA.

DNA Sequencing and Phylogenetic Analysis

The previously purified PCR- obtained product was sequenced using the forward and reverse primers employed in the PCR process. This sequencing was performed using Sanger's method and was conducted at Sci Genom Labs Private Ltd., Cochin, employing the ABI 3730XL automated sequencer. The obtained COI sequences from both forward and reverse directions were aligned using ClustalW, and this region was considered to be the final product sequence. This final sequence was then subjected to a species confirmation search on NCBI BLAST. After species confirmation, the aligned final COI gene sequence was submitted to GenBank (NCBI) for global access, which could serve as the molecular barcode of the species. The final nucleotide sequences were analysed through MEGA XI to understand the phylogenetic relationships using the neighbor-joining algorithm (Saitou and Nei, 1987). The inter- and intraspecific genetic diversity was calculated using the Kimura 2 parameter model.

Mosquito Colony Maintenance

A laboratory-reared *Ae. aegypti* mosquito colony from the Communicable Disease and Research Laboratory (CDRL)was used for the current study. Adult mosquitoes were reared in sterilized mosquito cages fitted with netting, maintaining 27±2°C constant temperature, 75–85% relative humidity, and a 14:10 light-dark photoperiod. Their nutrition included soaked raisins and blood feeding of adult female mosquitoes, which were started on the third day after emergence (Munstermann, 1997).

Screening of Plants

Thirty plants representing a broad spectrum of families and genera were collected from the Thrissur district of Kerala, India, under the direction of a detailed examination of the literature. The selection of plants was mainly based on their potential medicinal value, antimicrobial activity, aromatic nature and efficacy as pesticides or insecticides. A Soxhlet apparatus was used to perform extractions with different solvents during the initial screening of plants. A preliminary screening involved the preparation of a 1mg/ml (1000ppm) concentration of plant extract using three different solvents with varying polarities. 25 healthy fourth instar *Ae. aegypti* larvae were put into each beaker. Six replicates were arranged for each

extract. *Ae. aegypti* larvae were considered dead for evaluating mortality, if they did not show outward movement for respiration. The dead larvae from six test replicates were pooled and the percentage of larval mortality for each test concentration was calculated (Aivazi and Vijayan, 2009).

Plant Extraction for Bioassay

Plant leaves were collected, washed with clean water for the larval bioassay, and then dried in the shade. A hand mixer grinder was used to grind the dried plant parts into a fine powder. A Dionex ASE 150 accelerated solvent extractor was used for taking the plant extract. Solvents with different polarities were used for extraction. The collected extracts were then transferred to HS-2005V-N Rotary Flash evaporator and the remaining excess solvent contents were removed by evaporation. Accelerated solvent extraction, rotary evaporation and lyophilizaion were done at Central Instruments Laboratory, Kerala Veterinary and Animal Science University, Mannuthy, Kerala. Stock solution was prepared by dissolving 1mg extract in acetone (Kraujalis et al., 2013; Kettle et al., 2016)

Isolation and Identification of Bioactive Compound

Gas Chromatography-Mass Spectrometry (GC/MS) was used for the preliminary investigation of plant extract compounds. Based on the data collected, further appropriate separation techniques were selected. Column chromatography and Thin layer chromatography techniques were employed for the isolation of the bioactive compound of the selected plant. Chloroform and hexane in a 1:1 ratio was used as mobile phases for TLC separation. The most efficient fraction was selected. That fraction was then separated through column chromatography, in which a 1:1:5 ratio of three different solvents, hexane, chloroform, and ethyl acetate, was used as the mobile phase. Next, column chromatographic separation was conducted by following a modified technique by Bajpai et al. (2016). The final fraction obtained was then subjected to TLC using chloroform and ethyl acetate in a 2:1 ratio as the mobile phase. GC/MS analysis of the isolated compound was done at Care Keralam PVT. Ltd., Kinfra Park, Koratty, Thrissur, Kerala, India. NMR was done to understand the structure of the purified isolated compound.1H-NMR spectra and 13C-NMR spectra were documented on VNMRS-400 "Agilent-NMR" spectrophotometer using Dimethyl sulfoxide (DMSO) as solvent at Central

Instrumentation and Research Facility, Institution of Excellence, University of Mysore, Mysuru, India.

Plant Larval Bioassay

The larval bioassay was conducted following the WHO protocol (WHO, 2005a), adapted as needed for the particulars of the study. Test concentrations of the isolated bioactive compound were formulated by adding 1 ml of the compound concentration to the test containers and made up to 250 ml by adding 249 ml of dechlorinated tap water. For comparison, a control was established with 1 ml of acetone mixed with 249 ml of water in separate containers for each trial. Twenty-five *Ae. aegypti* larvae were placed into both the test concentrations and the control group in each setup, with six replicates per condition, and the experimental setup was left undisturbed for 24 hours. LC_{50} and LC_{90} values for the plant-isolated bioactive compound were determined through dosage-mortality regression analysis employing probit analysis (Finney, 1971).

Insecticide Larval Bioassay

Larval susceptibility determination was adhered to the established WHO protocol (Brown, 1986). The fourth instar larvae of *Ae. aegypti* were exposed to varying concentrations of insecticides, which were prepared by mixing stock solutions with distilled tap water. Stock solutions of 1mg/ml cypermethrin, lambda-cyhalothrin, malathion, and temephos were prepared in water. To prepare test concentrations, 1 ml of the insecticide solution was combined with 249 ml of water in a 500 ml beaker, and the test solution was vigorously mixed for 30 seconds with a glass rod. Test controls were made by adding 1 ml of solvent solution, instead of an insecticide solution, to 249 ml of dechlorinated water. A strainer was used to transfer 25 late third or early fourth instar larvae into each beaker, which held separate test and control setups. For the larval bioassay, six successive insecticide test concentrations were created. Mortality was documented after 24 hours, and larvae that were immobile were considered dead.

The LC_{50} and LC_{90} values for the pesticides were calculated using probit analysis in dosage-mortality regression analysis (Finney, 1971).

Synergistic and Co-toxicity Coefficient Assay of Bioactive Plant Isolate and Insecticides

Two separate experimental protocols were developed to determine the Synergistic factor (SF) and the Co-toxicity coefficient (CTC), both concentrating on investigating the interaction between the plant extract and the insecticide as amalgamated product (Kalyanasundaram and Das 1985). These experiments aimed to understand the possible combined effects of each chemical insecticide and the bioactive compound isolated from the plant against *Ae. aegypti* larvae.

In particular, the investigation into the Co-toxicity coefficient aimed to determine the extent to which the combined mixture influenced mortality rates in comparison to expected outcomes. Similarly, evaluating the Synergistic factor aimed to elucidate the degree of synergy or antagonism observed between the insecticide and plant isolate.

Selection Experiment

A susceptible laboratory-reared population of *Ae. aegypti* was exposed to the synergistic combination SC4 over five consecutive generations, and The F0 generation, selected as the susceptible strain, was established after 25 generations of laboratory colonization at CDRL without any previous insecticide exposure. The bioassay experiments were conducted following the WHO (Brown, 1986) protocol. Resistance Ratio (RR) assessment was performed in accordance with the WHO, 2016a protocol.

Quantitative Enzymatic Assay

Quantitative enzyme assays were employed to analyse the essential detoxifying enzymes. The primary objectives of these assays were to elucidate the mode of action and to monitor the potential development of resistance in the chosen mosquito species against SC4. The procedure outlined by Kranthi (2005) was employed to determine Acetylcholinesterase and Carboxylesterase activities. An altered methodology based on the technique established by Habig et al. (1974) was employed to evaluate the specific enzymatic activity of Glutathione S-transferase. The specific enzymatic activity of cytochrome P450 was assessed using the approach detailed by Khan et al. (2020).

Antimicrobial Assay of Plant-Isolated Compound

Antimicrobial susceptibility testing, based on the agar well diffusion method described by Murray et al., 1995 and modified by Olurinola, 1996, was conducted on solid agar media placed in petri plates against three strains of bacteria.

CHAPTER I

Molecular Identification of Important Vector Mosquito Species from Selected Sites of Thrissur District, Kerala, India

1.1 Introduction

Taxonomy is an analytical and dynamic branch in biology due to its requirement for constant renewal, as novel species are being revealed and some previous species demand changes in their classification. Nevertheless, it is also considered imprecise when the morphology of a specimen is the only consideration for its identification and classification. To overcome this issue, taxonomists are now relying on a further advanced approach that integrates a more accurate branch of the system, molecular biology, in which a specific region of DNA of the species is separated, amplified, and equated for identification. This technique, termed DNA barcoding, consists of the isolation, magnification, and identification of petite but exceedingly conservative sequences of DNA ranging in length from 400 to 800 base pairs (Hebert et al., 2003a; Ball and Armstrong, 2006). Not all taxonomically distinct groups that are defined exclusively by physical characters require barcoding. Although these characteristics may be adequate to differentiate between specific taxonomic groups, molecular barcoding provides a potent substitute. When physical characteristics are insufficient for accurate identification, such as in the case of subspecies where genetic differences may exist despite physical similarities or the classification of cultivated plant varieties bred for particular traits, this method becomes especially useful. Moreover, ecological variants of different populations of the same species adapted to certain environments can be studied using molecular barcoding. Molecular barcoding can even help to distinguish morphological mutants or organisms within a species with unique physical characteristics. By comparing the DNA barcode with sequences from other taxonomic groups, this technique enables precise identification and facilitates the preparation of a taxonomic record for a specimen. In discrepancies, molecular phylogenetic analyses employing molecular operational taxonomic units can help to establish accurate species identification (Floyd et al., 2002; Naddaf et al., 2012).

Mitochondrial DNA (mtDNA) has gained widespread use in animal phylogenetic research due to its rapid mutation rate, which far exceeds that of nuclear DNA. This accelerated mutation rate leads to the accumulation of genetic differences among

closely related species, as documented by various researchers (Moore, 1995; Mindell, 1997; Brown et al., 1999). The rapid pace of sequence evolution in mitochondrial DNA (mtDNA) leads to noticeable genetic differences even among groups that have experienced relatively short periods of isolation. The revolutionary work of scientist John Avise marked the initial recognition of these distinctions in mtDNA sequences, highlighting their significance as a repository of the evolutionary history of an organism within a species. This profound perception has played a pivotal role in connecting the domains of population genetics and systematics, ultimately giving rise to the flourishing field of phylogeography (Avise and Zink, 1988).

Because genetic sequence variations between species are usually much more pronounced than within species, mtDNA sequences are often used as genetic markers to identify separate species. This method increased the understanding of the evolutionary relationships between animal species and improved the accuracy of animal species classification. Over the past two decades, DNA sequencing technology has made major advances, transitioning from manual to automated sequencers. For example, an automated 96-capillary sequencer can produce over a thousand sequences with 1000 base pairs in a single day. This technical breakthrough has made DNA sequencing more accessible to everyone, even those without a background in genetics. The development of DNA barcoding is closely intertwined with these remarkable advancements (Hebert et al., 2003a; Hebert and Gregory, 2005; Schindel and Miller, 2005; Valentini et al., 2009).

Globally, mosquitoes exert a significant influence on public health. Although mosquitoes have received more diligence than most other insect varieties due to their role as disease vectors, the systematic understanding of these insects still needs to be completed. A considerable number of the species are marked as vectors owing to their competency to transmit pathogens from one living organism to another, making their identification crucial. Most species are primarily found within the *Anopheles*, *Aedes*, and *Culex* genera. These species are known for their role in transmitting a wide range of vector-borne diseases that cause substantial morbidity and mortality among humans, exceeding the impact of any other group of organisms (Murugan et al., 2016). These mosquito-borne diseases involve dengue fever, zika, malaria, Japanese encephalitis, West Nile fever, lymphatic filariasis, and yellow

fever. With the rising incidence rates and the absence of effective preventive measures and vaccines for some of these mosquito-borne illnesses, significant outbreaks of these diseases place a considerable strain on the healthcare systems and economies of various nations (Kuno and Chang, 2005; Weaver and Reisen, 2010; Liang et al., 2015; Atoni et al., 2019; Atoni et al., 2020).

For several important reasons, identifying vector mosquitoes is essential for disease control and public health. First, it is possible to apply focused prevention strategies targeted to specific mosquito species. Because of their diverse habits, breeding locations, and insecticide susceptibilities, mosquitoes must be precisely identified to implement effective interventions. Second, identifying vector mosquitoes is essential to disease surveillance since it keeps track of the quantity and presence of these vectors, which can be used to predict possible outbreaks and provide early warning systems. This proactive approach makes timely responses and resource allocation to high-risk areas possible. Because some sibling species have morphological characteristics that are closely related, it may become difficult to identify and distinguish them. Identification of mosquito species is mainly done by analysing the physical characteristics. This method can be challenging because distinguishing morphological traits are often lost in the process of being captured or preserved, or they may only be visible at some stages of development. Furthermore, only expert mosquito taxonomists can accurately distinguish between mosquito species due to the minor differences in physical characteristics. The vectorial status of mosquito species demands accurate and immediate identification to prepare effective control methods (Cook et al., 2005; Patsoula et al., 2006; Kumar et al., 2007; Verna and Munstermann, 2011; Versteirt et al., 2012; Versteirt et al., 2015).

Additionally, understanding the transmission dynamics of mosquito-borne diseases is greatly enhanced through vector identification. Researchers can reach into the behaviour, distribution, and feeding habits of specific vector species that aid in predicting disease transmission patterns and crafting preventive measures. Moreover, the knowledge of vector mosquitoes contributes to developing vaccines and treatments specifically targeting the pathogens carried by these vectors, potentially leading to more effective medical interventions. As climate change continues to alter the geographical range and behaviour of vector mosquitoes, identification becomes even more critical to adapt strategies to emerging disease

risks (Chaiphongpachara, 2018; Sumruayphol et al., 2020; Chaiphongpachara et al., 2022).

In India, the primary vector mosquitoes crucial for disease transmission belong mainly to four genera: *Culex*, *Aedes*, *Anopheles*, and *Armigeres* (Mandal, 2012; Dev and Sharma, 2013; Moirangthem and Singh, 2018; Manikandan et al., 2022). The increased rates of disease transmission carried by mosquito vectors are frequently documented in densely populated regions, with a notable concentration in urban and semi-urban areas. Densely populated areas, such as urban and semi-urban regions, create an advantageous environment for the intensified transmission of diseases by mosquito vectors. Several factors contribute to this phenomenon, including the proximity of individuals, increased human activities, and, often, inadequate sanitation and waste management practices. These conditions create a fertile ground for mosquitoes to thrive and transmit diseases efficiently (Dev et al., 2014; Dávalos-Becerril et al., 2019). Kerala has witnessed a continued prevalence of mosquito-borne diseases throughout the year for decades. The annual reports of casualties and deaths due to this indicate the need for attention in this area (Table 1.1). The integration of molecular techniques and an extensive understanding of vector mosquitoes play critical roles in addressing the challenges of mosquito vector-borne diseases, contributing to more operative public health strategies in an evolving global landscape.

Table 1.1: Confirmed cases of mosquito- vector borne diseases in Kerala 2010-2022 (dhs.kerala.gov.in, https://dhs.kerala.gov.in/data-on-communicable-diseases/)

Year	Dengue Fever		Malaria		Chikungunya		Japanese Encephalitis		West Nile fever		Zika	
	Case	Death	Case	Death	Case	Death	Case	Death	Case	Death	Case	Death
2010	2597	17	2299	7	210	0	20	5	0	0	0	0
2011	1304	10	1993	2	81	0	102	8	33	0	0	0
2012	4056	16	2036	3	62	0	3	1	0	0	0	0
2013	7938	29	1634	0	247	0	2	0	0	0	0	0
2014	2548	13	1751	6	264	0	3	2	0	0	0	0
2015	4114	29	1549	4	152	0	0	0	0	0	0	0
2016	7218	21	1540	3	124	0	1	0	0	0	0	0
2017	21993	165	1194	2	54	0	1	0	0	0	0	0
2018	4090	32	908	0	76	0	5	2	1	0	0	0
2019	4651	14	656	1	109	0	11	2	11	2	0	0
2020	2722	22	268	1	558	0	0	0	0	0	0	0
2021	3251	27	309	1	334	0	0	0	0	0	90	0
2022	4468	58	439	0	66	0	2	0	3	1	15	0

1.2 Methodology

1.2.1 Vector Mosquito Diversity

1.2.1.1 Study Site

Four distinct locations within Thrissur district, Kerala, India, were selected for the sampling of vector species. These selected regions included two urban areas and two semi-urban areas, namely SITE 1: Palakkal (10.474126°N latitude and 76.21513°E longitude), SITE 2: Thrissur Corporation Area (10.519682°N latitude and 76.228148 °E longitude), SITE 3: Muthuvara (10.553486°N latitude and 76.17716°E longitude), and SITE 4: Irinjalakuda Municipal Area (10.349122 °N latitude and 76.214167 °E longitude).

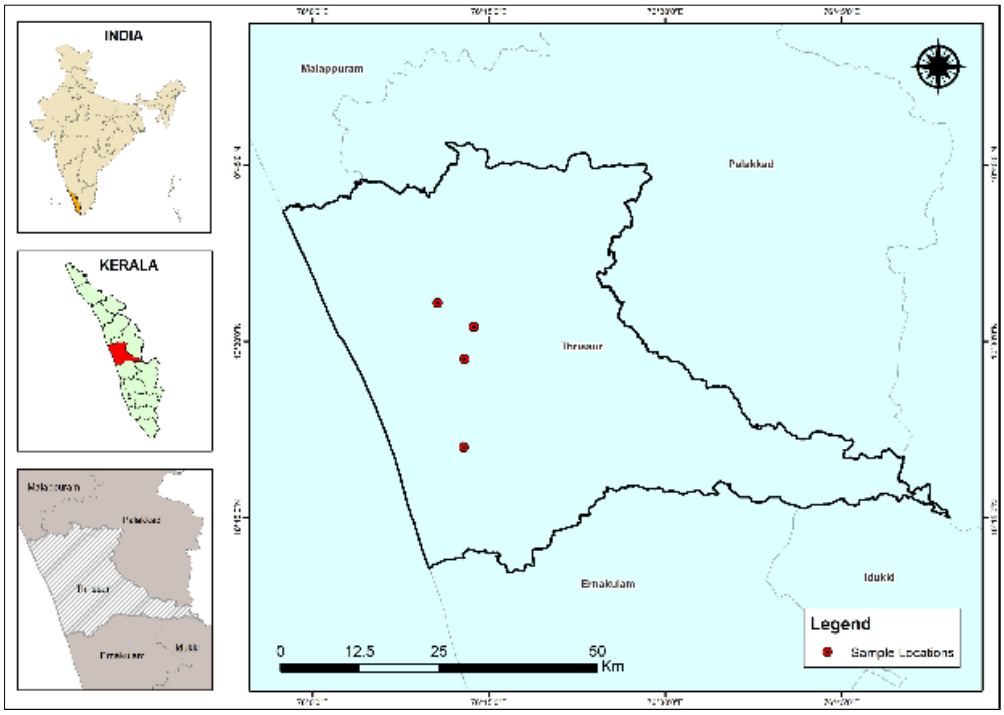

Figure 1.1 Location map of study sites

1.2.1.2 Sample Collection

Eggs: Mosquito eggs, the initial stage of the mosquito life cycle, were obtained from their natural breeding sites or artificial ovitraps. Floating eggs were collected using aquatic nets, pipettes, and dippers. These collected eggs were carefully labelled, transported to the laboratory, and carried out the larval and adult mosquito rearing and identification processes.

Larvae: Juvenile mosquito larvae were collected from diverse breeding sites employing pipettes, aquatic nets, and dippers. The specimens were placed in appropriately labelled plastic containers and transported to the laboratory. To facilitate identification, the larvae were subjected to hot water treatment at 40°C and examined using taxonomical keys (Barraud, 1934; Christopher, 1933; Black, 1968; Gilles, 1993; Das and Kaul, 1998; Nagpal and Sharma 1995; Tyagi et al., 2015) under a stereo-zoom microscope (Leica-M205C). The larvae, which were difficult for precise species-level identification at the juvenile stage, were reared into adults. Unidentified larvae of collected specimens were raised into adults under controlled laboratory conditions (temperature 26±2°C) using a larval diet. Adult female mosquitoes were fed on 5% sucrose solution and were allowed to take blood meal on the third day following their emergence.

Adult: Various attractant and non-attractant traps, including light and CO_2 traps, were used together with sweeping nets and an aspirator to gather adult mosquitoes in both resting and host-seeking states. After being collected, these adult mosquitoes were brought to the lab for identification. Adult specimens captured from the study field and those raised in the laboratory were carefully examined and dissected under a stereo-zoom microscope (Leica-M205C). Species identification was achieved using established morphological keys (Barraud, 1934; Christopher, 1933; Black, 1968; Gilles, 1993; Nagpal and Sharma, 1995; Das and Kaul, 1998; Tyagi et al., 2015). The identified species were carefully pinned, labelled, and stored in the Communicable Disease Research Laboratory at St. Joseph's College, Irinjalakuda.

1.2.1.3 Statistical Analysis

Different diversity index analyses were conducted to evaluate the mosquito collection data from four distinct sampling sites: Site 1 (Palakkal), Site 2 (Thrissur), Site 3 (Muthuvara) and Site 4 (Irinjalakkuda). The alpha diversity of the study area included Dominance (D), Simpsons (1-D), Shannon (H), Evenness (H/S), Margalef, Biodiversity index, and Berger-Parker indices. Jaccard and Sorenson diversity indices of beta diversity were used to compare the diversity between individual site pairs. Furthermore, gamma diversity was examined to measure species diversity throughout multiple sites within the ecosystem. PAST software was used to conduct all diversity analyses.

1.2.1.4 GIS Preparation

Throughout the diversity study, GPS coordinates were noted for each sampling site and mosquito species were recorded. After that, these mosquito data were used to create Geographic Information System (GIS) maps. ArcGIS software was used for the GIS mapping.

1.2.2 DNA Barcoding

1.2.2.1 Collection and Preservation

Mosquitoes were collected from different sampling sites using various collection techniques. Once morphologically identified, these mosquitoes were placed into individual glass vials containing 70% ethanol and preserved at -20º C. Each vial was marked with a specific code number and was kept as a voucher specimen for future reference.

1.2.2.2 Genomic DNA Extraction and PCR Amplification

Thoracic legs were used to extract Genomic DNA from a morphologically identified mosquito species, following the ORIGIN Genomic DNA isolation Kit instructions outlined by Shere-Kharwar et al. (2013). The DNA amplification was performed using a Takara DNA thermal cycler, amplifying approximately 2ng of genomic DNA of the mitochondrial cytochrome oxidase subunit I (COI) gene with forward and reverse primers (forward primer: 5'-GGTCAACAAATCATAAA GATATTGG-3', and reverse primer: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al., 1994). In the PCR reaction, 2 ng (1 µl) of genomic DNA including 1 µl each of forward and reverse primers (10mM concentration),1 µl of dNTPs (2mM), 5 µl of 10X reaction buffer with $MgCl_2$, 0.5 µl of Taq polymerase (5 U/µl), and 41.5 µl of water was used. Amplification consisted of 30 cycles, starting with an initial denaturation at 95°C for 5 minutes, followed by 10-second denaturation at 95°C, 1-minute annealing at 50°C, 45-second extension at 72°C for each cycle, and a final 3-minute extension at 72°C.

1.2.2.3 Agarose Gel Electrophoresis

The PCR samples were separated on a 2% TAE agarose gel, following the protocol outlined by Mahesh et al. in 2012. Afterwards, it was treated with EtBr, described by Sambrook and Russell in 2001, and photographed using a gel documentation

system. To recognize the product's size, a Gene Ruler (Thermo Scientific; GeneRuler 100bp DNA Ladder, Catalog #SM0242) was employed. EtBr was used as an intercalating agent within the DNA base pairs to emit an orange glow when exposed to ultraviolet light.

1.2.2.4 PCR Product Purification

The purification of PCR-obtained product was done using the Fermentas GeneJET PCR purification kit after completing COI fragment amplification by PCR. This purification kit is specially designed for the rapid purification of single-stranded or double-stranded PCR amplification products by removing additional DNA polymerase, primers, nucleotides, salts, oils, and other contaminants from the PCR-obtained product. After that, another evaluation on a 2% agarose gel of the purified product was done to ascertain the DNA present.

1.2.2.5 DNA Sequencing and Phylogenetic Analysis

The purified PCR product was then sequenced at Sci Genom Labs Private Ltd. An ABI 3730XL automated sequencer was employed for the sequencing. The methodology followed was Sanger's sequencing method, using the forward and reverse primers used for the PCR amplification. ClustalW was used to align the forward- and reverse-direction trimmed COI sequences. The aligned region of the sequence was considered the final product sequence (Thompson et al., 1994). The sequence was then run for similarity searches using the NCBI's BLAST n and BLAST p programs (Altschul et al., 1990). After that, the partial COI gene sequence was deposited into GenBank (NCBI) for global access. MEGAXI (Tamura et al., 2013) was used for the phylogenetic analysis of the final sequence. A phylogenetic tree was constructed using a neighbour-joining algorithm, and interspecific and intraspecific genetic diversity was analysed using the Kimura 2 parameter model (Saitou and Nei, 1987). Percentage nucleotide distance calculations were conducted using MEGAXI.

1.3 Result

1.3.1 Vector Mosquito Diversity and Diversity Analysis

During the data collection period, spanning from January 2016 to December 2016, a total of 24,778 mosquito specimens were collected from the research site. These

samples represented 11 distinct species distributed among four genera, *Anopheles*, *Aedes*, *Culex* and *Armigeres*. The mosquito collection was partitioned across the study sites as follows: Site 1 yielded 4,201 mosquitoes, Site 2 contributed 6,285, Site 3 yielded 6,606, and Site 4 had the highest count with 7,686 mosquitoes. The identified species were *Anopheles stephensi* with 157 individuals, *Anopheles subpictus* with 324, *Anopheles vagus* with 149, *Aedes aegypti* with 4,124, *Aedes albopictus* with 3,822, *Aedes vittatus* with 134, *Armigeres subalbatus* with 1,648, *Culex gelidus* with 2,200, *Culex pipiens* with 2,831, *Culex quinquefasciatus* with 3,944, and *Culex tritaeniorhynchus* with 5,445 individuals (Table 1.2).

Different diversity indices of the collected vector mosquito species during the study period were analysed and listed in Table 1.3. As indicated by the alpha diversity index, species richness varied across the four studied sites, Site 1 with 11species, Site 2 with 9 species, Site 3 having 11 species, and Site 4 with 10 species. This information provided an understanding of the diversity of species present within each Site, with Site 1 and Site 3 demonstrating higher species richness, indicating more diverse ecosystems. Site 2 and Site 4 showed slightly lower species richness but represented moderately diverse environments. The Dominance (D) index, which measured species dominance, differed across the four Sites, with Site 1 having a value of 0.1594, Site 2 at 0.1669, Site 3 at 0.1534, and Site 4 at 0.146. Lower Dominance (D) values suggested a more evenly distributed community, while higher values indicated a dominance of one or a few species. In this context, Site 4 exhibited the lowest Dominance (D) value, indicating a relatively even distribution of species, while Site 2 displayed the highest value, suggesting a higher degree of dominance by specific species.

The Simpson (1-D) index, reflecting species diversity and dominance, varied among the Sites, with Site 1 at 0.8406, Site 2 at 0.8331, Site 3 at 0.8466, and Site 4 at 0.854. Higher Simpson(1-D) values signified greater diversity and a reduced impact of dominant species, making Site 4 the most diverse among the Sites. In contrast, the Shannon (H) index, which assessed diversity and evenness, ranged from Site 1 at 1.973, Site 2 at 1.902, Site 3 at 1.992, and Site 4 at 2. Higher Shannon (H) values indicated increased species diversity and evenness, with Site 4 emerging as the most diverse Site overall. The Evenness(e^H/S) index differed across the four Sites, with Site 1 at 0.6541, Site 2 at 0.7445, Site 3 at 0.6665, and Site 4 at 0.7389. Evenness

assessed the even distribution of species, with higher values indicating a more balanced ecosystem. Site 2 exhibited the highest Evenness value, suggesting a relatively even distribution of species, while Site 3 had the lowest value, indicating a slightly less even distribution.

The Berger-Parker index, measured species dominance, varied with Site 1 at 0.2472, Site 2 at 0.262, Site 3 at 0.2187, and Site 4 at 0.1954. Lower Berger-Parker values indicated less dominance by a single species, indicating a more balanced community. Site 3 had the lowest Berger-Parker value, suggesting a more evenly distributed community, while Site 2 had the highest value, indicating a slightly higher dominance of a single species. The Margalef index, suggesting species richness, ranged from Site 1 at 1.118, Site 2 at 0.9594, Site 3 at 1.137, to Site 4 at 1.029. Higher Margalef values indicated greater species richness, reflecting a more diverse ecosystem. Site 3 exhibited the highest Margalef value, indicating the highest species richness, while Site 2 had the lowest value, suggesting slightly lower species richness.

Beta diversity indices were calculated for pairwise combinations of the four studied Sites. Jaccard's index values between Site 1 and Site 2, Site 1 and Site 3, Site 1 and Site 4, Site 2 and Site 3, Site 2 and Site 4, and Site 3 and Site 4 were 0.676, 0.634, 0.639, 0.697, 0.703, and 0.658, respectively. Sorensen's index values for the same pairwise combinations were 0.807, 0.776, 0.780, 0.821, 0.825, and 0.793, respectively. The beta diversity indices quantified the dissimilarity in species composition between pairs of ecological Sites. Higher values indicate more significant dissimilarity, suggesting that the species composition in those pairs of Sites was distinct. Jaccard's and Sorensen's indices provided insights into the ecological dissimilarity between different combinations of the studied Sites, aiding in understanding the variation in species composition among these Sites. The gamma diversity value was calculated as 11, which indicated the total species diversity across all the ecological Sites or areas under consideration. It represented the overall number of unique species found when considering all the Sites together as a single entity or ecosystem. A GIS map of vector mosquitoes according to their collection Sites was prepared, as shown in Figure 1.2. Figure 1.3 represents the Site wise abundance graph of collected vector mosquito species during the study time.

Table 1.2: Total number of vector mosquito species collected during the study period.

Sl No.	Mosquito species	Site 1	Site 2	Site 3	Site 4	Total
1	*An. stephensi*	41	41	41	34	157
2	*An. subpictus*	89	63	76	96	324
3	*An. vagus*	59	38	11	41	149
4	*Ae. aegypti*	587	1066	1445	1026	4124
5	*Ae. albopictus*	613	895	1164	1150	3822
6	*Ae. vittatus*	-	-	79	55	134
7	*Ar. subalbatus*	-	611	498	539	1648
8	*Cx.gelidus*	558	564	487	591	2200
9	*Cx. pipiens*	461	745	841	784	2831
10	*Cx. quinquefasciatus*	697	1034	743	1470	3944
11	*Cx. tritaeniorhynchus*	1096	1228	1221	1900	5445
TOTAL		**24778**				

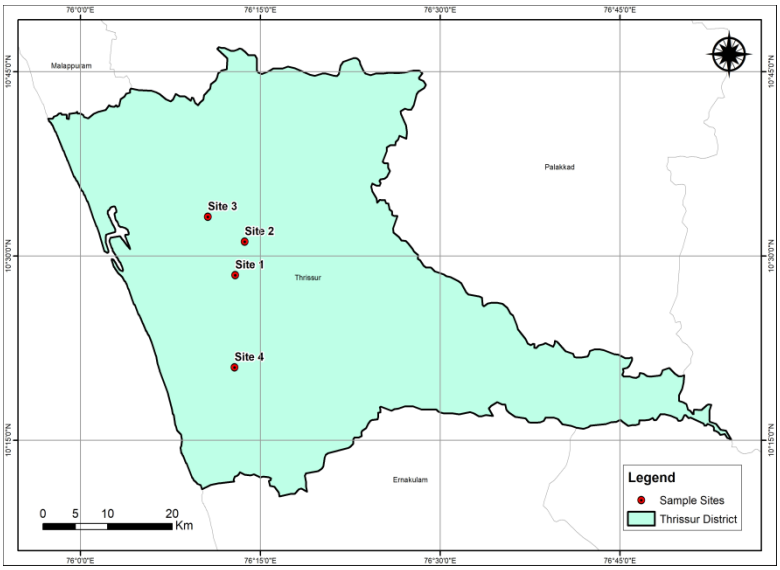

Figure 1.2 GIS map of sampling sites

List of mosquito species collected from the study area

Site 1 **PALAKKAL** 10.474126°N 76.21513°E
1.*An. stephensi*
2.*An. subpictus*
3.*An. vagus*
4. *Ae. aegypti*
5. *Ae. albopictus*
6. *Ae. vittatus*
7.*Ar. subalbatus*
8.*Cx. gelidus*
9.*Cx. pipiens*
10.*Cx. quinquefasciatus*
11.*Cx. tritaeniorhynchus*

Site 2 **THRISSUR CORPORATION AREA** 10.519682°N 76.228148 °E	**Site 3** **MUTHUVARA** 10.553486°N76.17716°E	**Site 4** **IRINJALAKUDA MUNCIPAL AREA** 10.349122°N76.214167 °E
1.*An. stephensi*	1.*An. stephensi*	1.*An. stephensi*
2.*An. subpictus*	2. *An. subpictus*	2.*An. subpictus*
3.*An. vagus*	3.*An. vagus*	3.*An. vagus*
4. *Ae. aegypti*	4. *Ae. aegypti*	4. *Ae. aegypti*
5. *Ae. albopictus*	5. *Ae. albopictus*	5. *Ae. albopictus*
6.*Cx. gelidus*	6. *Ae. vittatus*	6. *Ar. subalbatus*
7.*Cx. pipiens*	7.*Ar. subalbatus*	7. *Cx. gelidus*
8.*Cx. quinquefasciatus*	8.*Cx. gelidus*	8. *Cx. pipiens*
9.*Cx. tritaeniorhynchus*	9.*Cx. pipiens*	9. *Cx. quinquefasciatus*
	10.*Cx. quinquefasciatus*	10. *Cx. tritaeniorhynchus*
	11.*Cx. tritaeniorhynchus*	

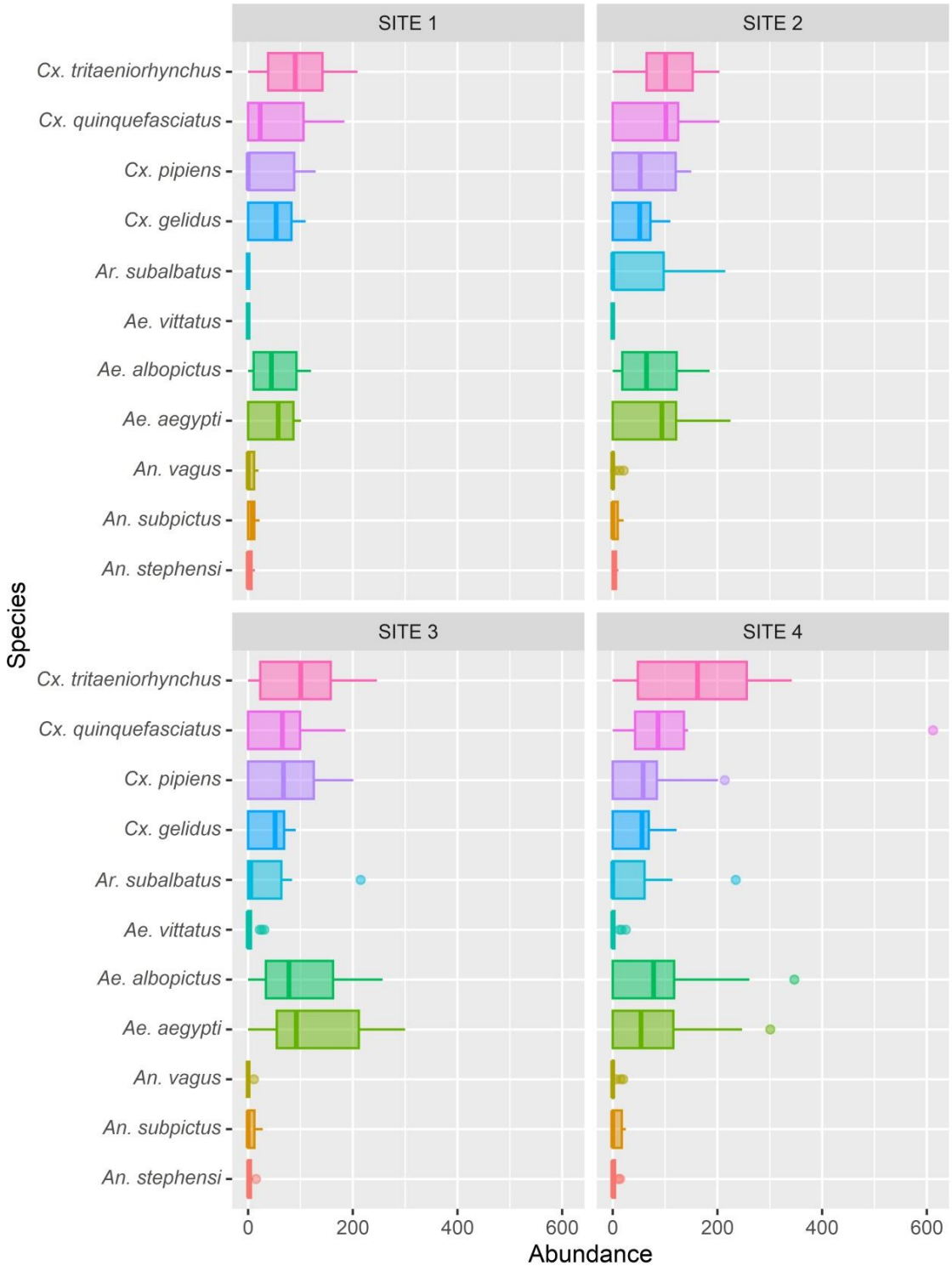

Figure 1.3 Site wise abundance graphs of collected vector mosquitoes

Table 1.3: Different diversity indices of collected vector mosquito species during the study period

Alpha Diversity Indices			**Site1**	**Site2**	**Site3**	**Site4**
Species Richness			11	9	11	10
Dominance (D)			0.1594	0.1669	0.1534	0.146
Simpson (1-D)			0.8406	0.8331	0.8466	0.854
Shannon (H)			1.973	1.902	1.992	2
Evenness (e^H/S)			0.6541	0.7445	0.6665	0.7389
Berger-Parker			0.2472	0.262	0.2187	0.1954
Margalef			1.118	0.9594	1.137	1.029
Beta Diversity Indices	**Site1& Site2**	**Site1& Site3**	**Site1& Site4**	**Site2& Site3**	**Site2 & Site4**	**Site3& Site4**
Jaccard's	0.676	0.634	0.639	0.697	0.703	0.658
Sorensen	0.807	0.776	0.780	0.821	0.825	0.793
Gamma Diversity =11						

1.3.2 Vector Mosquito Barcoding

The collected mosquitoes underwent morphological identification before being chosen for molecular identification. Partial sequencing was performed using PCR amplification, employing a forward primer with the DNA sequence 5'-GGTCAA CAAATCATAAAGATATTGG-3' and a reverse primer with the DNA sequence 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'. All the NCBI deposited species were assigned with accession number and their details are listed in Table 1.4.

PCR amplification of COI from *An. stephensi* resulted in a single 530 bp product. This sequence was submitted to the NCBI GenBank with Accession No. MT 899149.1. Figures 1.4a to 1.4f display the image of mosquito species collected, obtained DNA sequence, its conceptual translation product, the phylogenetic tree, electropherogram, and molecular barcode.

In the case of *An. stephensi*, the BLAST analysis of the 530 bp sequence demonstrated significant homology with other *Anopheles* species. Genetic divergence analysis revealed variations between geographically isolated *An. stephensi* species and related species. *An. stephensi* specimens from Thrissur, Kerala (GenBank Accession No. MT 899149.1), showed a 100% sequence similarity to specimens from Odisha and Tamil Nadu (MN329060.1 and LR736010.1) (Table

1.5a). In the phylogenetic tree, *Anopheles* species clustered into related clades, with *An. culicifacies* (KF406660.1, KF406658.1) and *An. varuna* (MT434331.1, MT434330.1) in a different clade closer to *An. stephensi*. The average nucleotide composition throughout the species seemed to be T=40.0%; C=15.3%; A=28.2%; G=16.5% and the nucleotide composition of *An. stephensi* collected from Thrissur district was T(U)= 38.9, C= 15.1, A=29.9, and G= 16.1(Table 1.5b).

For *An. subpictus*, PCR amplification yielded a single 678 bp product. This sequence has been deposited in the NCBI GenBank and received Accession No. MK603828.1. Figures 1.5a to 1.5f present the image of mosquito species collected, obtained DNA sequence, its conceptual translation product, the phylogenetic tree, electropherogram, and molecular barcode. The data in Table 1.6a indicated that *An. subpictus* from Thrissur, Kerala, exhibited a 0.34% genetic divergence from *An. subpictus* (MT508474.1) isolated from Vietnam, a 0.86% divergence from same species (MT258530.1) isolated from Kole wetlands of Thrissur, Kerala, and a considerable 15.67% evolutionary divergence from *An. nigerrimus* (MH330206.1) isolated from Sri Lanka. The phylogenetic tree analysis (NJ method) illustrated the phylogenetic relationship of *An. subpictus* isolated from Thrissur, Kerala with other species. The average nucleotide composition throughout the species seemed to be T=39.4%; C=15.3%; A=28.9%; G=16.4% and the nucleotide composition of *An. subpictus* collected from Thrissur district was T(U)= 38.9, C= 15.4, A=28.8, and G= 16.8 (Table 1.6b).

In the case of *An. vagus*, the gene fragment underwent PCR amplification of 613 bp product, which was subsequently deposited in the NCBI GenBank with Accession No. MW199166.1. Figures 1.6a to 1.6f presents the image of mosquito species collected, obtained DNA sequence, its conceptual translation product, the phylogenetic tree, electropherogram, and molecular barcode. The evolutionary divergence analysis of *An. vagus* conducted using MEGAXI (Table 1.7a) indicated that *An. vagus* isolated from Thrissur exhibited a close relationship with the same mosquito species (MH425409.1, MF179262.1) geographically isolated in Vietnam and China, showing divergences of 1.67% respectively, and 16.98%, from the *species An. culicifacies* (KF406658.1) from Pakistan. The phylogenetic tree, using the Neighbour Joining (NJ) method, illustrated the phylogenetic position of *An. vagus* isolated from Thrissur, Kerala. The average nucleotide composition

throughout the species seemed to be T=39.3%; C=15.5%; A=28.3%; G=16.9% and the nucleotide composition of *An. vagus* collected from Thrissur district was T(U)= 36.7, C= 17.3, A=29.2, and G= 16.8 (Table 1.7b).

Ae. aegypti's gene fragment underwent PCR amplification of 681 bp product, which was then deposited in the NCBI GenBank with Accession No. MK542380.1. Figures 1.7a to 1.7f presents the image of mosquito species collected, obtained DNA sequence, its conceptual translation product, the phylogenetic tree, electropherogram, and molecular barcode. The evolutionary analyses were conducted using MEGA XI, and the data in Table 1.8a revealed that, *Ae. aegypti* from Kerala exhibited a 9.47% evolutionary divergence from *Ae. vexans* (KP954638.1) isolated in the USA and an 11.06% evolutionary divergence from *Ae. lineatopennis* (HQ398909.1) isolated from Vietnam. The phylogenetic tree, constructed through the Neighbour Joining (NJ) method, illustrated the phylogenetic position of *Ae. aegypti* isolated from Thrissur, Kerala. The average nucleotide composition throughout the species seemed to be T=40.1%; C=16.0%; A=28.0%; G=15.8% and the nucleotide composition of *Ae. aegypti* collected from Thrissur district was T(U)= 39.4, C= 17.6, A=27.7, and G= 15.3 (Table 1.8b).

A single 677 bp product was obtained for *Ae. albopictus*, which was deposited in the NCBI GenBank with Accession No. MK297326.1. Figures 1.8a to 1.8f denotes the image of mosquito species collected, obtained DNA sequence, its conceptual translation product, the phylogenetic tree, electropherogram, and molecular barcode. The data in Table 1.9a revealed that, *Ae. albopictus* exhibited 0.00% evolutionary divergence with two specimens of *Ae. albopictus* (MF148287.1, MF148270.1) isolated from Malaysia. *Ae. vexans* (KP954638.1), isolated from Malaysia, showed only 9.48% evolutionary divergence from *Ae. albopictus* that was collected from Thrissur, Kerala. The phylogenetic tree, using the Neighbour Joining (NJ) method, illustrated the phylogenetic position of *Ae. albopictus* isolated from Thrissur, Kerala. The average nucleotide composition throughout the species seemed to be T=39.3%; C=16.0%; A=28.3%; G=16.4% and the nucleotide composition of *Ae. albopictus* collected from Thrissur district was T(U)= 38.6, C= 17.3, A=28.1, and G= 16.0 (Table 1.9b).

The gene fragments of *Ae. vittatus* produced a single 527 bp product, which was deposited in the NCBI GenBank with Accession No. MT858330.1. Figures 1.9a to 1.9f presents the image of mosquito species collected, obtained DNA sequence, its conceptual translation product, the phylogenetic tree, electropherogram, and molecular barcode. The BLAST search revealed that the partial COI nucleotide sequence of *Ae. vittatus* isolated from Thrissur, Kerala, was 99.81% similar to *Ae. vittatus* that was isolated from Sri Lanka (MH330198.1, MH330197.1). *Ae. vittatus* isolated from Thrissur, Kerala, exhibited a 7.62% evolutionary divergence from *Ae. lineatopennis* which was isolated from Japan (AB738145.1) and a 17.41% evolutionary divergence from *Ma. uniformis* isolated from Japan and Mozambique (LC473705.1, LC517293.1) (Table 1.10a). The phylogenetic tree, Neighbour Joining (NJ) method, illustrated the phylogenetic position of *Ae. vittatus* isolated from Thrissur, Kerala. The average nucleotide composition throughout the species seemed to be T=40.6%; C=15.6%; A=28.5%; G=15.4% and the nucleotide composition of *Ae. vittatus* collected from Thrissur district was T(U)= 39.9, C= 16.0, A=29.6, and G= 14.5 (Table 1.10b).

For *Ar. subalbatus*, the PCR amplified sequence of the CO1 gene fragment yielded a single 666 bp product, which was subsequently deposited in the NCBI GenBank with Accession No. MK297327.1. Figures 1.10a to 1.10f presented the image of mosquito species collected, obtained DNA sequence, its conceptual translation product, the phylogenetic tree, electropherogram, and molecular barcode. The data in Table 1.11a revealed that *Ar. subalbatus* exhibited an absolute 0.00% evolutionary divergence with *Ar. subalbatus* (KJ410334.1 & MW542319.1) isolated from the Cochin and Wayanad districts of Kerala. The average nucleotide composition throughout the species seemed to be T=39.8%; C=15.7%; A=28.4%; G=16.1% and the nucleotide composition of *Ar. subalbatus* collected from Thrissur district was T(U)= 40.0, C= 17.0, A=28.0, and G= 15.0 (Table 1.11b).

Cx. gelidus's CO1 gene fragment underwent PCR amplification of 677 bp product. The sequence deposited in the NCBI GenBank under Accession No. MK630238.1. Figures 1.11a to 1.11f present the image of mosquito species collected, obtained DNA sequence, its conceptual translation product, phylogenetic tree, electropherogram, and molecular barcode. Data from Table 1.12a indicated that *Cx. gelidus* exhibited only 0.29% evolutionary divergence with *Cx. gelidus*

(HQ398895.1) that was collected from Vietnam. *Cx. declarator* (KM593055.1, KM593051.1) isolated from Colombia showed a 3.90% evolutionary divergence with *Cx. gelidus* isolated from Kerala. The phylogenetic tree (NJ) showed that *Cx. gelidus* species (MK630238.1) isolated from Thrissur, Kerala, were the closest relatives of *Cx. gelidus* (HQ398895.1) from Vietnam, both belonging to the same clade. The average nucleotide composition throughout the species seemed to be T=40.3%; C=15.5%; A=28.2%; G=16.0% and the nucleotide composition of *Cx. gelidus* collected from Thrissur district was T(U)= 40.1, C= 16.2, A=28.8, and G= 14.9 (Table 1.12b).

The CO1 gene fragment of *Cx. tritaeniorhynchus* underwent PCR amplification of a 678 bp product. The obtained sequence was deposited in the NCBI GenBank with Accession No. MH745093.1. Figures 1.12a-1.12f presented the image of mosquito species collected, obtained DNA sequence, its conceptual translation product, phylogenetic tree, electropherogram, and molecular barcode of the species. *Cx. tritaeniorhynchus* isolated from Sri Lanka (MH330220.1) showed 0.36% divergence. A 6.64% divergence with *Cx. declarator* (KM593055.1, KM593051.1) isolated from Colombia was observed (Table 1.13a). The phylogenetic tree (NJ) revealed the phylogenetic status of *Cx. tritaeniorhynchus* isolated from Kerala with other species. The average nucleotide composition throughout the species seemed to be T=40.0%; C=15.6%; A=28.4%; G=16.1% and the nucleotide composition of *Cx. tritaeniorhynchus* collected from Thrissur district was T(U)= 38.8, C= 16.2, A=29.7, and G= 15.3 (Table 1.13b).

The CO1 gene fragment of 680 bp product of *Cx. pipiens* was subjected to PCR amplification. The sequence was deposited in the NCBI GenBank with Accession No. MK347224.1. Figures 1.13a-1.13f presents the image of mosquito species collected, obtained DNA sequence, along with its conceptual translation product, phylogenetic tree, electropherogram, and molecular barcode. Evolutionary analyses were performed in MEGA XI. The data in Table 1.14a revealed that *Cx. pipiens* exhibited 0.00% evolutionary divergence with *Cx. pipiens* (MK300247.1 & LC102133.1) isolated from Kenya and Portugal. *Cx. declarator* (KM593055.1 & KM593051.1) isolated from Colombia showed a 6.21% evolutionary divergence with *Cx. pipiens* isolated from Kerala. The phylogenetic tree (NJ) illustrated the phylogenetic position of *Cx. pipiens* isolated from Thrissur, Kerala, demonstrating

that *Cx. pipiens* (MK347224.1) was the closey related to *Cx. pipiens* (MK300247.1 & LC102133.1) isolated from Kenya and Portugal. The average nucleotide composition throughout the species seemed to be T=40.2%; C=15.4%; A=28.2%; G=16.2% and the nucleotide composition of *Cx. pipiens* collected from Thrissur district was T(U)= 39.6, C= 15.6, A=29.0, and G= 15.8 (Table14.b).

The CO1 gene fragment of *Cx. quinquefasciatus* yielded a single product of 544 bp. The sequence was deposited in the NCBI GenBank with Accession No. MW143512.1. Figures 1.14a-1.14f presented the image of mosquito species collected, the obtained DNA sequence, its conceptual translation product, phylogenetic tree, electropherogram, and molecular barcode. From the data given in Table 1.15a, it was clear that *Cx. quinquefasciatus* exhibited 0.00% evolutionary divergence with *Cx. quinquefasciatus* (MT895717.1 & MW509611.1) isolated from Thrissur Kole lands, Kerala, and the USA, as well as 5.94% with *Cx. declarator* (KM593055.1) from Colombia. *An. subpictus* (KJ461792.1) isolated from Sri Lanka showed a 15.80% evolutionary divergence with *Cx. quinquefasciatus* isolated from Kerala. The phylogenetic tree constructed showed the phylogenetic position of *Cx. quinquefasciatus* isolated from Kerala, with phylogenetically close species. The average nucleotide composition throughout the species seemed to be T=39.9%; C=15.2%; A=28.0%; G=16.9% and the nucleotide composition of *Cx. quinquefasciatus* collected from Thrissur district was T(U)= 39.4, C= 15.5, A=28.8, and G= 16.3 (Table 1.15b).

Table 1.4: Accession number of collected vector mosquito species provided by NCBI GenBank

Mosquito Species	Voucher Number	Sequence Length (Base pair)	Accession Number
An. stephensi	ST18	530 bp	MT899149.1
An. subpictus	ST05	678 bp	MK603828.1
An. vagus	ST08	613 bp	MW199166.1
Ae. aegypti	ST01	681 bp	MK542380.1
Ae. albopictus	ST02	677 bp	MK297326.1
Ae. vittatus	ST14	527 bp	MT858330.1
Ar. subalbatus	ST04	666 bp	MK297327.1
Cx. gelidus	ST06	677 bp	MK630238.1
Cx. tritaeniorhynchus	ST07	672 bp	MH745093.1
Cx. pipiens	ST09	680 bp	MK347224.1
Cx. quinquefasciatus	ST17	544 bp	MW143512.1

1.3.2.1 Species Name: *Anopheles stephensi*

GenBank Accession Number: MT 899149.1

Voucher Number: ST18

Systematic position

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Diptera
Suborder	:	Nematocera
Family	:	Culicidae
Subfamily	:	Anophelini
Genus	:	*Anopheles*
Subgenus	:	*Myzomyia*
Species	:	*Anopheles stephensi* (Figure 1.4a)

> MT 899149.1 *Anopheles stephensi*|530bp

TTAGGACACCCAGGAGCATTTATTGGAGACGATCAAATTTATAATGTAATTGTAACTGCTCATGC
TTTTATTATAATTTTCTTTATAGTTATACCTATTATAATTGGGGGATTTGGAAATTGATTAGTTCC
TTTAATATTAGGAGCACCAGATATAGCATTTCCTCGAATAAATAATATAAGATTTTGAATATTAC
CCCCCTCATTAACTCTTTTAATTTCTAGAAGTATAGTAGAAAATGGAGCAGGAACAGGATGAAC
TGTTTATCCGCCTTTATCGTCTGGAATTGCTCACGCTGGGGCTTCAGTAGATTTAGCAATTTTTTC
ATTACATTTAGCTGGAATTTCTTCAATTTTAGGAGCAGTTAATTTTATTACTACAGTAATTAATAT
ACGATCGCCAGGAATTACGTTAGACCGAATACCTTTATTCGTTTGATCTGTTGTAATTACTGCTA
TTTTATTATTATTATCATTACCTGTATTAGCTGGAGCTATTACTATATTACTTACAGACCGAAATT
TAAATAC

Figure 1.4b The DNA sequence of *An. stephensi* COI gene

> MT 899149.1 *Anopheles stephensi*

MVGTSLSILIRAELGHPGAFIGDDQIYNVIVTAHAFIMIFFMVMPIMIGGFGNWLVPLMLGAPDMAFP
RMNNMSFWMLPPSLTLLISSSMVENGAGTGWTVYPPLSSGIAHAGASVDLAIFSLHLAGISSILGAVN
FITTVINMRSPGITLDRMPLFVWSVVITAILLLLSLPVLAGAITMLLT

Figure 1.4c The protein sequence of *An. stephensi* COI gene

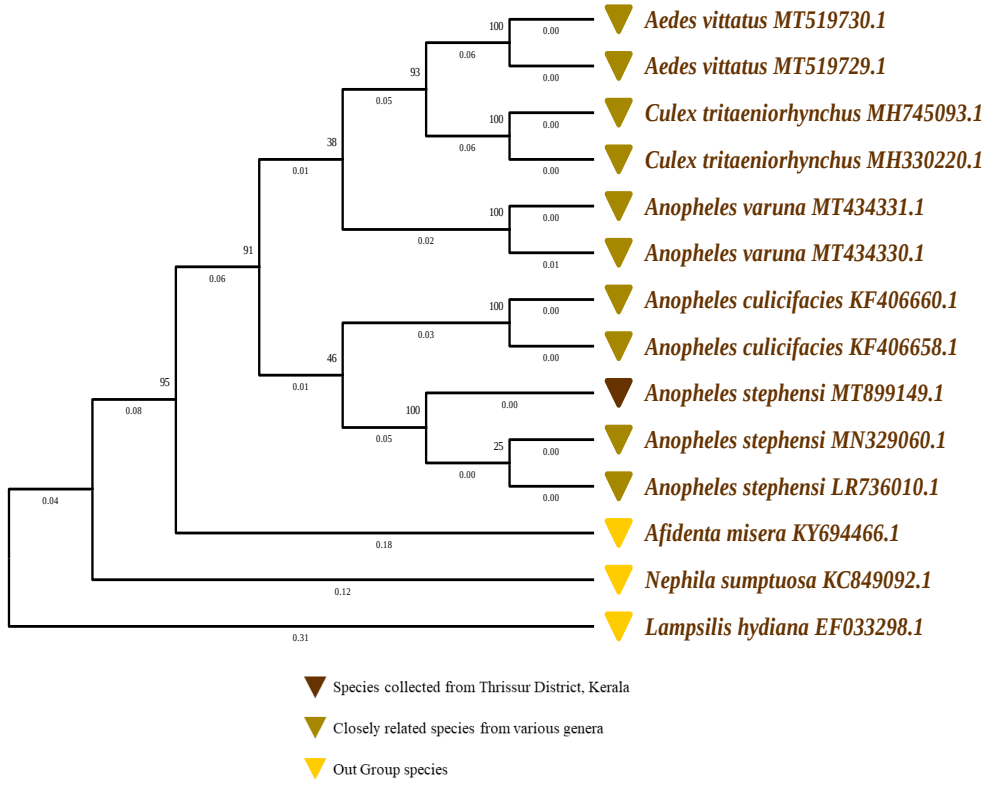

Figure 1.4d Phylogenetic tree of *An. stephensi*

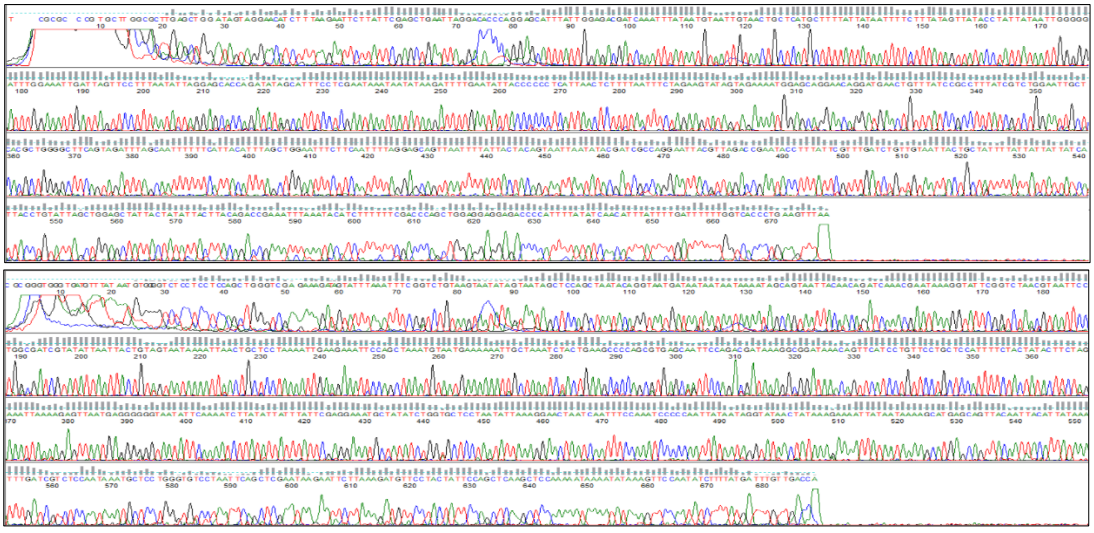

Figure 1.4e Electropherogram showing the nucleotide sequence of mitochondrial COI gene of *An. stephensi*

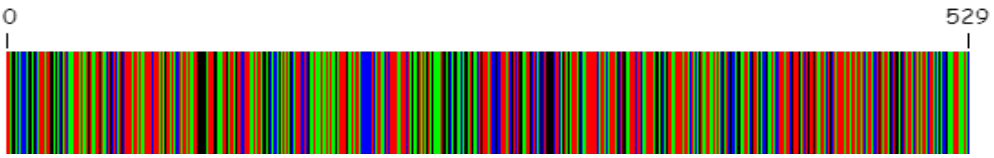

Figure 1.4f Molecular barcode of *An. stephensi*

Table 1.5a: Percentage of evolutionary divergence of *An. stephensi* with its closely related species accessible from NCBI GenBank

Accession Number	**Organism**	**Percentage of Divergence**
MT899149.1	*Anopheles stephensi*	0.00%
MN329060.1	*Anopheles stephensi*	0.00%
LR736010.1	*Anopheles stephensi*	0.00%
KF406660.1	*Anopheles culicifacies*	9.07%
KF406658.1	*Anopheles culicifacies*	9.07%
MT434331.1	*Anopheles varuna*	9.03%
MT434330.1	*Anopheles varuna*	9.05%
MT519730.1	*Aedes vittatus*	16.78%
MT519729.1	*Aedes vittatus*	16.78%
MH745093.1	*Culex tritaeniorhynchus*	16.72%
MH330220.1	*Culex tritaeniorhynchus*	16.76%
KC849092.1	*Nephila sumptuosa*	29.31%
KY694466.1	*Afidenta misera*	35.49%
EF033298.1	*Lampsilis hydiana*	67.90%

Table 1.5b: The nucleotide frequency comparison of *An. stephensi* COI gene sequence with its kin species

ACCESSION NO. AND NAME OF THE SPECIES	**NUCLEOTIDE FREQUENCIES (%)**																			
	T(U)	C	A	G	TOTAL	T-1	C-1	A-1	G-1	POS #1	T-2	C-2	A-2	G-2	POS #2	T-3	C-3	A-3	G-3	POS #3
MT899149.1 *Anopheles stephensi*	38.9	15.1	29.9	16.1	509.0	28.2	12.9	29.4	29.4	170.0	45.3	27.6	11.8	15.3	170.0	43.2	4.7	48.5	3.6	169.0
MN329060.1 *Anopheles stephensi*	38.9	15.1	29.9	16.1	509.0	28.2	12.9	29.4	29.4	170.0	45.3	27.6	11.8	15.3	170.0	43.2	4.7	48.5	3.6	169.0
LR736010.1 *Anopheles stephensi*	38.9	15.1	29.9	16.1	509.0	28.2	12.9	29.4	29.4	170.0	45.3	27.6	11.8	15.3	170.0	43.2	4.7	48.5	3.6	169.0
KF406660.1 *Anopheles culicifacies*	40.1	15.3	28.9	15.7	509.0	25.9	15.3	29.4	29.4	170.0	45.3	27.6	11.8	15.3	170.0	49.1	3.0	45.6	2.4	169.0
KF406658.1 *Anopheles culicifacies*	40.1	15.3	28.9	15.7	509.0	25.9	15.3	29.4	29.4	170.0	45.3	27.6	11.8	15.3	170.0	49.1	3.0	45.6	2.4	169.0
MT434331.1 *Anopheles varuna*	38.7	16.3	29.1	15.9	509.0	25.3	15.9	29.4	29.4	170.0	45.3	27.6	11.8	15.3	170.0	45.6	5.3	46.2	3.0	169.0
MT434330.1 *Anopheles varuna*	39.1	15.9	29.1	15.9	509.0	25.3	15.9	29.4	29.4	170.0	45.3	27.6	11.8	15.3	170.0	46.7	4.1	46.2	3.0	169.0
MT519730.1 *Aedes vittatus*	40.5	15.7	28.5	15.3	509.0	26.5	15.9	29.4	28.2	170.0	45.3	28.2	11.8	14.7	170.0	49.7	3.0	44.4	3.0	169.0
MT519729.1 *Aedes vittatus*	40.5	15.7	28.5	15.3	509.0	26.5	15.9	29.4	28.2	170.0	45.3	28.2	11.8	14.7	170.0	49.7	3.0	44.4	3.0	169.0
MH745093.1 *Culex tritaeniorhynchus*	39.7	15.5	29.3	15.5	509.0	24.7	17.6	28.8	28.8	170.0	45.3	27.6	11.8	15.3	170.0	49.1	1.2	47.3	2.4	169.0
MH330220.1 *Culex tritaeniorhynchus*	39.5	15.7	29.5	15.3	509.0	24.1	18.2	28.8	28.8	170.0	45.3	27.6	11.8	15.3	170.0	49.1	1.2	47.9	1.8	169.0
KY694466.1 *Afidenta misera*	38.1	18.7	27.7	15.5	509.0	24.7	16.5	33.5	25.3	170.0	45.3	26.5	12.4	15.9	170.0	44.4	13.0	37.3	5.3	169.0
EF033298.1 *Lampsilis hydiana*	45.2	12.0	17.9	25.0	509.0	34.7	11.8	24.7	28.8	170.0	45.9	20.0	13.5	20.6	170.0	55.0	4.1	15.4	25.4	169.0
KC849092.1 *Nephila sumptuosa*	41.9	12.2	28.0	17.9	508.0	34.1	11.2	25.3	29.4	170.0	47.6	24.7	12.9	14.7	170.0	44.0	0.6	45.8	9.5	168.0
Avg.	40.0	15.3	28.2	16.5	508.9	27.3	14.9	29.0	28.8	170.0	45.5	26.9	12.0	15.6	170.0	47.2	4.0	43.7	5.1	168.9

1.3.2.2 Species Name: *Anopheles subpictus*

GenBank Accession Number: MK603828.1.

Voucher Number: ST05

Systematic position

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Diptera
Suborder	:	Nematocera
Family	:	Culicidae
Subfamily	:	Anophelini
Genus	:	*Anopheles*
Subgenus	:	*Myzomyia*
Species	:	*Anopheles subpictus* (Figure 1.5a)

> MK603828.1 *Anopheles subpictus*| 678bp

AAAGATATTGGAACATTATATTTCATTTTTGGGGCATGAGCCGGAATAGTGGGTACTTCTTTAAGAA
TTCTTATTCGAGCAGAATTAGGTCACCCAGGAGCTTTTATTGGAGATGATCAAATTTATAATGTAAT
TGTTACAGCTCACGCATTTATTATAATTTTTTTTATAGTTATACCTATTATAATTGGAGGATTTGGAA
ATTGATTAGTGCCTTTAATGTTGGGAGCTCCAGATATAGCATTCCCACGAATAAATAATATAAGATT
TTGAATACTTCCTCCCTCATTAACCCTTTTAATTTCTAGAAGTATAGTAGAAAATGGGGCAGGAACA
GGTTGAACTGTTTATCCTCCTCTTTCTTCAGGAATTGCTCACGCAGGGGCTTCAGTAGATTTAGCTAT
TTTTTCTTTACATTTAGCGGGGATTTCATCAATTTTAGGTGCTGTAAATTTTATTACTACAGTTATTA
ATATACGATCACCAGGAATTACATTAGATCGAATACCTTTGTTTGTATGATCGGTAGTAATTACTGC
TATTTTATTATTATTATCATTGCCAGTATTAGCAGGAGCTATCACTATGTTACTTACAGATCGTAATT
TAAATACTTCTTTTTTCGATCCCGCGGGAGGAGGAGATCCGATTTTATATCAACACTTATTTTGATTT
TTG

Figure 1.5b The DNA sequence of *An. subpictus* COI gene

> MK603828.1 *Anopheles subpictus*

MLGAPDMAFPRMNNMSFWMLPPSLTLLISSSMVENGAGTGWTVYPPLSSGIAHAGASVDLAIFSLHLAG
ISSILGAVNFITTVINMRSPGITLDRMPLFVWSVVITAILLLLSLPVLAGAITMLLTDRNLNTSFFDPAGGG
DPILYQHLFWFL

Figure 1.5c The protein sequence of *An. subpictus* COI gene

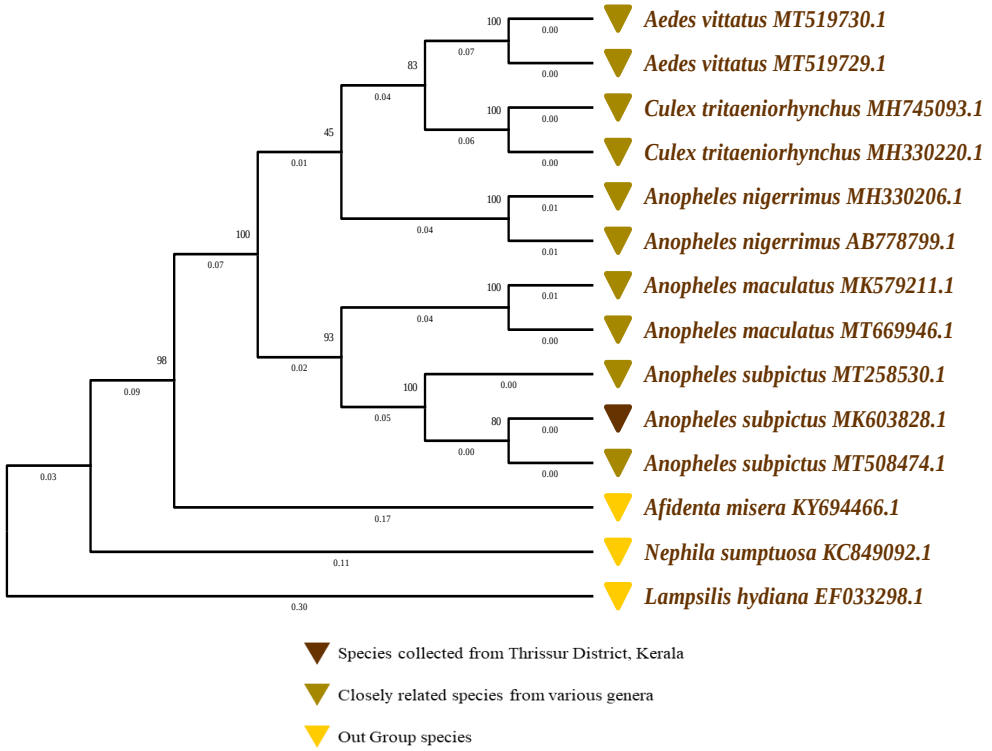

Figure 1.5d Phylogenetic tree of *An. subpictus*

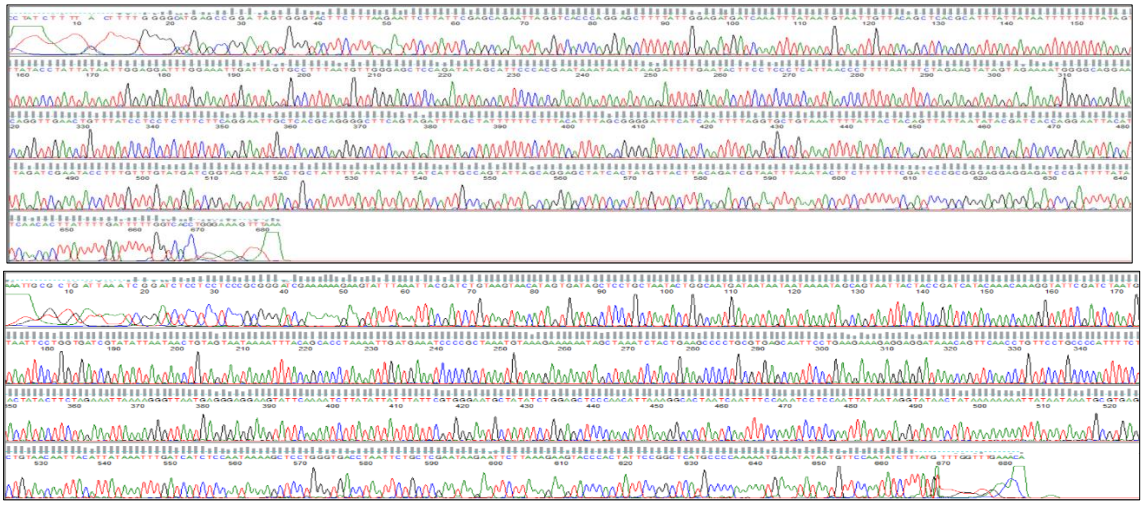

Figure 1.5e Electropherogram showing the nucleotide sequence of mitochondrial COI gene of *An. subpictus*

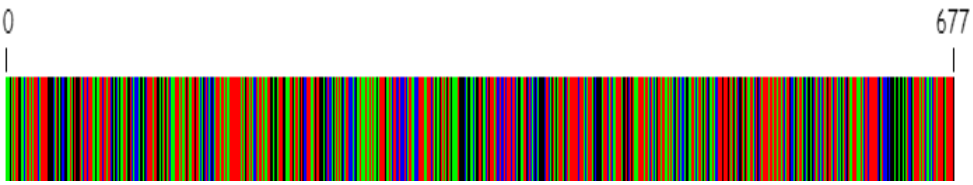

Figure 1.5f Molecular barcode of *An. subpictus*

Table 1.6a: Percentage of evolutionary divergence of *An. subpictus* with its closely related species accessible from NCBI GenBank

Accession Number	Organism	Percentage of Divergence
MK603828.1	*Anopheles subpictus*	0.00%
MT258530.1	*Anopheles subpictus*	0.86%
MT508474.1	*Anopheles subpictus*	0.34%
MT669946.1	*Anopheles maculatus*	10.80%
MK579211.1	*Anopheles maculatus*	11.26%
AB778799.1	*Anopheles nigerrimus*	15.35%
MH330206.1	*Anopheles nigerrimus*	15.67%
MT519730.1	*Aedes vittatus*	19.63%
MT519729.1	*Aedes vittatus*	19.63%
MH745093.1	*Culex tritaeniorhynchus*	19.66%
MH330220.1	*Culex tritaeniorhynchus*	19.72%
KC849092.1	*Nephila sumptuosa*	39.58%
KY694466.1	*Afidenta misera*	35.91%
EF033298.1	*Lampsilis hydiana*	78.71%

Table 1.6b: The nucleotide frequency comparison of *An. subpictus* COI gene sequence with its kin species

ACCESSION NO. AND NAME OF THE SPECIES	NUCLEOTIDE FREQUENCIES (%)																		
	T(U)	C	A	G	TOTAL	T-1	C-1	A-1	G-1	POS #1	T-2	C-2	A-2	G-2	POS #2	T-3	C-3	A-3	G-3	POS #3
MK603828.1 *Anopheles subpictus*	38.9	15.4	28.8	16.8	583.0	44.1	27.7	12.8	15.4	195.0	45.9	4.1	43.8	6.2	194.0	26.8	14.4	29.9	28.9	194.0
MT258530.1 *Anopheles subpictus*	38.8	15.6	29.2	16.5	583.0	44.1	27.7	12.8	15.4	195.0	45.4	4.6	44.8	5.2	194.0	26.8	14.4	29.9	28.9	194.0
MT508474.1 *Anopheles subpictus*	38.9	15.4	28.8	16.8	583.0	44.1	27.7	12.8	15.4	195.0	45.9	4.1	43.8	6.2	194.0	26.8	14.4	29.9	28.9	194.0
MH330206.1 *Anopheles nigerrimus*	37.2	15.3	31.7	15.8	583.0	44.1	27.7	12.8	15.4	195.0	40.2	4.1	53.1	2.6	194.0	27.3	13.9	29.4	29.4	194.0
AB778799.1 *Anopheles nigerrimus*	37.7	14.8	32.2	15.3	583.0	44.1	27.7	12.8	15.4	195.0	42.3	2.1	54.6	1.0	194.0	26.8	14.4	29.4	29.4	194.0
MK579211.1 *Anopheles maculatus*	39.1	15.3	30.0	15.6	583.0	44.1	27.7	12.8	15.4	195.0	45.9	4.1	47.4	2.6	194.0	27.3	13.9	29.9	28.9	194.0
MT669946.1 *Anopheles maculatus*	39.1	15.4	29.8	15.6	583.0	44.1	27.7	12.8	15.4	195.0	46.4	4.1	46.9	2.6	194.0	26.8	14.4	29.9	28.9	194.0
MT519730.1 *Aedes vittatus*	39.8	16.0	29.2	15.1	583.0	44.1	28.2	12.8	14.9	195.0	48.5	4.1	44.8	2.6	194.0	26.8	15.5	29.9	27.8	194.0
MT519729.1 *Aedes vittatus*	39.8	16.0	29.2	15.1	583.0	44.1	28.2	12.8	14.9	195.0	48.5	4.1	44.8	2.6	194.0	26.8	15.5	29.9	27.8	194.0
MH745093.1 *Culex tritaeniorhynchus*	39.3	15.8	29.7	15.3	583.0	44.1	27.7	12.8	15.4	195.0	49.0	2.1	46.9	2.1	194.0	24.7	17.5	29.4	28.4	194.0
MH330220.1 *Culex tritaeniorhynchus*	39.1	16.0	29.8	15.1	583.0	44.1	27.7	12.8	15.4	195.0	49.0	2.1	47.4	1.5	194.0	24.2	18.0	29.4	28.4	194.0
KY694466.1 *Afidenta misera*	37.9	18.7	28.3	15.1	583.0	44.6	26.2	13.3	15.9	195.0	44.3	13.4	37.6	4.6	194.0	24.7	16.5	34.0	24.7	194.0
EF033298.1 *Lampsilis hydiana*	44.8	12.2	18.5	24.5	583.0	45.6	20.0	14.4	20.0	195.0	54.1	4.6	16.0	25.3	194.0	34.5	11.9	25.3	28.4	194.0
KC849092.1 *Nephila sumptuosa*	41.4	12.4	28.7	17.5	582.0	46.2	25.1	13.8	14.9	195.0	45.1	0.5	45.6	8.8	193.0	33.0	11.3	26.8	28.9	194.0
Avg.	39.4	15.3	28.9	16.4	582.9	44.4	26.9	13.0	15.6	195.0	46.4	4.2	44.1	5.3	193.9	27.4	14.7	29.5	28.4	194.0

1.3.2.3 Species Name: *Anopheles vagus*

GenBank Accession Number: MW199166.1

Voucher Number: ST08

Systematic position

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Diptera
Suborder	:	Nematocera
Family	:	Culicidae
Subfamily	:	Anophelini
Genus	:	*Anopheles*
Subgenus	:	*Myzomyia*
Species	:	*Anopheles vagus* (Figure 1.6a)

> MW199166.1 *Anopheles vagus* |613bp

AGGAATAGTCGGAACATCTCTTAGAATTCTAATTCGAGCTGAACTAGGACATCCCGGAGCATTTATTG
GGGATGATCAAATTTATAATGTAATTGTTACAGCCCACGCTTTTATTATAATTTTTTTTATAGTAATACC
TATTATAATTGGAGGATTTGGAAACTGATTAGTTCCACTAATGCTAGGGGCTCCCGATATAGCATTCCC
ACGAATAAATAATATAAGTTTTTGAATATTACCCCCATCTCTTACTCTTTTAATTTCTAGTAGTATAGTA
GAAAATGGGGCAGGAACAGGTTGAACTGTATATCCCCCTCTTTCATCGGGGATTGCTCACGCTGGGGC
TTCAGTTGATTTAGCAATTTTCTCACTTCATTTAGCAGGAATTTCTTCAATTTTAGGAGCAGTAAATTTT
ATTACTACAGTAATTAATATACGATCTCCAGGAATTACGCTAGATCGAATACCTTTATTTGTTTGATCA
GTTGTAATTACTGCAGTCTTATTATTATTATCACTTCCAGTATTAGCAGGAGCTATTACTATACTATTAA
CTGATCGAAATTTAAATACTTCGTTCTTTGACCCTGCGGGAGGAGGAGACCCTATTTTA

Figure 1.6b The DNA sequence of *An. vagus* COI gene

> MW199166.1 *Anopheles vagus*

MVGTSLSILIRAELGHPGAFIGDDQIYNVIVTAHAFIMIFFMVMPIMIGGFGNWLVPLMLGAPDMAFPRMNN
MSFWMLPPSLTLLISSSMVENGAGTGWTVYPPLSSGIAHAGASVDLAIFSLHLAGISSILGAVNFITTVINMRS
PGITLDRMPLFVWSVVITAVLLLLSLPVLAGAITMLLTDRNLNTSFFDPAGGGDPIL

Figure 1.6c The protein sequence of *An. vagus* COI gene

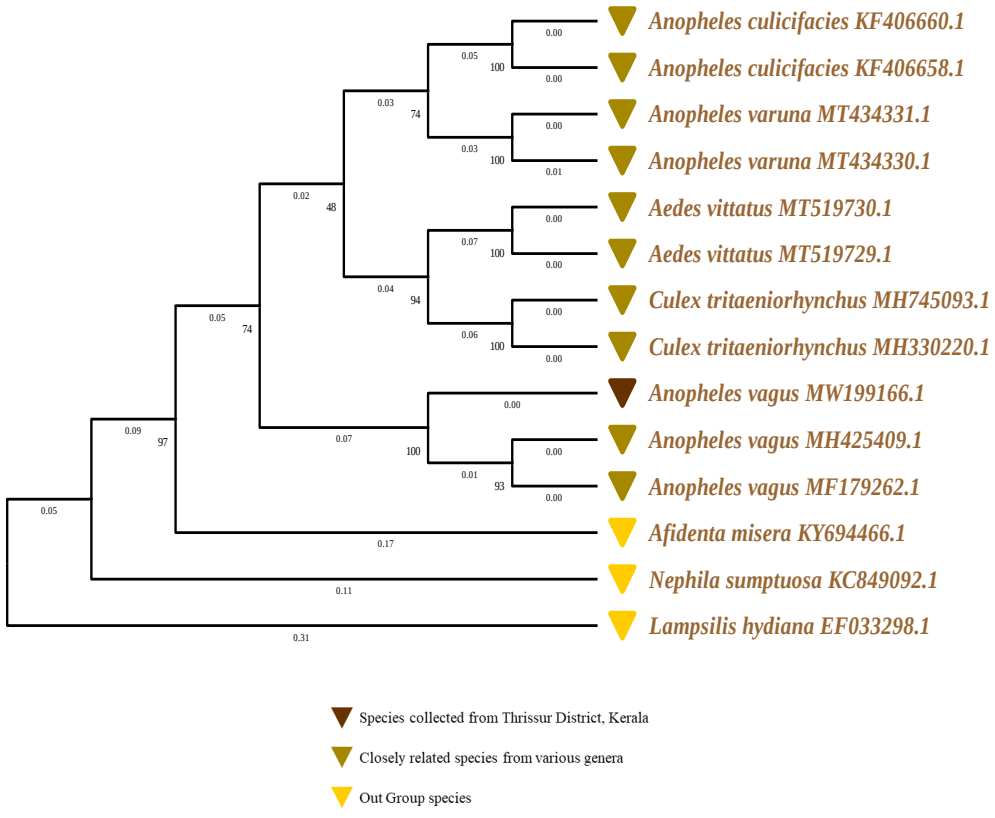

Figure 1.6d Phylogenetic tree of *An. vagus*

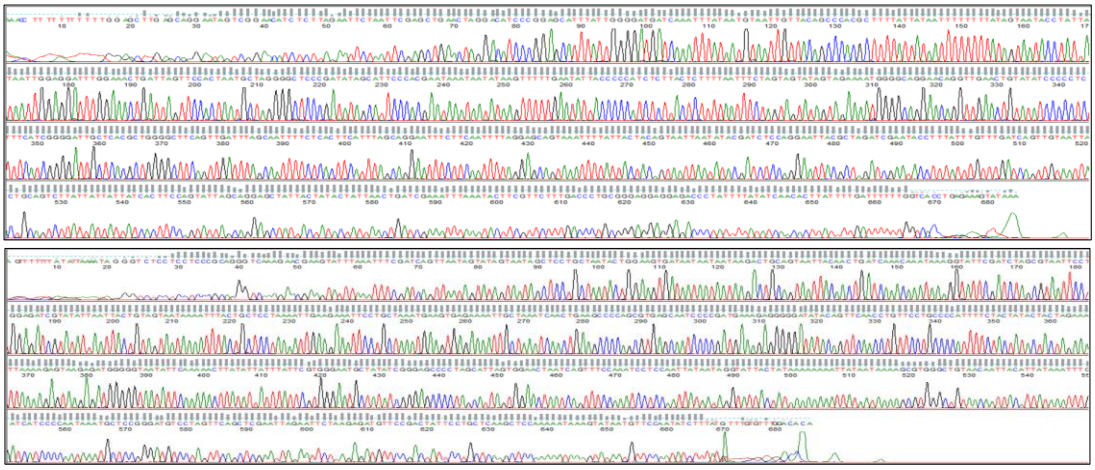

Figure 1.6e Electropherogram showing the nucleotide sequence of mitochondrial COI gene of *An. vagus*

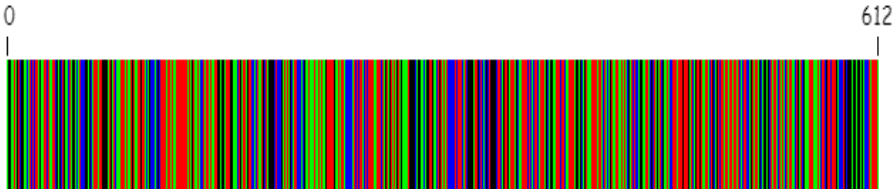

Figure 1.6f Molecular barcode of *An. vagus*

Table 1.7a: Percentage of evolutionary divergence of *An. vagus* with its closely related species accessible from NCBI GenBank

Accession Number	Organism	Percentage of Divergence
MW199166.1	*Anopheles vagus*	0.00%
MH425409.1	*Anopheles vagus*	1.67%
MF179262.1	*Anopheles vagus*	1.67%
MT434331.1	*Anopheles varuna*	14.71%
MT434330.1	*Anopheles varuna*	15.19%
KF406660.1	*Anopheles culicifacies*	17.23%
KF406658.1	*Anopheles culicifacies*	16.98%
MH745093.1	*Culex tritaeniorhynchus*	18.42%
MH330220.1	*Culex tritaeniorhynchus*	18.42%
MT519730.1	*Aedes vittatus*	18.56%
MT519729.1	*Aedes vittatus*	18.56%
KY694466.1	*Afidenta misera*	31.66%
KC849092.1	*Nephila sumptuosa*	38.15%
EF033298.1	*Lampsilis hydiana*	89.79%

Table 1.7b: The nucleotide frequency comparison of *An. vagus* COI gene sequence with its kin species

ACCESSION NO. AND NAME OF THE SPECIES	**NUCLEOTIDE FREQUENCIES (%)**																			
	T(U)	C	A	G	TOTAL	T-1	C-1	A-1	G-1	POS #1	T-2	C-2	A-2	G-2	POS #2	T-3	C-3	A-3	G-3	POS #3
MW199166.1 *Anopheles vagus*	36.7	17.3	29.2	16.8	548.0	22.4	18.0	29.5	30.1	183.0	45.4	27.3	11.5	15.8	183.0	42.3	6.6	46.7	4.4	182.0
MH425409.1 *Anopheles vagus*	36.9	17.3	29.0	16.8	548.0	22.4	18.0	29.5	30.1	183.0	45.4	27.3	11.5	15.8	183.0	42.9	6.6	46.2	4.4	182.0
MF179262.1 *Anopheles vagus*	36.5	17.7	29.0	16.8	548.0	22.4	18.0	29.5	30.1	183.0	45.4	27.3	11.5	15.8	183.0	41.8	7.7	46.2	4.4	182.0
KF406660.1 *Anopheles culicifacies*	39.6	15.1	29.2	16.1	548.0	25.1	15.3	30.1	29.5	183.0	45.4	27.3	11.5	15.8	183.0	48.4	2.7	46.2	2.7	182.0
KF406658.1 *Anopheles culicifacies*	39.6	15.1	29.2	16.1	548.0	25.1	15.3	30.1	29.5	183.0	45.4	27.3	11.5	15.8	183.0	48.4	2.7	46.2	2.7	182.0
MT434331.1 *Anopheles varuna*	38.3	16.1	29.4	16.2	548.0	24.6	15.8	30.1	29.5	183.0	45.4	27.3	11.5	15.8	183.0	45.1	4.9	46.7	3.3	182.0
MT434330.1 *Anopheles varuna*	38.7	15.7	29.4	16.2	548.0	24.6	15.8	30.1	29.5	183.0	45.4	27.3	11.5	15.8	183.0	46.2	3.8	46.7	3.3	182.0
MT519730.1 *Aedes vittatus*	40.3	15.3	28.8	15.5	548.0	26.2	15.3	30.1	28.4	183.0	45.4	27.9	11.5	15.3	183.0	49.5	2.7	45.1	2.7	182.0
MT519729.1 *Aedes vittatus*	40.3	15.3	28.8	15.5	548.0	26.2	15.3	30.1	28.4	183.0	45.4	27.9	11.5	15.3	183.0	49.5	2.7	45.1	2.7	182.0
MH745093.1 *Culex tritaeniorhynchus*	39.6	15.1	29.6	15.7	548.0	24.6	16.9	29.5	29.0	183.0	45.4	27.3	11.5	15.8	183.0	48.9	1.1	47.8	2.2	182.0
MH330220.1 *Culex tritaeniorhynchus*	39.4	15.3	29.7	15.5	548.0	24.0	17.5	29.5	29.0	183.0	45.4	27.3	11.5	15.8	183.0	48.9	1.1	48.4	1.6	182.0
KY694466.1 *Afidenta misera*	37.8	18.4	28.3	15.5	548.0	24.6	16.4	33.9	25.1	183.0	45.9	25.7	12.0	16.4	183.0	42.9	13.2	39.0	4.9	182.0
EF033298.1 *Lampsilis hydiana*	45.1	11.5	17.9	25.5	548.0	35.0	11.5	24.6	29.0	183.0	47.0	19.1	13.1	20.8	183.0	53.3	3.8	15.9	26.9	182.0
KC849092.1 *Nephila sumptuosa*	41.0	12.1	28.9	18.1	547.0	32.8	10.9	26.8	29.5	183.0	47.5	24.6	12.6	15.3	183.0	42.5	0.6	47.5	9.4	181.0
Avg.	39.3	15.5	28.3	16.9	547.9	25.7	15.7	29.5	29.0	183.0	45.7	26.5	11.7	16.1	183.0	46.4	4.3	43.8	5.4	181.9

1.3.2.4 Species Name: *Aedes aegypti*

GenBank Accession Number: MK542380.1

Voucher Number: ST01

Systematic position

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Diptera
Suborder	:	Nematocera
Family	:	Culicidae
Subfamily	:	Culicini
Genus	:	*Aedes*
Subgenus	:	*Stegomyia*
Species	:	*Aedes aegypti* (Figure 1.7a)

> MK542380 *Aedes aegypti*|681bp

AAAGATATTGGAACTTTATATTTCATTTTTGGAGTATGATCCGGAATAGTCGGAACTTCTCTAAG
AATTTTAATTCGTGCTGAACTTAGCCACCCTGGTATATTTATTGGGAATGACCAAATTTATAATGT
AATTGTAACAGCTCATGCATTTATTATAATTTTCTTTATAGTAATACCAATTATAATTGGAGGATT
TGGAAATTGATTAGTTCCTTTAATATTAGGAGCCCCTGATATAGCCTTTCCTCGAATAAATAATAT
AAGTTTTTGAATACTACCTCCTTCATTGACTCTTCTATTATCAAGCTCAATAGTAGAAAATGGGGC
AGGAACTGGGTGAACAGTTTATCCTCCTCTCTCTTCAGGAACAGCTCATGCTGGAGCTTCTGTTG
ATTTAGCTATTTTTTCTCTTCATTTAGCTGGAATTTCCTCAATTTTAGGGGCAGTAAATTTTATTAC
AACTGTAATTAATATACGATCGTCAGGAATTACTTTAGATCGACTACCTTTATTTGTTTGATCTGT
AGTTATTACAGCTATCTTATTACTTCTTTCTCTTCCTGTTTTAGCTGGAGCTATTACTATGTTATTA
ACAGACCGAAACTTAAATACATCTTTCTTTGATCCAATCGGAGGAGGAGATCCTATTTTATACCA
ACACTTATTCTGATTTTTGGTC

Figure 1.7b The DNA sequence of *Ae. aegypti* COI gene

> MK542380 *Aedes aegypti*

MGTLYFIFGVWSGMVGTSLSILIRAELSHPGMFIGNDQIYNVIVTAHAFIMIFFMVMPIMIGGFGNWLV
PLMLGAPDMAFPRMNNMSFWMLPPSLTLLLSSSMVENGAGTGWTVYPPLSSGTAHAGASVDLAIFSL
HLAGISSILGAVNFITTVINMRSSGITLDRLPLFVWSVVITAILLLLSLPVLAGAITMLLTDRNLNTSFFDP
IGGGDPILYQHLFWFLV

Figure 1.7c The protein sequence of *Ae. aegypti* COI gene

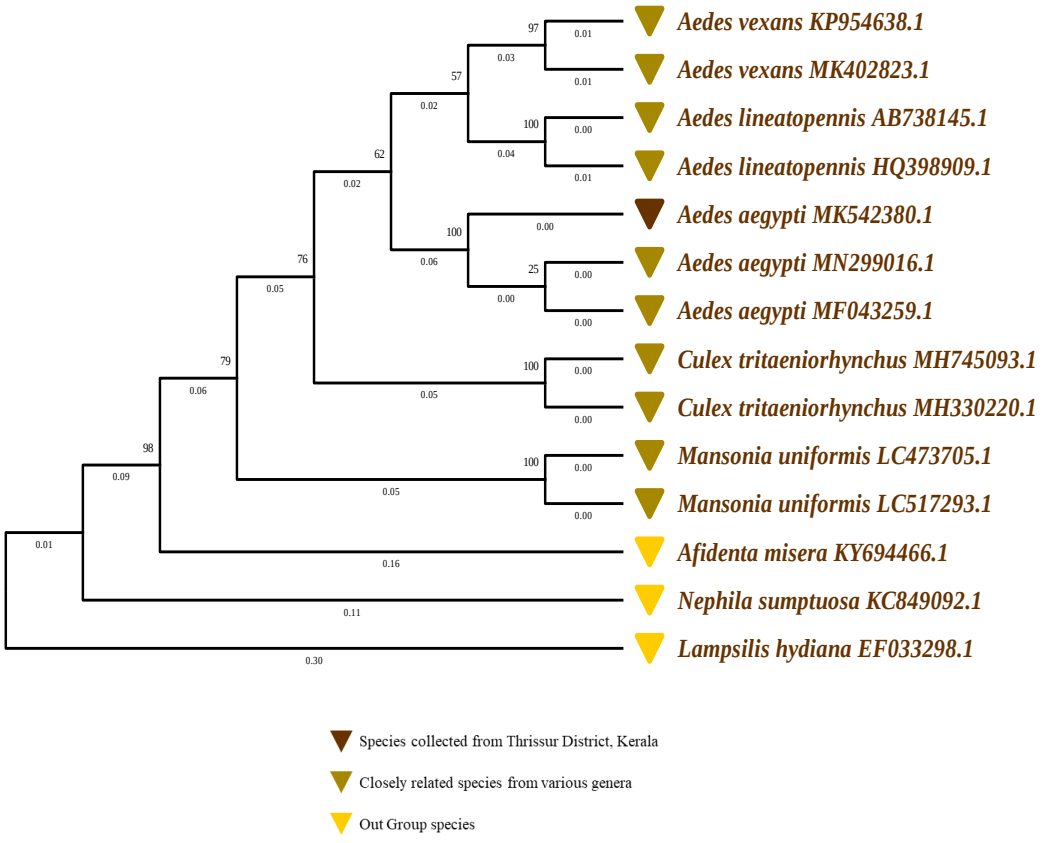

Figure 1.7d Phylogenetic tree of *Ae. aegypti*

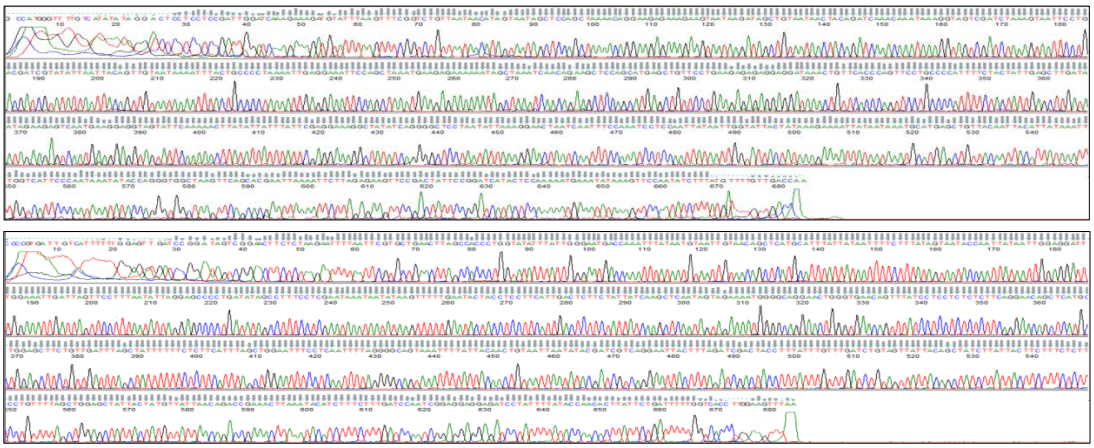

Figure 1.7e Electropherogram showing the nucleotide sequence of mitochondrial COI gene of *Ae. aegypti*

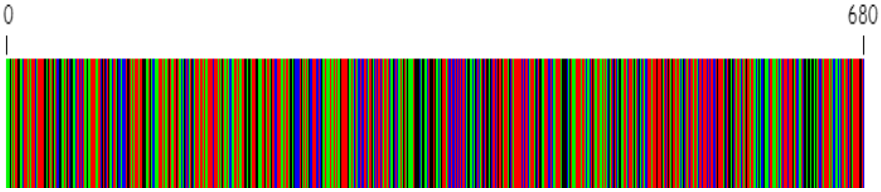

Figure 1.7f Molecular barcode of *Ae. aegypti*

Table 1.8a: Percentage of evolutionary divergence of *Ae. aegypti* with its closely related species accessible from NCBI GenBank

Accession Number	Organism	Percentage of Divergence
MK542380.1	*Aedes aegypti*	0.00%
MN299016.1	*Aedes aegypti*	0.00%
MF043259.1	*Aedes aegypti*	0.00%
KP954638.1	*Aedes vexans*	9.74%
MK402823.1	*Aedes vexans*	10.39%
AB738145.1	*Aedes lineatopennis*	10.83%
HQ398909.1	*Aedes lineatopennis*	11.06%
MH745093.1	*Culex tritaeniorhynchus*	13.85%
MH330220.1	*Culex tritaeniorhynchus*	13.84%
LC473705.1	*Mansonia uniformis*	15.51%
LC517293.1	*Mansonia uniformis*	15.51%
KY694466.1	*Afidenta misera*	28.68%
KC849092.1	*Nephila sumptuosa*	31.07%
EF033298.1	*Lampsilis hydiana*	58.19%

Table 1.8b: The nucleotide frequency comparison of *Ae. aegypti* COI gene sequence with its kin species

ACCESSION NO. AND NAME OF THE SPECIES	**NUCLEOTIDE FREQUENCIES (%)**																		
	T(U)	C	A	G	TOTAL	T-1	C-1	A-1	G-1	POS #1	T-2	C-2	A-2	G-2	POS #2	T-3	C-3	A-3	G-3	POS #3
MK542380.1 *Aedes aegypti*	39.4	17.6	27.7	15.3	556.0	43.5	28.5	13.4	14.5	186.0	48.6	7.0	40.5	3.8	185.0	25.9	17.3	29.2	27.6	185.0
MN299016.1 *Aedes aegypti*	39.4	17.6	27.7	15.3	556.0	43.5	28.5	13.4	14.5	186.0	48.6	7.0	40.5	3.8	185.0	25.9	17.3	29.2	27.6	185.0
MF043259.1 *Aedes aegypti*	39.4	17.6	27.7	15.3	556.0	43.5	28.5	13.4	14.5	186.0	48.6	7.0	40.5	3.8	185.0	25.9	17.3	29.2	27.6	185.0
KP954638.1 *Aedes vexans*	40.6	16.0	28.8	14.6	556.0	43.5	28.5	13.4	14.5	186.0	50.8	3.8	43.8	1.6	185.0	27.6	15.7	29.2	27.6	185.0
MK402823.1 *Aedes vexans*	39.9	16.0	29.3	14.7	556.0	43.5	28.5	13.4	14.5	186.0	48.6	3.8	45.4	2.2	185.0	27.6	15.7	29.2	27.6	185.0
AB738145.1 *Aedes lineatopennis*	40.3	16.2	28.8	14.7	556.0	43.5	28.0	13.4	15.1	186.0	51.4	3.8	43.8	1.1	185.0	25.9	16.8	29.2	28.1	185.0
HQ398909.1 *Aedes lineatopennis*	39.9	16.7	28.6	14.7	556.0	43.5	28.0	13.4	15.1	186.0	50.3	5.4	43.2	1.1	185.0	25.9	16.8	29.2	28.1	185.0
LC473705.1 *Mansonia uniformis*	40.1	14.9	30.0	14.9	556.0	44.1	27.4	14.0	14.5	186.0	49.2	2.7	45.9	2.2	185.0	27.0	14.6	30.3	28.1	185.0
LC517293.1 *Mansonia uniformis*	40.1	14.9	30.0	14.9	556.0	44.1	27.4	14.0	14.5	186.0	49.2	2.7	45.9	2.2	185.0	27.0	14.6	30.3	28.1	185.0
MH745093.1 *Culex tritaeniorhynchus*	38.8	16.2	29.7	15.3	556.0	43.5	28.0	13.4	15.1	186.0	48.6	2.2	47.0	2.2	185.0	24.3	18.4	28.6	28.6	185.0
MH330220.1 *Culex tritaeniorhynchus*	38.7	16.4	29.9	15.1	556.0	43.5	28.0	13.4	15.1	186.0	48.6	2.2	47.6	1.6	185.0	23.8	18.9	28.6	28.6	185.0
KY694466.1 *Afidenta misera*	38.1	18.7	28.1	15.1	556.0	44.1	26.3	14.0	15.6	186.0	45.4	13.0	36.8	4.9	185.0	24.9	16.8	33.5	24.9	185.0
EF033298.1 *Lampsilis hydiana*	44.8	12.6	18.5	24.1	556.0	44.6	20.4	15.1	19.9	186.0	55.7	4.9	15.1	24.3	185.0	34.1	12.4	25.4	28.1	185.0
KC849092.1 *Nephila sumptuosa*	42.0	12.6	27.9	17.5	555.0	45.7	25.3	14.5	14.5	186.0	46.7	0.5	43.5	9.2	184.0	33.5	11.9	25.9	28.6	185.0
Avg.	40.1	16.0	28.0	15.8	555.9	43.9	27.2	13.7	15.1	186.0	49.3	4.7	41.4	4.6	184.9	27.1	16.0	29.1	27.8	185.0

1.3.2.5 Species Name: *Aedes albopictus*

GenBank Accession Number: MK297326.1

Voucher Number: ST02

Systematic position

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Diptera
Suborder	:	Nematocera
Family	:	Culicidae
Subfamily	:	Culicini
Genus	:	*Aedes*
Subgenus	:	*Stegomyia*
Species	:	*Aedes albopictus* (Figure 1.8a)

> MK297326.1 *Aedes albopictus* |677bp

AAGATATTGGAACATTATACTTTATTTTCGGTATTTGATCTGGGATAGTCGGGACTTCACTAAGAG
TTTTAATTCGTATTGAACTTAGACATCCTGGTATATTTATTGGAAATGATCAAATTTATAATGTAAT
TGTTACTGCTCATGCTTTTATTATAATTTTTTTTATAGTAATACCTATCATAATTGGAGGATTTGGA
AACTGACTAGTACCCTTAATACTAGGAGCCCCTGATATAGCTTTTCCTCGAATAAATAATATAAGT
TTTTGAATATTACCCCCCTCTTTAACACTGCTGCTTTCTAGTTCTATAGTAGAAAACGGAGCTGGAA
CAGGGTGAACGGTTTATCCTCCCCTTTCTTCTGGAACAGCTCATGCTGGGGCTTCAGTTGATTTAGC
AATTTTTTCTTTACATTTAGCGGGAATCTCATCTATTTTAGGAGCAGTAAATTTTATTACAACTGTA
ATTAATATACGATCAGCTGGTATTACTCTTGATCGACTACCTTTATTTGTGTGATCAGTAGTAATTA
CAGCTATTTTATTACTTCTTTCTCTACCCGTATTAGCCGGAGCTATTACTATATTATTAACAGACCG
AAATTTAAATACATCTTTTTTTGATCCAATTGGAGGGGGAGACCCTATTTTATATCAACATTTATTT
TGATTTTTG

Figure 1.8b The DNA sequence of *Ae. albopictus* COI gene

> MK297326.1 *Aedes albopictus*

MGTLYFIFGIWSGMVGTSLSVLIRIELSHPGMFIGNDQIYNVIVTAHAFIMIFFMVMPIMIGGFGNWLVPL
MLGAPDMAFPRMNNMSFWMLPPSLTLLLSSSMVENGAGTGWTVYPPLSSGTAHAGASVDLAIFSLHL
AGISSILGAVNFITTVINMRSAGITLDRLPLFVWSVVITAILLLLSLPVLAGAITMLLTDRNLNTSFFDPIG
GGDPILYQHLFWFL

Figure 1.8c The protein sequence of *Ae. albopictus* COI gene

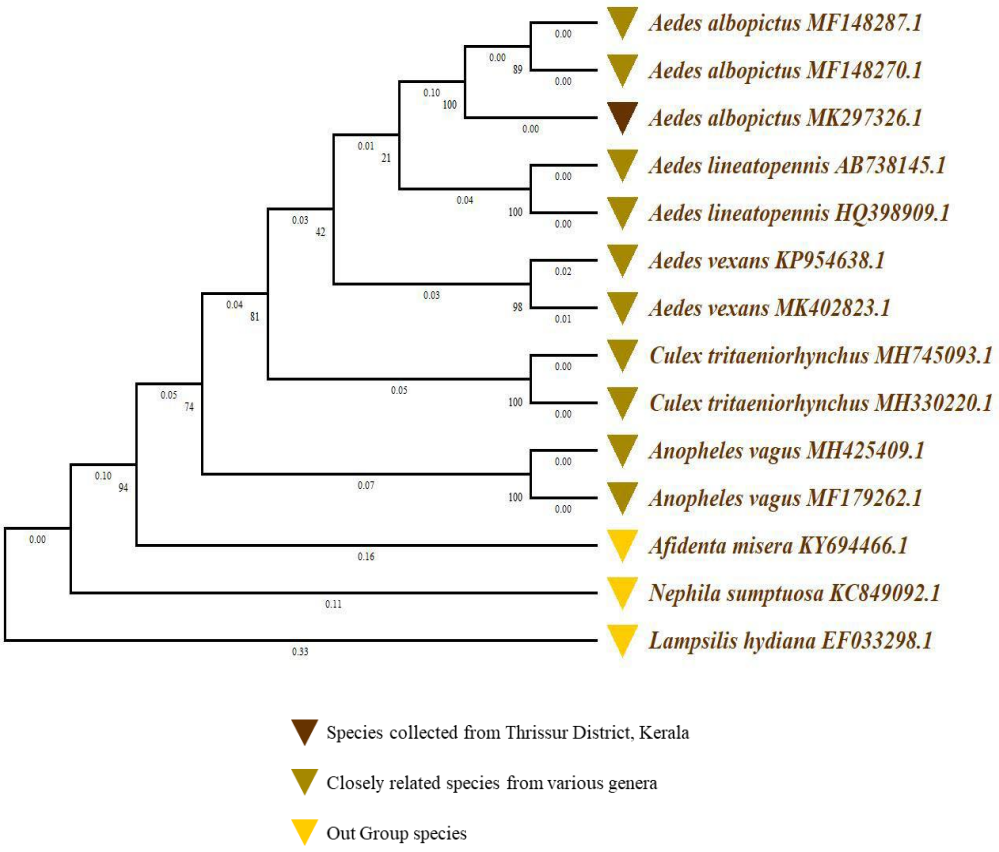

Figure 1.8d Phylogenetic tree of *Ae. albopictus*

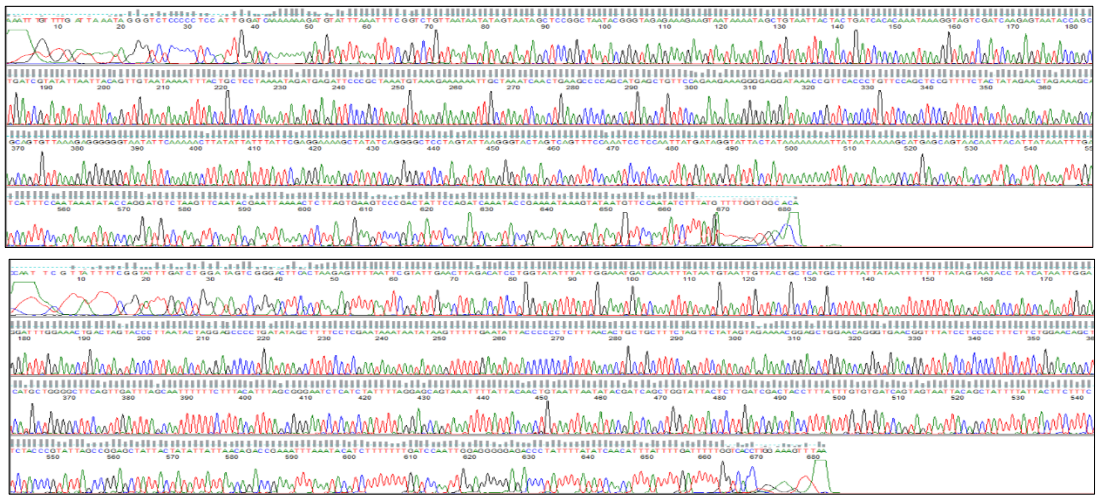

Figure 1.8e Electropherogram showing the nucleotide sequence of mitochondrial COI gene of *Ae. albopictus*

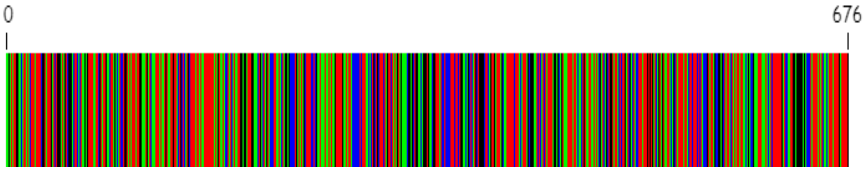

Figure 1.8f Molecular barcode of *Ae. albopictus*

Table 1.9a: Percentage of evolutionary divergence of *Ae. albopictus* with its closely related species accessible from NCBI GenBank

Accession Number	Organism	Percentage of Divergence
MK297326.1	*Aedes albopictus*	0.00%
MF148287.1	*Aedes albopictus*	0.23%
MF148270.1	*Aedes albopictus*	0.23%
KP954638.1	*Aedes vexans*	9.48%
MK402823.1	*Aedes vexans*	9.76%
AB738145.1	*Aedes lineatopennis*	9.78%
HQ398909.1	*Aedes lineatopennis*	10.24%
MH330220.1	*Culex tritaeniorhynchus*	10.79%
MH745093.1	*Culex tritaeniorhynchus*	11.08%
MH425409.1	*Anopheles vagus*	13.61%
MF179262.1	*Anopheles vagus*	13.30%
KY694466.1	*Afidenta misera*	19.65%
KC849092.1	*Nephila sumptuosa*	21.47%
EF033298.1	*Lampsilis hydiana*	33.82%

Table 1.9b: The nucleotide frequency comparison of *Ae. albopictus* COI gene sequence with its kin species

ACCESSION NO. AND NAME OF THE SPECIES	**NUCLEOTIDE FREQUENCIES (%)**																		
	T(U)	C	A	G	TOTAL	T-1	C-1	A-1	G-1	POS #1	T-2	C-2	A-2	G-2	POS #2	T-3	C-3	A-3	G-3	POS #3
MK297326.1 *Aedes albopictus*	38.6	17.3	28.1	16.0	588.0	47.4	6.6	41.3	4.6	196.0	24.0	17.9	30.1	28.1	196.0	44.4	27.6	12.8	15.3	196.0
MF148287.1 *Aedes albopictus*	38.6	17.3	28.4	15.6	588.0	47.4	6.6	42.3	3.6	196.0	24.0	17.9	30.1	28.1	196.0	44.4	27.6	12.8	15.3	196.0
MF148270.1 *Aedes albopictus*	38.6	17.3	28.4	15.6	588.0	47.4	6.6	42.3	3.6	196.0	24.0	17.9	30.1	28.1	196.0	44.4	27.6	12.8	15.3	196.0
KP954638.1 *Aedes vexans*	40.1	15.5	29.4	15.0	588.0	49.0	3.6	45.4	2.0	196.0	27.6	14.8	30.1	27.6	196.0	43.9	28.1	12.8	15.3	196.0
MK402823.1 *Aedes vexans*	39.5	15.5	30.1	15.0	588.0	46.9	3.6	47.4	2.0	196.0	27.6	14.8	30.1	27.6	196.0	43.9	28.1	12.8	15.3	196.0
AB738145.1 *Aedes lineatopennis*	40.0	15.6	29.3	15.1	588.0	50.0	3.6	44.9	1.5	196.0	26.0	15.8	30.1	28.1	196.0	43.9	27.6	12.8	15.8	196.0
HQ398909.1 *Aedes lineatopennis*	39.6	16.2	29.3	15.0	588.0	49.0	5.1	44.9	1.0	196.0	26.0	15.8	30.1	28.1	196.0	43.9	27.6	12.8	15.8	196.0
MH425409.1 *Anopheles vagus*	36.7	17.3	29.3	16.7	588.0	43.4	6.6	45.4	4.6	196.0	23.0	17.9	29.6	29.6	196.0	43.9	27.6	12.8	15.8	196.0
MF179262.1 *Anopheles vagus*	36.4	17.7	29.3	16.7	588.0	42.3	7.7	45.4	4.6	196.0	23.0	17.9	29.6	29.6	196.0	43.9	27.6	12.8	15.8	196.0
MH745093.1 *Culex tritaeniorhynchus*	39.1	15.6	29.8	15.5	588.0	49.0	2.0	46.9	2.0	196.0	24.5	17.3	29.6	28.6	196.0	43.9	27.6	12.8	15.8	196.0
MH330220.1 *Culex tritaeniorhynchus*	38.9	15.8	29.9	15.3	588.0	49.0	2.0	47.4	1.5	196.0	24.0	17.9	29.6	28.6	196.0	43.9	27.6	12.8	15.8	196.0
KY694466.1 *Afidenta misera*	37.6	18.5	28.4	15.5	588.0	43.9	13.3	37.8	5.1	196.0	24.5	16.3	34.2	25.0	196.0	44.4	26.0	13.3	16.3	196.0
EF033298.1 *Lampsilis hydiana*	44.6	12.1	18.4	25.0	588.0	53.6	4.6	15.8	26.0	196.0	34.7	11.7	25.0	28.6	196.0	45.4	19.9	14.3	20.4	196.0
KC849092.1 *Nephila sumptuosa*	41.2	12.4	28.8	17.5	587.0	45.1	0.5	45.6	8.7	195.0	32.7	11.2	27.0	29.1	196.0	45.9	25.5	13.8	14.8	196.0
Avg.	39.3	16.0	28.3	16.4	587.9	47.4	5.2	42.4	5.1	195.9	26.1	16.1	29.7	28.2	196.0	44.3	26.8	13.0	15.9	196.0

1.3.2.6 Species Name: *Aedes vittatus*

GenBank Accession Number: MT858330.1

Voucher Number: ST14

Systematic position

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Diptera
Suborder	:	Nematocera
Family	:	Culicidae
Subfamily	:	Culicini
Genus	:	*Aedes*
Subgenus	:	*Stegomyia*
Species	:	*Aedes vittatus* (Figure 1.9a)

> MT858330.1 *Aedes vittatus* |527bp

ATTGGAAATGACCAAATTTATAATGTTATTGTAACAGCTCATGCATTTATTATAATTTTCTTTATAGTA
ATACCAATTATAATTGGTGGATTTGGAAATTGATTAGTTCCTTTAATATTAGGAGCTCCTGATATAGC
TTTCCCTCGAATAAATAATATAAGTTTTTGAATATTACCTCCTTCATTAACACTACTACTTTCTAGTTC
TATAGTAGAAAACGGAGCAGGAACAGGTTGAACAGTTTATCCTCCTCTATCTTCTGGGACTGCTCATG
CTGGAGCATCAGTTGATTTAGCTATTTTTTCTCTTCATTTAGCAGGGATTTCTTCAATTTTAGGAGCAG
TAAATTTTATTACTACTGTAATTAATATACGATCAGCAGGAATTACTTTAGATCGTTTACCTTTATTTG
TTTGATCTGTTGTAATTACAGCTATTCTATTACTTTTATCATTACCAGTATTAGCAGGGGCTATTACTA
TATTATTAACAGATCGAAATTTAAATACTTCATTCTTCGACCCAAT

Figure 1.9b The DNA sequence of *Ae. vittatus* COI gene

> MT858330.1 *Aedes vittatus*

IGNDQIYNVIVTAHAFIMIFFMVMPIMIGGFGNWLVPLMLGAPDMAFPRMNNMSFWMLPPSLTLLLSSSM
VENGAGTGWTVYPPLSSGTAHAGASVDLAIFSLHLAGISSILGAVNFITTVINMRSAGITLDRLPLFVWSVV
ITAILLLLSLPVLAGAITMLLTDRNLNTSFFDP

Figure 1.9c The protein sequence of *Ae. vittatus* COI gene

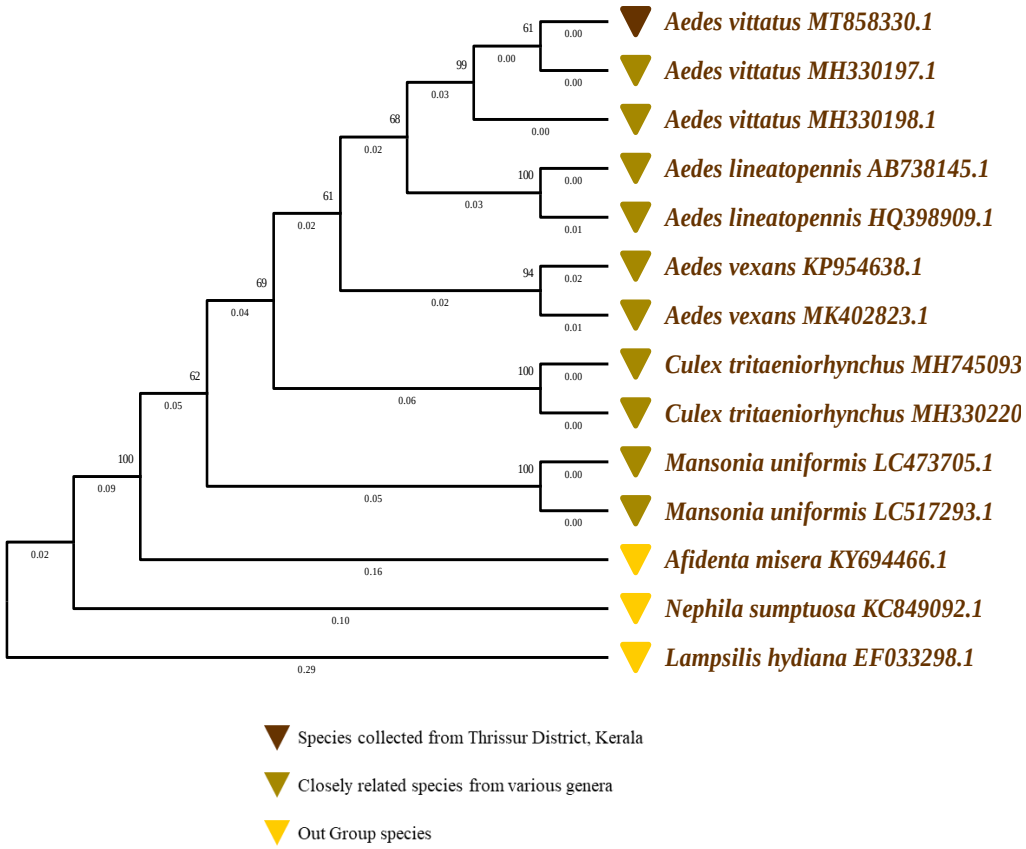

Figure 1.9d Phylogenetic tree of *Ae. vittatus*

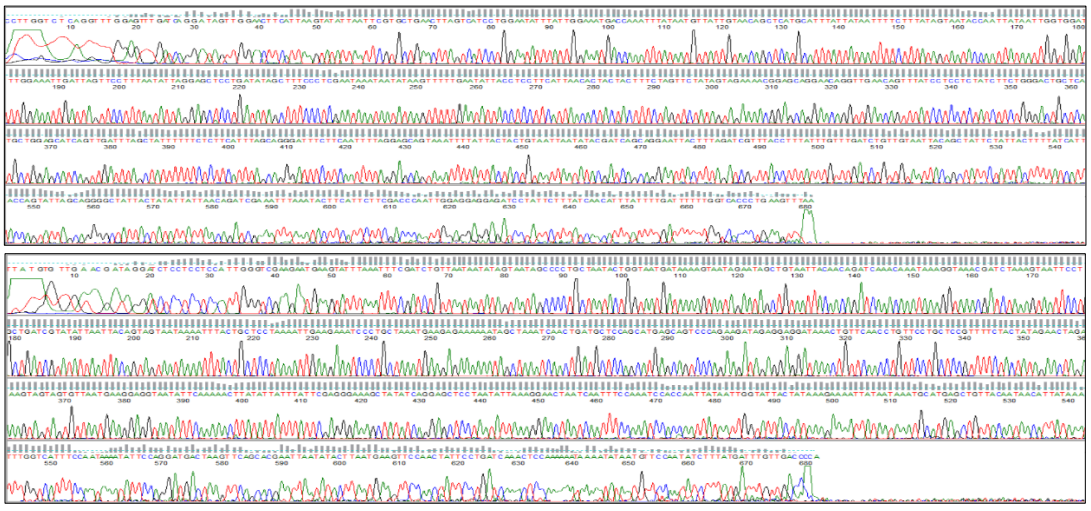

Figure 1.9e Electropherogram showing the nucleotide sequence of mitochondrial COI gene of *Ae. vittatus*

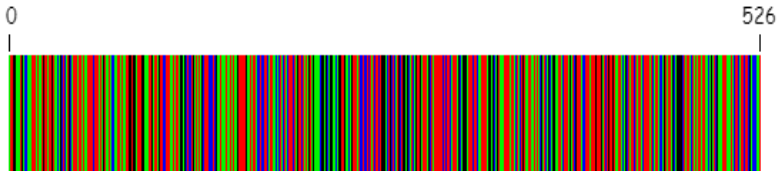

Figure 1.9f Molecular barcode of *Ae. vittatus*

Table 1.10a: Percentage of evolutionary divergence of *Ae. vittatus* with its closely related species accessible from NCBI GenBank

Accession Number	Organism	Percentage of Divergence
MT858330.1	*Aedes vittatus*	0.00%
MH330198.1	*Aedes vittatus*	0.19%
MH330197.1	*Aedes vittatus*	0.19%
AB738145.1	*Aedes lineatopennis*	7.62%
HQ398909.1	*Aedes lineatopennis*	8.16%
MK402823.1	*Aedes vexans*	9.63%
KP954638.1	*Aedes vexans*	10.24%
MH745093.1	*Culex tritaeniorhynchus*	14.03%
MH330220.1	*Culex tritaeniorhynchus*	14.10%
LC473705.1	*Mansonia uniformis*	17.41%
LC517293.1	*Mansonia uniformis*	17.41%
KY694466.1	*Afidenta misera*	43.00%
KC849092.1	*Nephila sumptuosa*	45.16%
EF033298.1	*Lampsilis hydiana*	102.36%

Table 1.10b: The nucleotide frequency comparison of *Ae. vittatus* COI gene sequence with its kin species

ACCESSION NO. AND NAME OF THE SPECIES	**NUCLEOTIDE FREQUENCIES (%)**																			
	T(U)	C	A	G	TOTAL	T-1	C-1	A-1	G-1	POS #1	T-2	C-2	A-2	G-2	POS #2	T-3	C-3	A-3	G-3	POS #3
MT858330.1 *Aedes vittatus*	39.9	16.0	29.6	14.5	524.0	27.4	14.9	29.7	28.0	175.0	44.0	29.1	13.1	13.7	175.0	48.3	4.0	46.0	1.7	174.0
MH330198.1 *Aedes vittatus*	39.9	16.0	29.8	14.3	524.0	27.4	14.9	29.7	28.0	175.0	44.0	29.1	13.1	13.7	175.0	48.3	4.0	46.6	1.1	174.0
MH330197.1 *Aedes vittatus*	39.9	16.0	29.4	14.7	524.0	27.4	14.9	29.7	28.0	175.0	44.0	29.1	13.1	13.7	175.0	48.3	4.0	45.4	2.3	174.0
AB738145.1 *Aedes lineatopennis*	40.6	16.2	28.6	14.5	524.0	26.3	16.0	29.7	28.0	175.0	44.0	28.6	13.1	14.3	175.0	51.7	4.0	43.1	1.1	174.0
HQ398909.1 *Aedes lineatopennis*	40.3	16.8	28.4	14.5	524.0	26.3	16.0	29.7	28.0	175.0	44.0	28.6	13.1	14.3	175.0	50.6	5.7	42.5	1.1	174.0
KP954638.1 *Aedes vexans*	41.0	15.8	29.0	14.1	524.0	28.0	14.9	29.7	27.4	175.0	44.0	29.1	13.1	13.7	175.0	51.1	3.4	44.3	1.1	174.0
MK402823.1 *Aedes vexans*	40.5	15.8	29.4	14.3	524.0	28.0	14.9	29.7	27.4	175.0	44.0	29.1	13.1	13.7	175.0	49.4	3.4	45.4	1.7	174.0
LC473705.1 *Mansonia uniformis*	40.3	14.9	30.2	14.7	524.0	27.4	13.7	30.9	28.0	175.0	44.0	28.6	13.7	13.7	175.0	49.4	2.3	46.0	2.3	174.0
LC517293.1 *Mansonia uniformis*	40.3	14.9	30.2	14.7	524.0	27.4	13.7	30.9	28.0	175.0	44.0	28.6	13.7	13.7	175.0	49.4	2.3	46.0	2.3	174.0
MH745093.1 *Culex tritaeniorhynchus*	39.5	16.0	29.6	14.9	524.0	24.6	17.7	29.7	28.0	175.0	44.0	28.6	13.1	14.3	175.0	50.0	1.7	46.0	2.3	174.0
MH330220.1 *Culex tritaeniorhynchus*	39.3	16.2	29.8	14.7	524.0	24.0	18.3	29.7	28.0	175.0	44.0	28.6	13.1	14.3	175.0	50.0	1.7	46.6	1.7	174.0
KY694466.1 *Afidenta misera*	38.4	18.9	28.1	14.7	524.0	24.6	16.6	34.3	24.6	175.0	44.6	26.3	14.3	14.9	175.0	46.0	13.8	35.6	4.6	174.0
EF033298.1 *Lampsilis hydiana*	45.6	12.4	18.7	23.3	524.0	35.4	11.4	25.7	27.4	175.0	45.1	21.1	14.9	18.9	175.0	56.3	4.6	15.5	23.6	174.0
KC849092.1 *Nephila sumptuosa*	43.0	12.2	27.7	17.0	523.0	34.9	10.3	26.3	28.6	175.0	46.9	25.7	14.3	13.1	175.0	47.4	0.6	42.8	9.2	173.0
Avg.	40.6	15.6	28.5	15.4	523.9	27.8	14.9	29.7	27.7	175.0	44.3	27.9	13.5	14.3	175.0	49.7	4.0	42.3	4.0	173.9

1.3.2.7 Species Name: *Armigeres subalbatus*

GenBank Accession Number: MK297327.1

Voucher Number: ST04

Systematic position

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Diptera
Suborder	:	Nematocera
Family	:	Culicidae
Subfamily	:	Culicini
Genus	:	*Armigeres*
Species	:	*Armigeres subalbatus* (Figure 1.10a)

> MK297327.1 *Armigeres subalbatus* |666bp

GATATTGGAACTTTATATTTTATTTTTGGTGCTTGAGCTGGAATAGTGGGAACTTCTTTAAGTATTTTA
ATTCGAACAGAATTAAATCACCCTGGAGTATTTATTGGAAATGATCAAATTTATAATGTAATTGTAA
CAGCTCATGCTTTTATTATAATTTTTTTTATAGTTATACCAATTATAATTGGAGGATTTGGAAATTGAT
TAGTACCCCTTATACTTGGAGCTCCAGATATAGCCTTCCCTCGAATAAATAATATAAGTTTTTGAATA
TTACCCCCTTCATTAACTCTACTAATTTCAAGTTCTTTAGTAGAAACAGGAGCTGGAACTGGATGAAC
CGTTTATCCTCCTTTATCTTCTGGAACTGCCCATGCTGGAGCTTCTGTTGATTTAGCTATTTTCTCTCTT
CATTTAGCAGGTATTTCTTCTATTTTGGGAGCAGTAAATTTTATTACAACTGTAATTAATATACGATC
ATCAGGGATTACTCTTGATCGATTACCCTTATTTGTTTGATCTGTTGTTATTACAGCTATTTTACTTCT
TCTTTCTTTACCAGTTTTAGCAGGAGCTATTACTATACTATTAACTGATCGGAATTTAAATACCTCATT
CTTTGACCCAATTGGAGGAGGAGATCCGATCTTATACCAACATTTATTT

Figure 1.10b The DNA sequence of *Ar. subalbatus* COI gene

> MK297327.1 *Armigeres subalbatus*

MGTLYFIFGAWAGMVGTSLSILIRTELNHPGVFIGNDQIYNVIVTAHAFIMIFFMVMPIMIGGFGNWLVPLM
LGAPDMAFPRMNNMSFWMLPPSLTLLISSSLVETGAGTGWTVYPPLSSGTAHAGASVDLAIFSLHLAGISSI
LGAVNFITTVINMRSSGITLDRLPLFVWSVVITAILLLLSLPVLAGAITMLLTDRNLNTSFFDPIGGGDPILYQ
HLF

Figure 1.10c The protein sequence of *Ar. subalbatus* COI gene

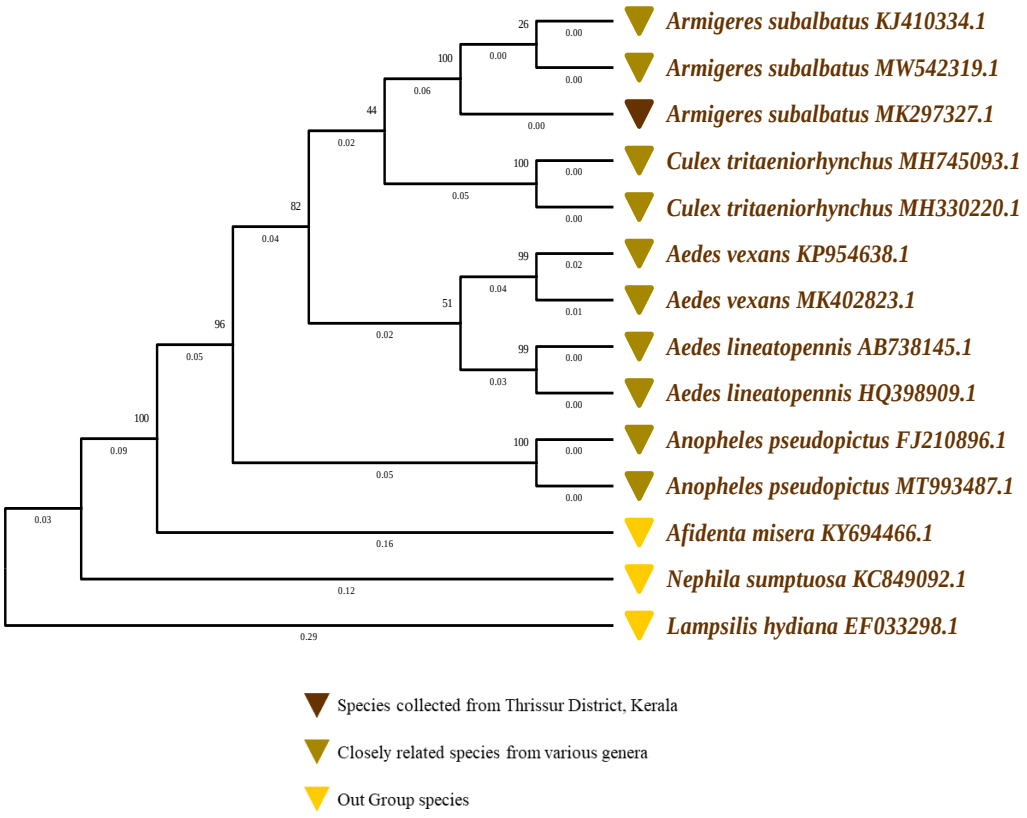

Figure 1.10d Phylogenetic tree of *Ar. subalbatus*

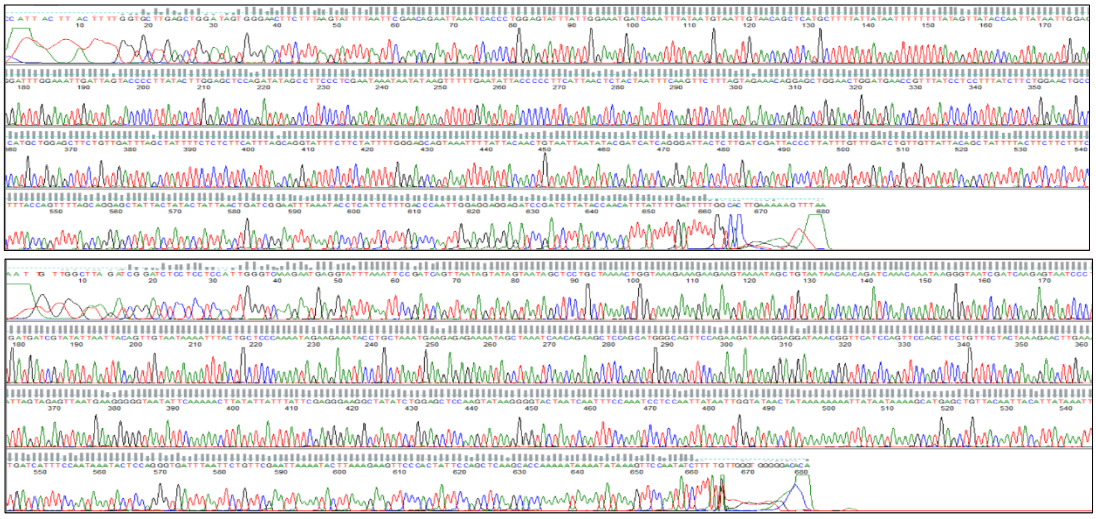

Figure 1.10e Electropherogram showing the nucleotide sequence of mitochondrial COI gene of *Ar. subalbatus*

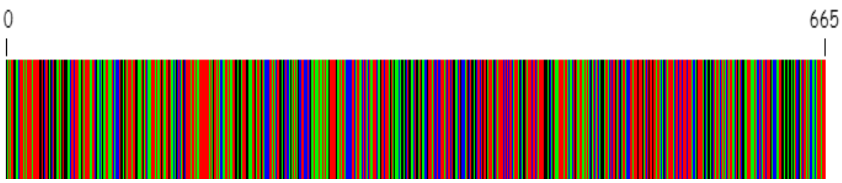

Figure 1.10f Molecular barcode of *Ar. subalbatus*

Table 1.11a: Percentage of evolutionary divergence of *Ar. subalbatus* with its closely related species accessible from NCBI GenBank

Accession Number	Organism	Percentage of Divergence
MK297327.1	*Armigeres subalbatus*	0.00%
KJ410334.1	*Armigeres subalbatus*	0.00%
MW542319.1	*Armigeres subalbatus*	0.00%
AB738145.1	*Aedes lineatopennis*	9.55%
HQ398909.1	*Aedes lineatopennis*	10.32%
MH745093.1	*Culex tritaeniorhynchus*	11.32%
MH330220.1	*Culex tritaeniorhynchus*	11.32%
KP954638.1	*Aedes vexans*	13.15%
MK402823.1	*Aedes vexans*	14.15%
FJ210896.1	*Anopheles pseudopictus*	15.16%
MT993487.1	*Anopheles pseudopictus*	15.38%
KY694466.1	*Afidenta misera*	27.89%
KC849092.1	*Nephila sumptuosa*	29.65%
EF033298.1	*Lampsilis hydiana*	48.23%

Table 1.11b: The nucleotide frequency comparison of *Ar. subalbatus* COI gene sequence with its kin species

ACCESSION NO. AND NAME OF THE SPECIES	**NUCLEOTIDE FREQUENCIES (%)**																		
	T(U)	C	A	G	TOTAL	T-1	C-1	A-1	G-1	POS #1	T-2	C-2	A-2	G-2	POS #2	T-3	C-3	A-3	G-3	POS #3
MK297327.1 *Armigeres subalbatus*	40.0	17.0	28.0	15.0	593.0	26.3	16.2	29.8	27.8	198.0	43.4	28.8	12.6	15.2	198.0	50.3	6.1	41.6	2.0	197.0
KJ410334.1 *Armigeres subalbatus*	40.0	17.0	28.0	15.0	593.0	26.3	16.2	29.8	27.8	198.0	43.4	28.8	12.6	15.2	198.0	50.3	6.1	41.6	2.0	197.0
MW542319.1 *Armigeres subalbatus*	40.0	17.0	28.0	15.0	593.0	26.3	16.2	29.8	27.8	198.0	43.4	28.8	12.6	15.2	198.0	50.3	6.1	41.6	2.0	197.0
KP954638.1 *Aedes vexans*	40.1	15.5	29.3	15.0	593.0	28.3	14.6	29.8	27.3	198.0	43.4	28.3	12.6	15.7	198.0	48.7	3.6	45.7	2.0	197.0
MK402823.1 *Aedes vexans*	39.5	15.5	30.0	15.0	593.0	28.3	14.6	29.8	27.3	198.0	43.4	28.3	12.6	15.7	198.0	46.7	3.6	47.7	2.0	197.0
AB738145.1 *Aedes lineatopennis*	40.0	15.7	29.2	15.2	593.0	26.8	15.7	29.8	27.8	198.0	43.4	27.8	12.6	16.2	198.0	49.7	3.6	45.2	1.5	197.0
HQ398909.1 *Aedes lineatopennis*	39.6	16.2	29.2	15.0	593.0	26.8	15.7	29.8	27.8	198.0	43.4	27.8	12.6	16.2	198.0	48.7	5.1	45.2	1.0	197.0
FJ210896.1 *Anopheles pseudopictus*	38.4	15.3	30.7	15.5	593.0	26.3	14.6	29.3	29.8	198.0	43.4	27.8	12.6	16.2	198.0	45.7	3.6	50.3	0.5	197.0
MT993487.1 *Anopheles pseudopictus*	38.3	15.3	30.9	15.5	593.0	26.3	14.6	29.3	29.8	198.0	42.9	27.8	13.1	16.2	198.0	45.7	3.6	50.3	0.5	197.0
MH745093.1 *Culex tritaeniorhynchus*	39.0	15.7	29.7	15.7	593.0	24.7	17.2	29.3	28.8	198.0	43.4	27.8	12.6	16.2	198.0	48.7	2.0	47.2	2.0	197.0
MH330220.1 *Culex tritaeniorhynchus*	38.8	15.9	29.8	15.5	593.0	24.2	17.7	29.3	28.8	198.0	43.4	27.8	12.6	16.2	198.0	48.7	2.0	47.7	1.5	197.0
KY694466.1 *Afidenta misera*	37.4	18.5	28.3	15.7	593.0	24.7	16.2	33.8	25.3	198.0	43.9	26.3	13.1	16.7	198.0	43.7	13.2	38.1	5.1	197.0
EF033298.1 *Lampsilis hydiana*	44.5	12.0	18.4	25.1	593.0	35.4	11.6	24.7	28.3	198.0	44.9	19.7	14.1	21.2	198.0	53.3	4.6	16.2	25.9	197.0
KC849092.1 *Nephila sumptuosa*	41.0	12.5	28.7	17.7	592.0	32.8	11.1	26.8	29.3	198.0	45.5	25.8	13.6	15.2	198.0	44.9	0.5	45.9	8.7	196.0
Avg.	39.8	15.7	28.4	16.1	592.9	27.4	15.2	29.4	28.1	198.0	43.7	27.2	12.9	16.2	198.0	48.2	4.5	43.2	4.1	196.9

1.3.2.8 Species Name: *Culex gelidus*

GenBank Accession Number: MK630238.1

Voucher Number: ST06

Systematic position

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Diptera
Suborder	:	Nematocera
Family	:	Culicidae
Subfamily	:	Culicini
Genus	:	*Culex*
Subgenus	:	*Culex*
Species	:	*Culex gelidus* (Figure 1.11a)

> MK630238.1 *Culex gelidus*|677bp

AAGATATTGGAACATTATATTTTATTTTTGGGGCTTGAGCAGGAATAATTGGAACTTCATTAAGAATTC
TAATTCGAGCAGAACTAAGTCAACCTGGAGTATTTATTGGAAATGATCAAATTTATAATGTTATTGTAA
CTGCTCACGCTTTTATTATAATTTTTTTTATAGTTATACCTATTATAATTGGAGGATTTGGAAATTGATT
AGTTCCTTTAATACTAGGAGCTCCTGATATAGCATTTCCTCGAATAAATAATATAAGTTTTTGAATACT
TCCTCCTTCATTAACTTTACTACTTTCAAGTAGTTTAGTTGAAAATGGGGCTGGAACTGGATGAACAGT
TTATCCCCCTCTTTCATCAGGTACAGCTCATGCTGGAGCTTCAGTTGATTTAGCTATTTTTTCTTTACAT
TTAGCTGGGATTTCATCAATTTTAGGAGCAGTAAATTTTATTACAACAGTAATTAATATACGATCTTCA
GGAATTACACTTGATCGAATACCTTTATTTGTTTGATCTGTAGTTATTACTGCTGTTTTATTACTCCTTTC
ATTACCCGTATTAGCTGGAGCTATTACAATATTATTAACTGATCGAAACCTAAATACTTCATTTTTTGA
CCCTATTGGAGGAGGAGATCCTATTTTATACCAACATTTATTTTGATTTTTT

Figure 1.11b The DNA sequence of *Cx. gelidus* COI gene

> MK630238.1 *Culex gelidus*

MGTLYFIFGAWAGMIGTSLSILIRAELSQPGVFIGNDQIYNVIVTAHAFIMIFFMVMPIMIGGFGNWLVPLML
GAPDMAFPRMNNMSFWMLPPSLTLLLSSSLVENGAGTGWTVYPPLSSGTAHAGASVDLAIFSLHLAGISSIL
GAVNFITTVINMRSSGITLDRMPLFVWSVVITAVLLLLSLPVLAGAITMLLTDRNLNTSFFDPIGGGDPILYQ
HLFWFF

Figure 1.11c The protein sequence of *Cx. gelidus* COI gene

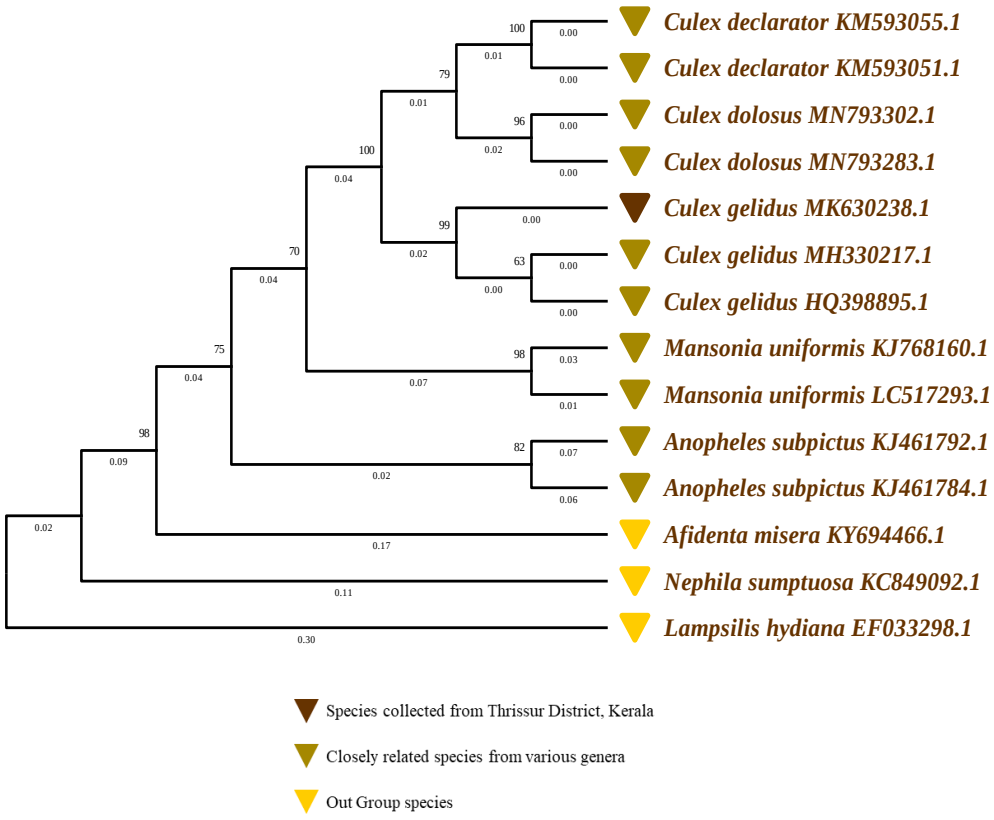

Figure 1.11d Phylogenetic tree of *Cx. gelidus*

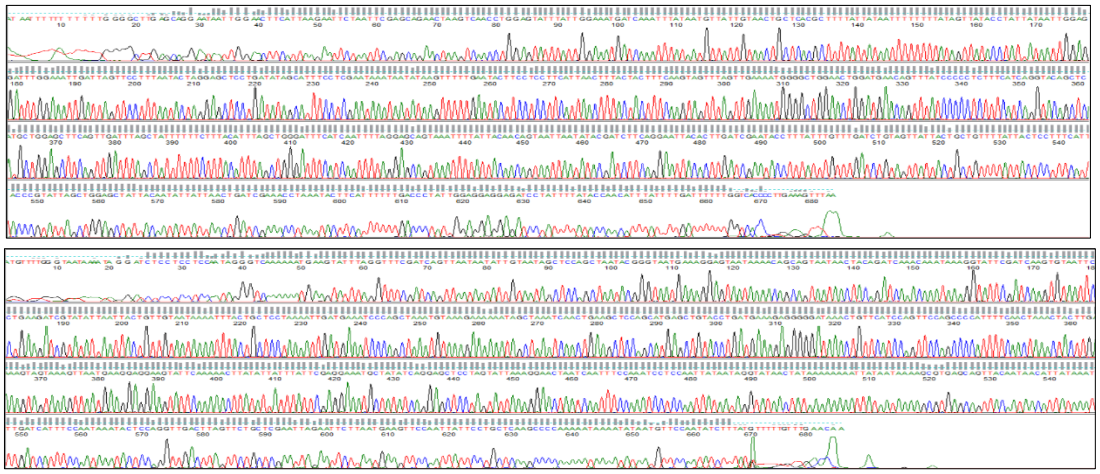

Figure 1.11e Electropherogram showing the nucleotide sequence of mitochondrial COI gene of *Cx. gelidus*

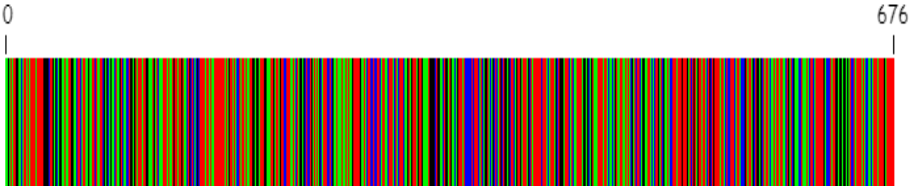

Figure 1.11f Molecular barcode of *Cx. gelidus*

Table 1.12a: Percentage of evolutionary divergence of *Cx. gelidus* with its closely related species accessible from NCBI GenBank

Accession Number	Organism	Percentage of Divergence
MK630238.1	*Culex gelidus*	0.00%
MH330217.1	*Culex gelidus*	0.43%
HQ398895.1	*Culex gelidus*	0.29%
KM593055.1	*Culex declarator*	3.90%
KM593051.1	*Culex declarator*	3.90%
MN793302.1	*Culex dolosus*	4.70%
MN793283.1	*Culex dolosus*	4.53%
LC517293.1	*Mansonia uniformis*	11.82%
KJ768160.1	*Mansonia uniformis*	12.53%
KJ461792.1	*Anopheles subpictus*	12.79%
KJ461784.1	*Anopheles subpictus*	14.17%
KC849092.1	*Nephila sumptuosa*	22.78%
KY694466.1	*Afidenta misera*	23.87%
EF033298.1	*Lampsilis hydiana*	39.93%

Table 1.12b: The nucleotide frequency comparison of *Cx. gelidus* COI gene sequence with its kin species

ACCESSION NO. AND NAME OF THE SPECIES	**NUCLEOTIDE FREQUENCIES (%)**																			
	T(U)	C	A	G	TOTAL	T-1	C-1	A-1	G-1	POS #1	T-2	C-2	A-2	G-2	POS #2	T-3	C-3	A-3	G-3	POS #3
MK630238.1 *Culex gelidus*	40.1	16.2	28.8	14.9	556.0	43.5	28.0	13.4	15.1	186.0	51.4	3.2	44.3	1.1	185.0	25.4	17.3	28.6	28.6	185.0
MH330217.1 *Culex gelidus*	40.3	16.0	28.8	14.9	556.0	43.5	28.0	13.4	15.1	186.0	51.9	2.7	44.3	1.1	185.0	25.4	17.3	28.6	28.6	185.0
HQ398895.1 *Culex gelidus*	40.1	16.2	28.8	14.9	556.0	43.5	28.0	13.4	15.1	186.0	51.4	3.2	44.3	1.1	185.0	25.4	17.3	28.6	28.6	185.0
KM593055.1 *Culex declarator*	41.2	15.3	29.0	14.6	556.0	43.5	28.0	13.4	15.1	186.0	54.1	1.6	44.3	0.0	185.0	25.9	16.2	29.2	28.6	185.0
KM593051.1 *Culex declarator*	41.2	15.3	29.0	14.6	556.0	43.5	28.0	13.4	15.1	186.0	54.1	1.6	44.3	0.0	185.0	25.9	16.2	29.2	28.6	185.0
MN793302.1 *Culex dolosus*	40.1	15.8	28.8	15.3	556.0	43.5	28.0	13.4	15.1	186.0	50.8	3.2	43.8	2.2	185.0	25.9	16.2	29.2	28.6	185.0
MN793283.1 *Culex dolosus*	40.1	15.6	29.1	15.1	556.0	43.5	28.0	13.4	15.1	186.0	50.8	2.7	44.9	1.6	185.0	25.9	16.2	29.2	28.6	185.0
KJ461792.1 *Anopheles subpictus*	38.7	15.6	29.0	16.7	556.0	43.5	28.0	13.4	15.1	186.0	45.4	4.3	44.3	5.9	185.0	27.0	14.6	29.2	29.2	185.0
KJ461784.1 *Anopheles subpictus*	38.1	16.2	29.1	16.5	556.0	43.5	28.0	13.4	15.1	186.0	44.9	4.9	44.9	5.4	185.0	25.9	15.7	29.2	29.2	185.0
KJ768160.1 *Mansonia uniformis*	39.2	16.4	29.5	14.9	556.0	44.1	27.4	14.0	14.5	186.0	47.0	6.5	44.3	2.2	185.0	26.5	15.1	30.3	28.1	185.0
LC517293.1 *Mansonia uniformis*	40.1	14.9	30.0	14.9	556.0	44.1	27.4	14.0	14.5	186.0	49.2	2.7	45.9	2.2	185.0	27.0	14.6	30.3	28.1	185.0
KY694466.1 *Afidenta misera*	38.1	18.7	28.1	15.1	556.0	44.1	26.3	14.0	15.6	186.0	45.4	13.0	36.8	4.9	185.0	24.9	16.8	33.5	24.9	185.0
EF033298.1 *Lampsilis hydiana*	44.8	12.6	18.5	24.1	556.0	44.6	20.4	15.1	19.9	186.0	55.7	4.9	15.1	24.3	185.0	34.1	12.4	25.4	28.1	185.0
KC849092.1 *Nephila sumptuosa*	42.0	12.6	27.9	17.5	555.0	45.7	25.3	14.5	14.5	186.0	46.7	0.5	43.5	9.2	184.0	33.5	11.9	25.9	28.6	185.0
Avg.	40.3	15.5	28.2	16.0	555.9	43.9	27.0	13.7	15.3	186.0	49.9	3.9	41.8	4.4	184.9	27.1	15.6	29.0	28.3	185.0

1.3.2.9 Species Name: Culex tritaeniorhynchus

GenBank Accession Number: MH745093.1

Voucher Number: ST07

Systematic position

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Diptera
Suborder	:	Nematocera
Family	:	Culicidae
Subfamily	:	Culicini
Genus	:	*Culex*
Subgenus	:	*Culex*
Species	:	*Culex tritaeniorhynchus* (Figure 1.12a)

> MH745093.1 *Culex tritaeniorhynchus*|672bp

AGATATTGGAACATTATATTTTATTTTTGGGGCTTGAGCTGGAATAGTAGGTACTTCTTTAAG
TATTTTAATTCGAGCAGAATTAAGTCAACCCGGAGTATTTATTGGAAATGATCAAATTTATA
ATGTTATTGTAACTGCTCATGCTTTTATTATAATTTTTTTTATAGTAATACCAATTATAATTGG
TGGATTTGGAAATTGATTAGTTCCTTTAATACTTGGAGCTCCTGATATGGCCTTTCCACGAAT
AAATAATATAAGTTTTTGAATACTACCTCCTTCATTAACTCTACTACTTTCAAGTAGTTTAGT
AGAAAATGGAGCTGGGACTGGATGAACAGTTTATCCACCTCTATCATCTGGAACAGCACATG
CTGGAGCTTCAGTTGATTTAGCTATTTTTTCTTTACATTTAGCTGGGATTTCATCAATTTTAGG
GGCAGTAAATTTTATTACAACAGTAATTAATATACGATCTTCAGGAATTACACTTGATCGAA
TACCTTTATTTGTTTGATCAGTAGTAATTACTGCTGTTTTATTACTTCTTTCACTACCAGTTTT
AGCAGGAGCTATTACTATACTATTAACAGATCGAAATCTTAATACTTCATTCTTTGACCCAAT
TGGAGGAGGAGACCCAATTCTTTATCAACACTTATTCTGATT

Figure 1.12b The DNA sequence of *Cx. tritaeniorhynchus* COI gene

> MH745093.1 *Culex tritaeniorhynchus*

MGTLYFIFGAWAGMVGTSLSILIRAELSQPGVFIGNDQIYNVIVTAHAFIMIFFMVMPIMIGGFGNWLVPLM
LGAPDMAFPRMNNMSFWMLPPSLTLLLSSSLVENGAGTGWTVYPPLSSGTAHAGASVDLAIFSLHLAGISS
ILGAVNFITTVINMRSSGITLDRMPLFVWSVVITAVLLLLSLPVLAGAITMLLTDRNLNTSFFDPIGGGDPILY
QHLFWFL

Figure 1.12c The protein sequence of *Cx. tritaeniorhynchus* COI gene

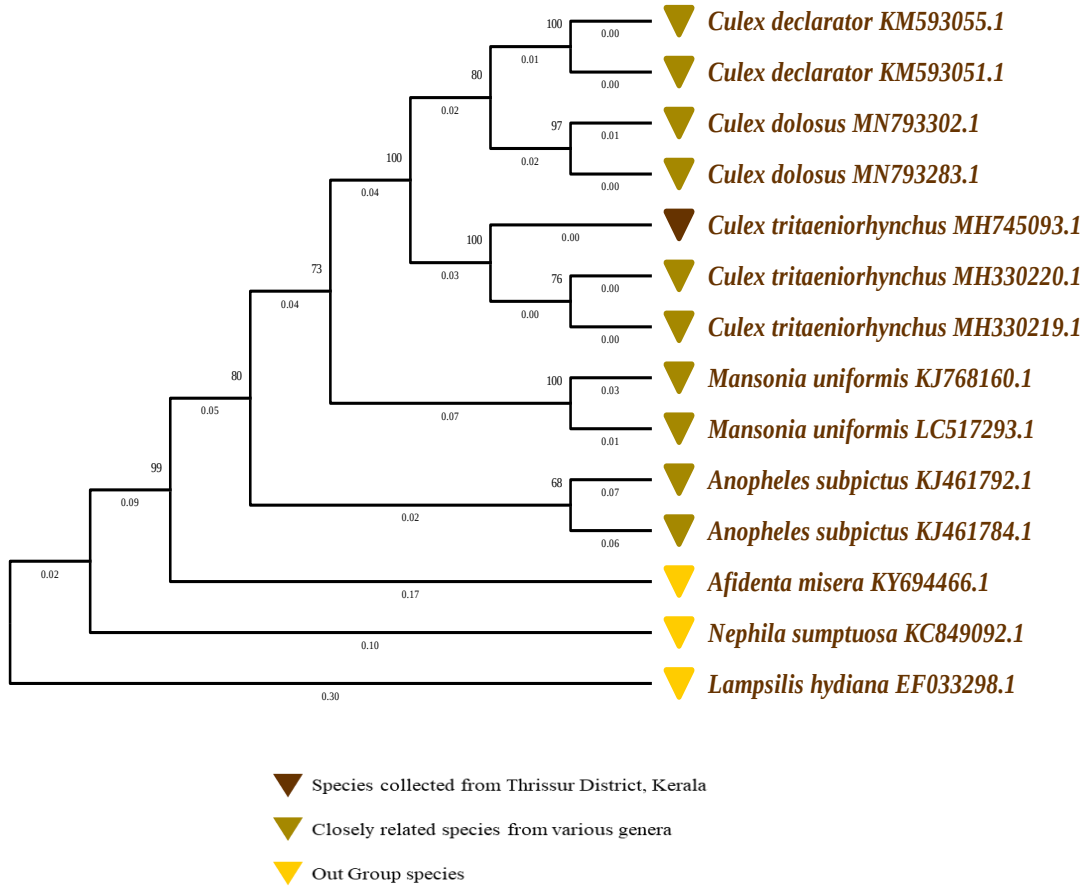

Figure 1.12d Phylogenetic tree of *Cx. tritaeniorhynchus*

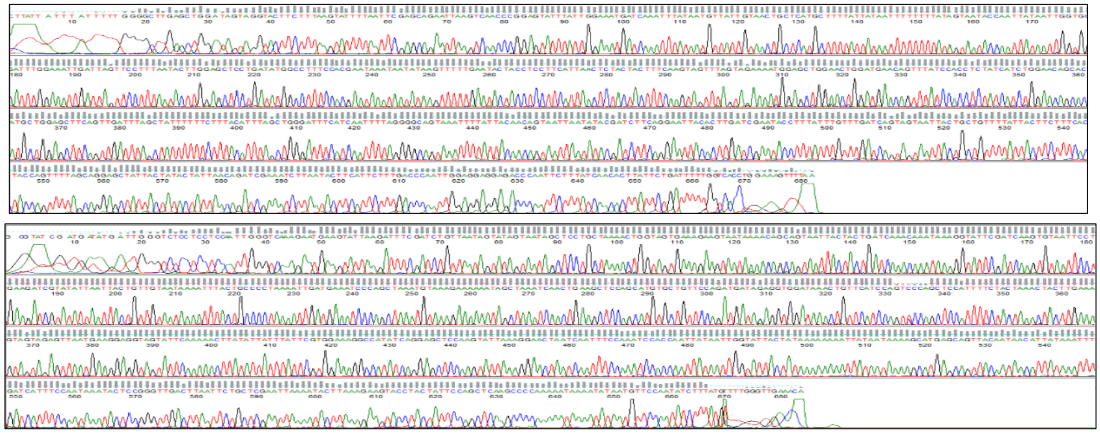

Figure 1.12e Electropherogram showing the nucleotide sequence of mitochondrial COI gene of *Cx. tritaeniorhynchus*

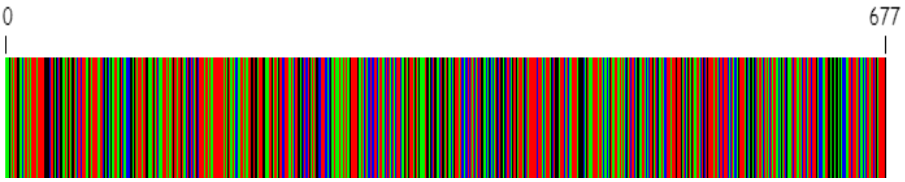

Figure 1.12f Molecular barcode of *Cx. tritaeniorhynchus*

Table 1.13a: Percentage of evolutionary divergence of *Cx. tritaeniorhynchus* with its closely related species accessible from NCBI GenBank

Accession Number	Organism	Percentage of Divergence
MH745093.1	*Culex tritaeniorhynchus*	0.00%
MH330220.1	*Culex tritaeniorhynchus*	0.36%
MH330219.1	*Culex tritaeniorhynchus*	0.54%
KM593055.1	*Culex declarator*	6.64%
KM593051.1	*Culex declarator*	6.64%
MN793302.1	*Culex dolosus*	7.76%
MN793283.1	*Culex dolosus*	7.77%
LC517293.1	*Mansonia uniformis*	17.04%
KJ461784.1	*Anopheles subpictus*	18.52%
KJ768160.1	*Mansonia uniformis*	19.38%
KJ461792.1	*Anopheles subpictus*	19.61%
KC849092.1	*Nephila sumptuosa*	36.06%
KY694466.1	*Afidenta misera*	39.39%
EF033298.1	*Lampsilis hydiana*	79.59%

Table 1.13b: The nucleotide frequency comparison of *Cx. tritaeniorhynchus* COI gene sequence with its kin species

ACCESSION NO. AND NAME OF THE SPECIES	**NUCLEOTIDE FREQUENCIES (%)**																			
	T(U)	C	A	G	TOTAL	T-1	C-1	A-1	G-1	POS #1	T-2	C-2	A-2	G-2	POS #2	T-3	C-3	A-3	G-3	POS #3
MH745093.1 *Culex tritaeniorhynchus*	38.8	16.2	29.7	15.3	556.0	43.5	28.0	13.4	15.1	186.0	48.6	2.2	47.0	2.2	185.0	24.3	18.4	28.6	28.6	185.0
MH330220.1 *Culex tritaeniorhynchus*	38.7	16.4	29.9	15.1	556.0	43.5	28.0	13.4	15.1	186.0	48.6	2.2	47.6	1.6	185.0	23.8	18.9	28.6	28.6	185.0
MH330219.1 *Culex tritaeniorhynchus*	38.7	16.4	29.7	15.3	556.0	43.5	28.0	13.4	15.1	186.0	48.6	2.2	47.0	2.2	185.0	23.8	18.9	28.6	28.6	185.0
KM593055.1 *Culex declarator*	41.2	15.3	29.0	14.6	556.0	43.5	28.0	13.4	15.1	186.0	54.1	1.6	44.3	0.0	185.0	25.9	16.2	29.2	28.6	185.0
KM593051.1 *Culex declarator*	41.2	15.3	29.0	14.6	556.0	43.5	28.0	13.4	15.1	186.0	54.1	1.6	44.3	0.0	185.0	25.9	16.2	29.2	28.6	185.0
MN793302.1 *Culex dolosus*	40.1	15.8	28.8	15.3	556.0	43.5	28.0	13.4	15.1	186.0	50.8	3.2	43.8	2.2	185.0	25.9	16.2	29.2	28.6	185.0
MN793283.1 *Culex dolosus*	40.1	15.6	29.1	15.1	556.0	43.5	28.0	13.4	15.1	186.0	50.8	2.7	44.9	1.6	185.0	25.9	16.2	29.2	28.6	185.0
KJ461792.1 *Anopheles subpictus*	38.7	15.6	29.0	16.7	556.0	43.5	28.0	13.4	15.1	186.0	45.4	4.3	44.3	5.9	185.0	27.0	14.6	29.2	29.2	185.0
KJ461784.1 *Anopheles subpictus*	38.1	16.2	29.1	16.5	556.0	43.5	28.0	13.4	15.1	186.0	44.9	4.9	44.9	5.4	185.0	25.9	15.7	29.2	29.2	185.0
KJ768160.1 *Mansonia uniformis*	39.2	16.4	29.5	14.9	556.0	44.1	27.4	14.0	14.5	186.0	47.0	6.5	44.3	2.2	185.0	26.5	15.1	30.3	28.1	185.0
LC517293.1 *Mansonia uniformis*	40.1	14.9	30.0	14.9	556.0	44.1	27.4	14.0	14.5	186.0	49.2	2.7	45.9	2.2	185.0	27.0	14.6	30.3	28.1	185.0
KY694466.1 *Afidenta misera*	38.1	18.7	28.1	15.1	556.0	44.1	26.3	14.0	15.6	186.0	45.4	13.0	36.8	4.9	185.0	24.9	16.8	33.5	24.9	185.0
EF033298.1 *Lampsilis hydiana*	44.8	12.6	18.5	24.1	556.0	44.6	20.4	15.1	19.9	186.0	55.7	4.9	15.1	24.3	185.0	34.1	12.4	25.4	28.1	185.0
KC849092.1 *Nephila sumptuosa*	42.0	12.6	27.9	17.5	555.0	45.7	25.3	14.5	14.5	186.0	46.7	0.5	43.5	9.2	184.0	33.5	11.9	25.9	28.6	185.0
Avg.	40.0	15.6	28.4	16.1	555.9	43.9	27.0	13.7	15.3	186.0	49.3	3.7	42.4	4.6	184.9	26.8	15.9	29.0	28.3	185.0

1.3.2.10 Species Name: *Culex pipiens*

GenBank Accession Number: MK347224.1

Voucher Number: ST09

Systematic position

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Diptera
Suborder	:	Nematocera
Family	:	Culicidae
Subfamily	:	Culicini
Genus	:	*Culex*
Subgenus	:	*Culex*
Species	:	*Culex pipiens* (Figure 1.13a)

> MK347224.1 *Culex pipiens* |680bp

AAAGATATTGGAACATTATATTTTATTTTTGGGGCTTGAGCTGGAATAGTTGGAACTTCTTTAAGTTTACT
AATTCGAGCAGAATTAAGTCAACCAGGTGTATTTATTGGAAATGATCAAATTTATAATGTTATTGTAACTG
CTCATGCTTTTATTATAATTTTTTTTATAGTAATACCAATCATAATTGGAGGATTTGGAAATTGATTAGTTC
CTTTAATGTTAGGAGCTCCAGATATGGCCTTTCCTCGAATAAATAATATAAGTTTTTGAATACTACCTCCT
TCATTGACACTACTACTTTCAAGTAGTTTAGTAGAAAATGGGGCTGGGACTGGATGAACAGTGTATCCCC
CTCTTTCATCTGGAACAGCTCATGCTGGAGCTTCAGTAGACTTAGCTATTTTTTCTTTACATTTAGCAGGAA
TTTCATCAATTTTAGGTGCAGTAAATTTTATTACAACAGTAATTAATATACGATCTTCAGGAATTACTCTT
GATCGAATACCTTTATTTGTTTGATCAGTAGTAATTACTGCAGTTTTATTACTTCTTTCTTTACCTGTTTTAG
CTGGTGCTATTACTATGTTATTAACAGATCGAAATTTAAATACTTCATTCTTTGATCCAATTGGAGGAGGA
GATCCAATTTTATATCAACATTTATTTTGATTTTTTGG

Figure 1.13b The DNA sequence of *Cx. pipiens* COI gene

> MK347224.1 *Culex pipiens*

MGTLYFIFGAWAGMVGTSLSLLIRAELSQPGVFIGNDQIYNVIVTAHAFIMIFFMVMPIMIGGFGNWLVPLMLGA
PDMAFPRMNNMSFWMLPPSLTLLLSSSLVENGAGTGWTVYPPLSSGTAHAGASVDLAIFSLHLAGISSILGAVNF
ITTVINMRSSGITLDRMPLFVWSVVITAVLLLLSLPVLAGAITMLLTDRNLNTSFFDPIGGGDPILYQHLFWF

Figure 1.13c The protein sequence of *Cx. pipiens* COI gene

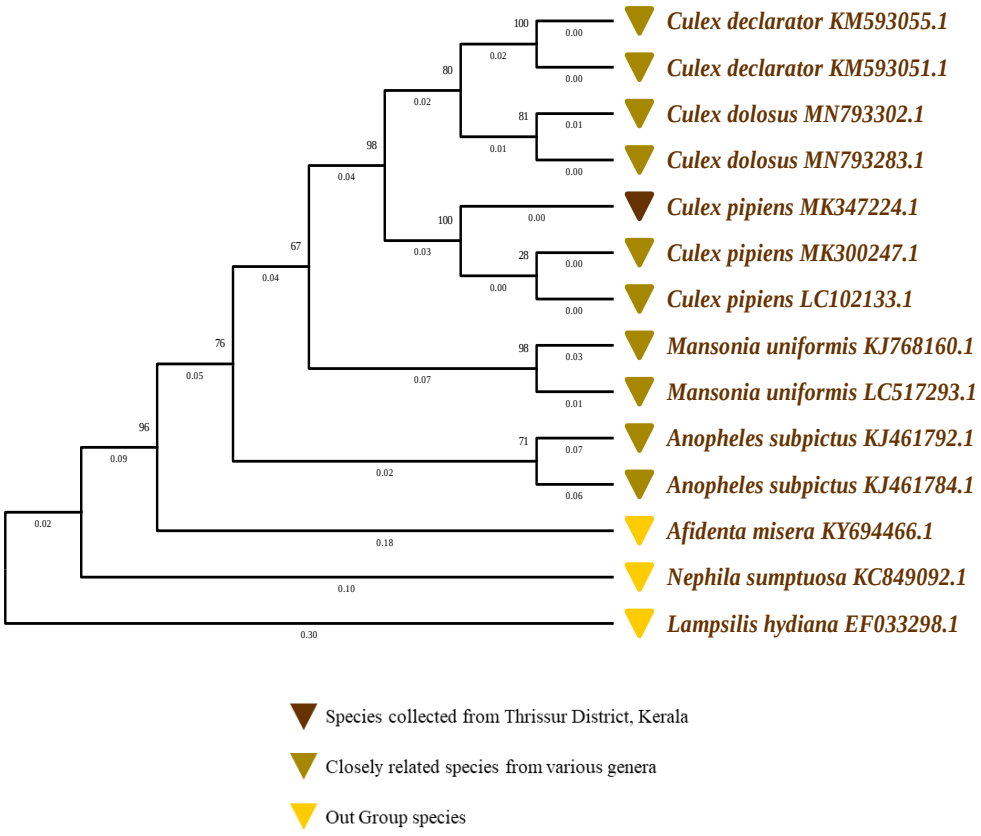

Figure 1.13d Phylogenetic tree of *Cx. pipiens*

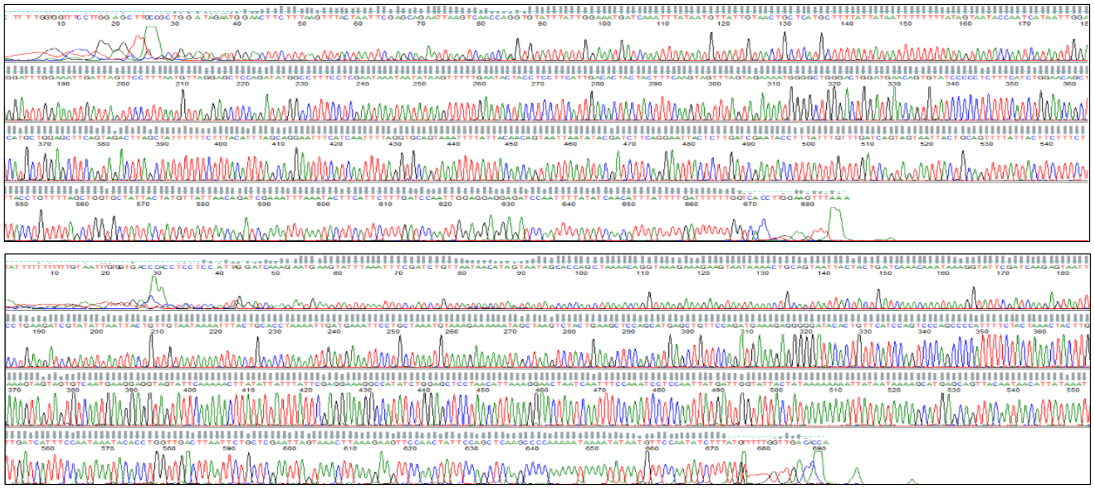

Figure 1.13e Electropherogram showing the nucleotide sequence of mitochondrial COI gene of *Cx. pipiens*

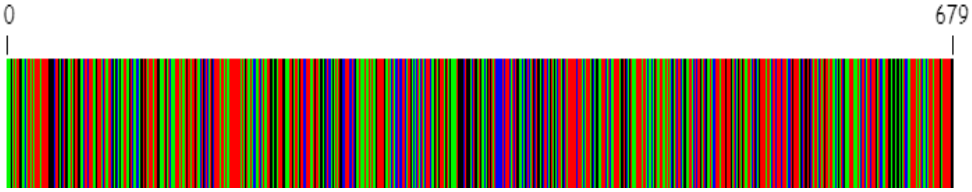

Figure 1.13f Molecular barcode of *Cx. pipiens*

Table 1.14a: Percentage of evolutionary divergence of *Cx. pipiens* with its closely related species accessible from NCBI GenBank

Accession Number	Organism	Percentage of Divergence
MK347224.1	*Culex pipiens*	0.00%
MK300247.1	*Culex pipiens*	0.00%
LC102133.1	*Culex pipiens*	0.00%
MN793283.1	*Culex dolosus*	5.80%
MN793302.1	*Culex dolosus*	5.99%
KM593055.1	*Culex declarator*	6.21%
KM593051.1	*Culex declarator*	6.21%
LC517293.1	*Mansonia uniformis*	14.31%
KJ461792.1	*Anopheles subpictus*	15.48%
KJ768160.1	*Mansonia uniformis*	16.34%
KJ461784.1	*Anopheles subpictus*	17.09%
KC849092.1	*Nephila sumptuosa*	26.99%
KY694466.1	*Afidenta misera*	30.65%
EF033298.1	*Lampsilis hydiana*	47.11%

Table 1.14b: The nucleotide frequency comparison of *Cx. pipiens* COI gene sequence with its kin species

ACCESSION NO. AND NAME OF THE SPECIES	**NUCLEOTIDE FREQUENCIES (%)**																		
	T(U)	C	A	G	TOTAL	T-1	C-1	A-1	G-1	POS #1	T-2	C-2	A-2	G-2	POS #2	T-3	C-3	A-3	G-3	POS #3
MK347224.1 *Culex pipiens*	39.6	15.6	29.0	15.8	556.0	43.5	28.0	13.4	15.1	186.0	48.6	2.7	44.9	3.8	185.0	26.5	16.2	28.6	28.6	185.0
MK300247.1 *Culex pipiens*	39.6	15.6	29.0	15.8	556.0	43.5	28.0	13.4	15.1	186.0	48.6	2.7	44.9	3.8	185.0	26.5	16.2	28.6	28.6	185.0
LC102133.1 *Culex pipiens*	39.6	15.6	29.0	15.8	556.0	43.5	28.0	13.4	15.1	186.0	48.6	2.7	44.9	3.8	185.0	26.5	16.2	28.6	28.6	185.0
KM593055.1 *Culex declarator*	41.2	15.3	29.0	14.6	556.0	43.5	28.0	13.4	15.1	186.0	54.1	1.6	44.3	0.0	185.0	25.9	16.2	29.2	28.6	185.0
KM593051.1 *Culex declarator*	41.2	15.3	29.0	14.6	556.0	43.5	28.0	13.4	15.1	186.0	54.1	1.6	44.3	0.0	185.0	25.9	16.2	29.2	28.6	185.0
MN793302.1 *Culex dolosus*	40.1	15.8	28.8	15.3	556.0	43.5	28.0	13.4	15.1	186.0	50.8	3.2	43.8	2.2	185.0	25.9	16.2	29.2	28.6	185.0
MN793283.1 *Culex dolosus*	40.1	15.6	29.1	15.1	556.0	43.5	28.0	13.4	15.1	186.0	50.8	2.7	44.9	1.6	185.0	25.9	16.2	29.2	28.6	185.0
KJ461792.1 *Anopheles subpictus*	38.7	15.6	29.0	16.7	556.0	43.5	28.0	13.4	15.1	186.0	45.4	4.3	44.3	5.9	185.0	27.0	14.6	29.2	29.2	185.0
KJ461784.1 *Anopheles subpictus*	38.1	16.2	29.1	16.5	556.0	43.5	28.0	13.4	15.1	186.0	44.9	4.9	44.9	5.4	185.0	25.9	15.7	29.2	29.2	185.0
KJ768160.1 *Mansonia uniformis*	39.2	16.4	29.5	14.9	556.0	44.1	27.4	14.0	14.5	186.0	47.0	6.5	44.3	2.2	185.0	26.5	15.1	30.3	28.1	185.0
LC517293.1 *Mansonia uniformis*	40.1	14.9	30.0	14.9	556.0	44.1	27.4	14.0	14.5	186.0	49.2	2.7	45.9	2.2	185.0	27.0	14.6	30.3	28.1	185.0
KY694466.1 *Afidenta misera*	38.1	18.7	28.1	15.1	556.0	44.1	26.3	14.0	15.6	186.0	45.4	13.0	36.8	4.9	185.0	24.9	16.8	33.5	24.9	185.0
EF033298.1 *Lampsilis hydiana*	44.8	12.6	18.5	24.1	556.0	44.6	20.4	15.1	19.9	186.0	55.7	4.9	15.1	24.3	185.0	34.1	12.4	25.4	28.1	185.0
KC849092.1 *Nephila sumptuosa*	42.0	12.6	27.9	17.5	555.0	45.7	25.3	14.5	14.5	186.0	46.7	0.5	43.5	9.2	184.0	33.5	11.9	25.9	28.6	185.0
Avg.	40.2	15.4	28.2	16.2	555.9	43.9	27.0	13.7	15.3	186.0	49.3	3.9	41.9	4.9	184.9	27.3	15.3	29.0	28.3	185.0

1.3.2.11 Species Name: *Culex quinquefasciatus*

GenBank Accession Number: MW143512.1

Voucher Number: ST17

Systematic position

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Diptera
Suborder	:	Nematocera
Family	:	Culicidae
Subfamily	:	Culicini
Genus	:	*Culex*
Subgenus	:	*Culex*
Species	:	*Culex quinquefasciatus* (Figure1.14a)

> MW143512.1 *Culex quinquefasciatus* |544bp

TTACTAATTCGAGCAGAATTAAGTCAACCAGGTGTATTTATTGGAAATGATCAAATTTATAATGT
TATTGTAACTGCTCATGCTTTTATTATAATTTTTTTTATAGTAATACCAATCATAATTGGAGGATT
TGGAAATTGATTAGTTCCTTTAATGTTAGGAGCTCCAGATATGGCCTTTCCTCGAATAAATAATA
TAAGTTTTTGAATACTACCTCCTTCATTGACACTACTACTTTCAAGTAGTTTAGTAGAAAATGGA
GCTGGGACTGGATGAACAGTGTATCCCCCTCTTTCATCTGGAACAGCTCATGCTGGAGCTTCAGT
AGACTTAGCTATTTTTTCTTTACATTTAGCAGGAATTTCATCAATTTTAGGTGCAGTAAATTTTAT
TACAACAGTAATTAATATACGATCTTCAGGAATTACTCTTGATCGAATACCTTTATTTGTTTGAT
CAGTAGTAATTACTGCAGTTTTATTACTTCTTTCTTTACCTGTTTTAGCTGGTGCTATTACTATGT
TATTAACAGATCGAAATTTAA

Figure 1.14b The DNA sequence of *Cx. quinquefasciatus* COI gene

> MW143512.1 *Culex quinquefasciatus*

LLIRAELSQPGVFIGNDQIYNVIVTAHAFIMIFFMVMPIMIGGFGNWLVPLMLGAPDMAFPRMNNMS
FWMLPPSLTLLLSSSLVENGAGTGWTVYPPLSSGTAHAGASVDLAIFSLHLAGISSILGAVNFITTVIN
MRSSGITLDRMPLFVWSVVITAVLLLLSLPVLAGAITMLLTDRNL

Figure 1.14c The protein sequence of *Cx. quinquefasciatus* COI gene

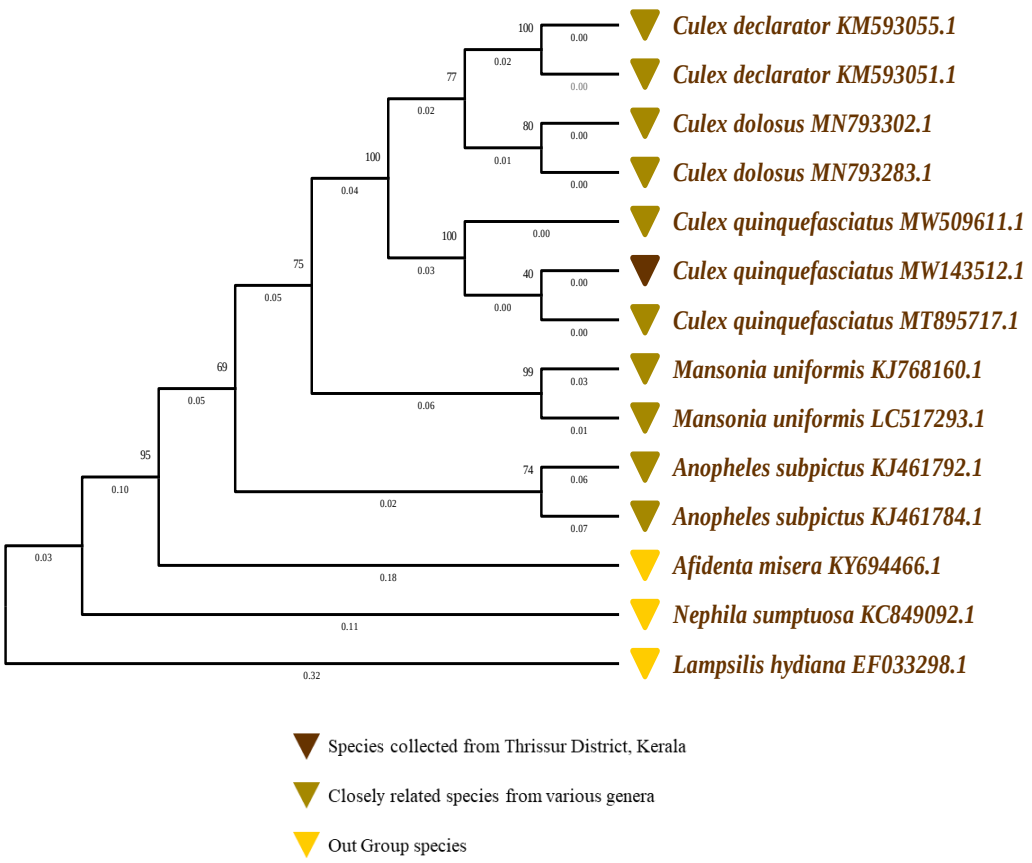

Figure 1.14d Phylogenetic tree of *Cx. quinquefasciatus*

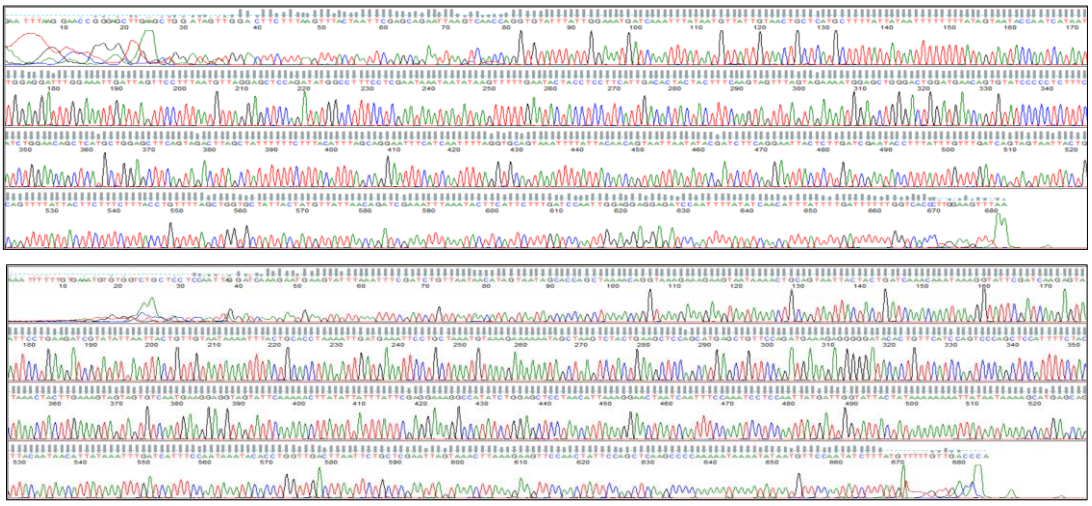

Figure 1.14e Electropherogram showing the nucleotide sequence of mitochondrial COI gene of *Cx. quinquefasciatus*

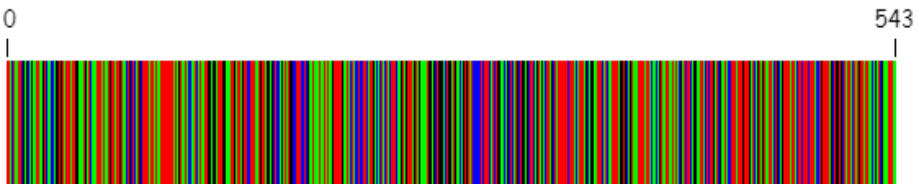

Figure 1.14f Molecular barcode of *Cx.quinquefasciatus*

Table 1.15a: Percentage of evolutionary divergence of *Cx. quinquefasciatus* with its closely related species accessible from NCBI GenBank

Accession Number	Organism	Percentage of Divergence
MW143512.1	*Culex quinquefasciatus*	0.00%
MT895717.1	*Culex quinquefasciatus*	0.00%
MW509611.1	*Culex quinquefasciatus*	0.00%
MN793302.1	*Culex dolosus*	5.29%
MN793283.1	*Culex dolosus*	5.29%
KM593055.1	*Culex declarator*	5.94%
KM593051.1	*Culex declarator*	5.94%
LC517293.1	*Mansonia uniformis*	13.34%
KJ461792.1	*Anopheles subpictus*	15.80%
KJ768160.1	*Mansonia uniformis*	15.99%
KJ461784.1	*Anopheles subpictus*	18.08%
KC849092.1	*Nephila sumptuosa*	28.50%
KY694466.1	*Afidenta misera*	31.35%
EF033298.1	*Lampsilis hydiana*	50.77%

Table 1.15b: The nucleotide frequency comparison of *Cx. quinquefasciatus* COI gene sequence with its kin species

ACCESSION NO. AND NAME OF THE SPECIES	NUCLEOTIDE FREQUENCIES (%)																			
	T(U)	C	A	G	TOTAL	T-1	C-1	A-1	G-1	POS #1	T-2	C-2	A-2	G-2	POS #2	T-3	C-3	A-3	G-3	POS #3
MW143512.1 *Culex quinquefasciatus*	39.4	15.5	28.8	16.3	510.0	44.1	27.6	12.4	15.9	170.0	48.8	2.4	45.9	2.9	170.0	25.3	16.5	28.2	30.0	170.0
MT895717.1 *Culex quinquefasciatus*	39.4	15.5	28.8	16.3	510.0	44.1	27.6	12.4	15.9	170.0	48.8	2.4	45.9	2.9	170.0	25.3	16.5	28.2	30.0	170.0
MW509611.1 *Culex quinquefasciatus*	39.4	15.5	28.8	16.3	510.0	44.1	27.6	12.4	15.9	170.0	48.8	2.4	45.9	2.9	170.0	25.3	16.5	28.2	30.0	170.0
KM593055.1 *Culex declarator*	40.8	15.3	28.6	15.3	510.0	44.1	27.6	12.4	15.9	170.0	53.5	1.8	44.7	0.0	170.0	24.7	16.5	28.8	30.0	170.0
KM593051.1 *Culex declarator*	40.8	15.3	28.6	15.3	510.0	44.1	27.6	12.4	15.9	170.0	53.5	1.8	44.7	0.0	170.0	24.7	16.5	28.8	30.0	170.0
MN793302.1 *Culex dolosus*	39.8	15.7	28.6	15.9	510.0	44.1	27.6	12.4	15.9	170.0	50.6	2.9	44.7	1.8	170.0	24.7	16.5	28.8	30.0	170.0
MN793283.1 *Culex dolosus*	39.8	15.5	28.8	15.9	510.0	44.1	27.6	12.4	15.9	170.0	50.6	2.4	45.3	1.8	170.0	24.7	16.5	28.8	30.0	170.0
KJ461792.1 *Anopheles subpictus*	38.0	15.3	29.2	17.5	510.0	44.1	27.6	12.4	15.9	170.0	43.5	4.1	46.5	5.9	170.0	26.5	14.1	28.8	30.6	170.0
KJ461784.1 *Anopheles subpictus*	37.6	15.9	29.0	17.5	510.0	44.1	27.6	12.4	15.9	170.0	44.1	4.1	45.9	5.9	170.0	24.7	15.9	28.8	30.6	170.0
KJ768160.1 *Mansonia uniformis*	39.2	15.7	29.4	15.7	510.0	44.7	27.1	12.9	15.3	170.0	47.1	5.3	45.3	2.4	170.0	25.9	14.7	30.0	29.4	170.0
LC517293.1 *Mansonia uniformis*	40.0	14.3	30.0	15.7	510.0	44.7	27.1	12.9	15.3	170.0	48.8	1.8	47.1	2.4	170.0	26.5	14.1	30.0	29.4	170.0
KY694466.1 *Afidenta misera*	37.6	18.4	28.0	15.9	510.0	44.7	25.9	12.9	16.5	170.0	43.5	12.9	38.2	5.3	170.0	24.7	16.5	32.9	25.9	170.0
EF033298.1 *Lampsilis hydiana*	44.9	12.0	17.6	25.5	510.0	45.3	19.4	14.1	21.2	170.0	55.9	4.1	14.1	25.9	170.0	33.5	12.4	24.7	29.4	170.0
KC849092.1 *Nephila sumptuosa*	41.3	12.4	27.9	18.5	509.0	46.5	24.7	13.5	15.3	170.0	44.4	0.6	45.0	10.1	169.0	32.9	11.8	25.3	30.0	170.0
Avg.	39.9	15.2	28.0	16.9	509.9	44.5	26.6	12.7	16.2	170.0	48.7	3.5	42.8	5.0	169.9	26.4	15.3	28.6	29.7	170.0

1.4 Discussion

1.4.1 Vector Mosquito Diversity

Mosquito-borne diseases constitute a significant global health concern. They exact a heavy toll on public health, causing illness and death, particularly in regions where mosquito vectors are prevalent. Beyond the immediate health impacts, these diseases also place substantial economic burdens on affected communities and healthcare systems (Ligsay et al., 2021).

A total of 11 vector mosquito species under four genera were collected and identified using molecular barcoding during the study, conducted at four distinct sites within Thrissur district. Among these sites, two were in urban areas, and the other two were in semi-urban areas. Various diversity indices, encompassing alpha, beta, and gamma diversity, were employed for analysis. The assessment of diversity indices for vector mosquito species holds significance, as it can offer valuable insights into implementing effective vector control measures within the area. Mosquitoes inhabit various water-related environments, including sewage, stagnant, and septic tanks. It is imperative to promptly address the proliferation of both vector and non-vector mosquito populations. This action is essential for mitigating vector-borne diseases and minimizing the annoyance caused by mosquitoes by applying suitable control methods. The breeding habitat is pivotal in mosquito population dynamics, serving as the site for numerous critical life cycle processes (Reuben, 1978). Rajavel et al. (2001) reported that mosquito management required the collection of adequate information concerning species diversity and how they were distributed within a specific region. This information would be essential for meaningfully formulating and implementing suitable strategies to control the mosquito population. Consequently, these efforts contributed to a decrease in the menace posed by mosquitoes and a reduction in the incidence of associated diseases. This information would be necessary to formulate and implement appropriate strategies to control the mosquito population effectively. As a result, these measures have contributed to reducing both the threat that mosquitoes present and the frequency of diseases that are linked to them. Kumar and Nattuthurai (2011) analysed mosquito fauna diversity in Dindigul district, Tamil Nadu. They found that in addition to measuring overall organism diversity, the widely used diversity

indices in environmental assessment could effectively be used as instruments to monitor mosquito species. Through the implementation of longitudinal databases, habitat type, latitude, land use, and mosquito vector species monitoring across multiple sites, it became feasible to predict the impact of environmental changes on mosquito populations. Furthermore, since the breeding sites had a major impact on changes in adult mosquito abundance in the study habitats, it was essential to learn about the dynamics of immature mosquitoes.

1.4.2 Vector Mosquito Barcoding

The mosquito population in India is extremely varied, consisting of 393 species spread over 49 genera and 41 subgenera. Among this extensive array of mosquito species, 31 have been identified as capable of transmitting a wide range of agents responsible for human and animal diseases (Prakash et al., 2014; Benelli et al., 2016). Mosquito-borne diseases have remained a persistent health challenge in Kerala, with continuous concerns about their impact. Kerala has witnessed a notable burden of mosquito-borne diseases (Vanaja and Sumodan, 2019), as evident from the reported cases spanning from 2010 to 2022, as documented in the data sourced from dhs.kerala.gov.in. In this study, a total of 11 mosquito species from four genera (*Anopheles*, *Aedes*, *Culex*, and *Armigeres*) were collected and analysed from four distinct study sites from Thrissur district, Kerala, India. These species have displayed strong vectorial capabilities and are recognized as primary, secondary, or potential vectors of numerous pathogens that impact human and animal health.

The collected species from the *Anopheles* genus comprised *An. stephensi*, *An. subpictus*, and *An. vagus. An. stephensi* is a widely recognized mosquito species in Asia, particularly in India, plays a significant role as a vector for *P. falciparum* and *P. vivax*, two parasites responsible for causing malaria (Surendran et al., 2019; Ahmed et al., 2021). *An. subpictus* is acknowledged as a primary or secondary malaria vector, a disease of substantial socio-economic significance across the globe. It has been implicated in malaria transmission in India and has also been identified as a malaria vector in various locations, including the Maldives Islands, Celebes, South Java, Portuguese Timor, and Malaysia. Additionally, it assumes a secondary role in malaria transmission in Sri Lanka (Panicker, 1981; Chandra et al., 2010; Singh et al., 2014). In Asia, West Nile virus has been isolated from *An. subpictus*, with documented cases in India (Hubálek and Halouzka, 1999).

Furthermore, a study conducted by Thenmozhi et al. (2006) reported *An. subpictus* as a major vector of JEV in Cuddalore, an endemic zone for the disease in Tamil Nadu, India.*An. vagus* is a widely documented mosquito species found in malaria-endemic regions of the Indian subcontinent. This species plays a significant and notable role in the transmission of malaria, particularly in countries such as Bangladesh, Laos, and Cambodia. In these regions, *An. vagus* is recognized as a primary vector responsible for transmitting the malaria parasite to humans (Alam et al., 2010; Rueda et al., 2011; Bashar and Tuno, 2014; Dhiman et al., 2016).

Three species belonging to the *Aedes* genus were collected from the study site, specifically *Ae. aegypti*, *Ae. albopictus*, and *Ae. vittatus*. *Ae. albopictus* and *Ae. aegypti* are among the highest abundant mosquito vectors globally. These two species are distributed across nearly every continent, except for Antarctica, and have significantly contributed to numerous outbreaks of vector-borne diseases throughout the past century. These mosquitoes are acknowledged as major carriers of viruses responsible for several significant diseases, including dengue, yellow fever, chikungunya, and Zika (Bhatt et al., 2013; Kraemer et al., 2015; Girard et al., 2020; Lwande et al., 2020; Laporta et al., 2023). The *Ae. vittatus* mosquito has gained significant public notice lately due to its link to the Zika virus. Furthermore, this mosquito species is acknowledged for its crucial involvement in preserving and spreading viruses that hold considerable public health significance, the yellow fever virus, dengue virus, and chikungunya virus. These viruses have consistently been detected in mosquitoes captured in the wild, underscoring their role in the natural preservation of these viral pathogens (Jupp and McIntosh, 1990; Sudeep and Shil, 2017; Mulwa et al., 2018).

In this study, *Ar. subalbatus* was identified as the sole species from the *Armigeres* genus. *Ar. subalbatus*, a species of mosquito, plays a crucial role in both human and veterinary health due to its function as a carrier for filarial worms, including *Brugia pahangi*, a significant human pathogen and *Dirofilaria repens*, a parasite of veterinary concern. This mosquito species has been identified as a carrier of the Japanese Encephalitis Virus. Furthermore, reports have indicated its involvement as a vector for the filarial worm *Wuchereria bancrofti* in India (Das et al., 1983; Chen et al., 2000; Lee et al., 2007; Liu et al., 2013; Muslim et al., 2013).

Several species of *Culex* mosquitoes serve as vectors, facilitating the transmission of pathogens and parasites responsible for numerous significant illnesses, including JE, WNV, St. Louis encephalitis, and filariasis (Karthika et al., 2018). Throughout the study period, four species from the *Culex* genus were collected: *Cx. gelidus*, *Cx. pipiens*, *Cx. quinquefasciatus*, and *Cx. tritaeniorhynchus*. Studies have reported a substantial rise in the population of *Cx. gelidus* in countries like India, Sri Lanka, and Malaysia, displacing previously dominant mosquito species such as *Cx. tritaeniorhynchus*. This mosquito species plays a prominent role as a vector for the JEV in Malaysia and contributes to the secondary transmission of JEV in countries like India, Sri Lanka, and others, as evidenced by repeated virus isolations from mosquitoes captured in their natural habitat (Murty et al., 2010; Arunachalam et al., 2014; Sudeep, 2014, Sudeep etal., 2015). *Cx. pipiens* mosquitoes are essential in public health as they are vectors for filarial worms, WNV, and encephalitis viruses. Among their roles, *Cx. pipiens* mosquitoes are particularly notable as the primary vectors of WNV in Europe, making them a crucial component of WNV transmission cycles in the region (Hubálek, 2008; Farajollahi et al., 2011; Kilpatrick, 2011; Kioulos et al., 2014). Research findings have indicated that *Cx. quinquefasciatus* is the primary vector for bancroftian filariasis and is also considered a potential carrier of WNV. Additionally, this species has been shown to possess the ability to transmit the JE virus and Chikungunya virus (Goddard et al., 2002; Sudomo et al., 2010; Bhattacharya et al., 2016). *Cx. tritaeniorhynchus* holds the primary status as the vector responsible for transmitting JEV and is found across Southeast Asia, the Middle East, Africa, and Europe. Additionally, this mosquito species is involved in transmitting other viral diseases affecting humans and animals, such as Dengue fever and Rift Valley fever (Kanojia, 2007; Shi et al., 2014; Sanisuriwong et al., 2021; Tong et al., 2023).

1.5 Conclusion

The molecular identification and diversity analysis of vector mosquitoes conducted in this study has yielded valuable insights into the composition and genetic variability of important disease-transmitting vectors. The findings revealed the presence of a diverse array of vector mosquito species, spanning different genera such as *Anopheles*, *Aedes*, *Culex*, and *Armigeres*. This diversity underscores the complex nature of vector-borne diseases and highlights the need for comprehensive

surveillance and control measures. The application of molecular techniques has enabled the precise identification and classification of these mosquito species, providing a better understanding of their vectorial capabilities and potential contributions to disease transmission. Furthermore, identifying primary and secondary vectors among these mosquito species underscores the complexity of disease transmission dynamics. Understanding the genetic diversity within these populations is crucial for developing targeted control strategies and alleviating disease outbreaks. Overall, this chapter has discussed valuable information in the field of vector-borne disease research, emphasizing the importance of continued monitoring and research efforts to combat the threats presented by these disease-transmitting mosquitoes.

CHAPTER II

Screening of Locally Available Plants for Their Larvicidal Effect on Fourth Instar Larvae of *Ae. aegypti* and Bioactive Compound Isolation from the Selected Plant

2.1 Introduction

Mosquitoes serve as vital dipteran vectors, playing a crucial role in transmitting numerous diseases. Their importance goes beyond mere nuisances, as they are highly efficient carriers of various pathogens. As a result, mosquitoes hold considerable economic significance in the field of public health. WHO has emphasized the gravity of mosquito-borne diseases, which comprise a spectrum of dreadful conditions and life-threatening illnesses. These diseases have a profound global impact, affecting over a billion people across the world and causing a significant threat to human populations. Dengue fever has become one of the most widespread infectious diseases, spreading to 129 countries. Due to its high transmission rates and wide geographic distribution, it is a compelling problem that needs immediate attention as well as efficient preventive and control measures. The global impact of dengue necessitates wide-ranging research and interventions to combat the disease and abate its burden on affected populations (Farrar et al., 2007; Guzman et al., 2010; Mahalingam et al., 2013; Gubler et al., 2014).

As the female mosquitoes that typically feed on their hosts from early morning to evening, *Ae. aegypti* are generally known as daytime mosquitoes due to their feeding time preference. They mostly prefer fresh water for their breeding and breed in natural or artificial sites like pools, tree holes, leaf axils, pots, tanks, coconut shells, and plastic containers. The females oviposit on hard surfaces and just above the water surface, and after getting embryonated, they can survive desiccation for up to around a year. Female mosquitoes spread the dengue virus, primarily those of the species *Ae. aegypti* and, to a slightly lesser extent, *Ae. albopictus*. These mosquitoes have also been reported to transmit Zika, yellow fever, and chikungunya viruses (Russell et al., 2001).

Ae. aegypti is commonly grouped as the primary urban vector of dengue viruses globally. An intensification in the distribution of *Ae. aegypti* and the dengue virus epidemic have been observed all over the world in recent decades (Mackenzie et al., 2004). WHO reports that more than forty per cent of the global human population is

now at risk of dengue and assesses there could be dengue infections ranging from a hundred million every year. Approximately five hundred thousand people suffer from severe dengue symptoms and necessitate hospitalization each year, including children. Supposedly, 2.5% of the infected population has been reported dead. Although several countries are susceptible to infection, India bears the paramount burden, with a high prevalence and endemicity found in many major cities (Cecilia, 2014).

Chemical insecticides are predominantly and extensively used to control pests concerning economy and health. The indiscriminate global practice of these potent chemicals has elevated many environmental issues, and it has altered the chemical composition of natural habitats and resources and even directly affected human health due to its highly lethal nature. Besides all these detrimental effects, many of these compounds have induced resistance in both the targeted and non-targeted species. This resistance can occur when an organism is repeatedly exposed to a specific insecticide, causing it to develop mechanisms to counteract the lethal effects. Over time, this can lead to the inefficiency of that insecticide against the targeted pest and may require higher doses or alternative chemicals to achieve the same level of control. Developing cross-resistance, in which the target organism acquires resistance against similar compounds of treated ones without exposure, is also an important hurdle in dealing with chemical insecticides (Baldacchino et al., 2015).

Plants act as natural sources of a wide variety of chemicals, making them invaluable reservoirs of essential compounds that can act against pest species. Over the years, human communities have relied on traditional plant-based products as safe and effective methods to control insect pests and vectors (Mandal, 2010). Earlier reports suggest that phytochemicals have been used for mosquito control since the 1920s. Approximately 2000 plant species were recognized for their effective properties in pest management, with hundreds of these species demonstrating significant activity against mosquitoes. An earlier survey, primarily focused on agriculture, identified thousands of plant species documented in the literature as having potential insecticidal properties. These studies have revealed numerous plant metabolites that harbour a wide range of bioactive compounds effective against fungi, nematodes, insect pests, and even cancer (Roark, 1947). India boasts an incredibly diverse flora,

offering a wealth of biologically active and environmentally safe agents for resisting mosquitoes and other pests (Khare and Manjusha, 2007). Numerous studies have been conducted worldwide to explore the potential of phytoextracts in controlling mosquito vectors, but many of these studies have focused only on preliminary screening (Prajapati et al., 2005; Shaalan et al., 2005b; Amer and Mehlhorn, 2006; Chaiyasit et al., 2006; Pavela, 2016).

In the past, botanicals were almost entirely replaced by chemical insecticides, particularly with the introduction of DDT in the 1940s as part of the Malaria eradication program. The marked rise of synthetic chemical insecticides, such as organochlorides, organophosphates, carbamates, and pyrethroids, for mosquito control led to a decline in phytoextracts. However, extensive research since then revealed the detrimental effects of these chemical treatments on various non-target aquatic and terrestrial life forms, raising concerns within the scientific community (Desneux et al., 2007). One of the major concerns was the emergence of pesticide resistance, even in response to low concentrations of these chemicals, which posed a significant threat to pest control efforts. This phenomenon impaired the challenge of dealing with chemical insecticides and emphasised the urgent need for alternative approaches (Brattsten et al., 1986; Chareonviriyaphap et al.,1999; Cui et al., 2006; Garcia et al., 2018).

The escalating resistance and adverse environmental effects caused by chemical insecticides have sparked interest in seeking a more sustainable and effective alternative for mosquito control. This pursuit has once again led researchers to explore the potential of secondary metabolites derived from plants, which have demonstrated significant insecticidal activity against vector mosquitoes. Many plant species have been identified for their insecticidal properties and have been successfully employed in pest management strategies (Shaalan et al., 2005b; Thiyagarajan et al., 2014; Siegwart et al., 2015; Pilaquinga et al., 2019). Exploring the insecticidal potential of plant secondary metabolites has gathered attention due to their numerous advantages over chemical insecticides. These botanical compounds offer a sustainable and renewable source of mosquito control agents, promoting eco-friendly pest management practices. Unlike chemical insecticides, which often persist in the environment and harm non-target species, plant-derived insecticides pose a lower risk to beneficial organisms and ecosystems. Additionally,

the use of botanicals may help alleviate the development of resistance in mosquito populations, as the complex chemical composition of these natural products may make it more challenging for mosquitoes to develop resistance mechanisms. As a result, plant-based insecticides have emerged as a promising avenue against mosquito-borne diseases and reducing the environmental impact associated with conventional chemical control methods (Isman, 2014; Hazra et al., 2017; Hikal et al., 2017).

In contemporary pest management practices, plant isolates are increasingly employed either individually or in combination with other plant metabolites or chemical insecticides as part of integrated strategies. This approach capitalizes on the diverse properties of plant-derived compounds, enhancing their efficacy in controlling mosquito populations. Numerous plant families, such as Asteraceae, Solanaceae, Oocystaceae, Meliaceae, Rutaceae and Euphorbiaceae, have been identified for their remarkable ability to act as both larvicidal and repellent agents against various mosquito species, as reported by Shaalan et al. (2005b). Among the various classes of phytochemicals with well-documented insecticidal properties are alkaloids, alkanes, terpenoids, steroids, phenolics, and essential oils. The insecticidal potency of plant extracts is influenced by several factors, including the extraction technique, the polarity of the solvents used, specific plant species, regional variations, and the parts of the plant utilised (Pitasawat et al., 2007; Oliveira et al., 2010; Ghosh et al., 2012; Şengül and Canpolat, 2022). As a result, diverse plant species, ranging from herbs to shrubs and trees, are harnessed to isolate and exploit mosquitocidal compounds, thus expanding the repertoire of effective mosquito control solutions. This broad diversity of botanical sources offers excellent potential for developing novel and sustainable mosquito control products and strategies, fostering a harmonious coexistence between human populations and mosquito vectors.

2.2 Methodology

2.2.1 Collection of Plants

For the present study, a total of thirty plants were collected from the Thrissur district of Kerala, India, representing diverse families and genera. The selection process was based on careful consideration of the existing literature survey conducted in the

field. Plants were chosen based on their potential medicinal importance, antimicrobial activity, or efficacy as insecticides or pesticides. These selection criteria ensured that the plants included in the study possessed desirable properties and relevance to the research objectives.

2.2.2 Mosquito Colony Maintenance

The present research utilised laboratory-reared *Ae. aegypti* larvae, which were maintained in an insectary at the CDRL. The larvae were bred under controlled conditions to ensure uniformity and consistency in their development. Specifically, the adult mosquiitoes were kept and reared in sterilized cages equipped with netting to prevent any external interference. A constant rearing environment was sustained with 27±2°C temperature, 75–85% relative humidity, and a photoperiod cycle of 14 hours of light and 10 hours of darkness (14:10 L/D). These carefully controlled conditions provided an ideal setting for the growth and development of mosquitoes. Freshly water-soaked raisins were provided as the primary food source for the nutrition and sustenance of the adult mosquitoes. This nutritionally rich diet served as a suitable energy source for the mosquitoes to thrive in the insectary. The consistent temperature, humidity, and photoperiod cycle created a stable and supportive environment for the mosquitoes, allowing them to reach their reproductive stage. As the study focused on female *Ae. aegypti* mosquitoes, they were fed on blood placed in a separate mosquito-resting cage on the third day post-emergence. This step was crucial for stimulating the reproductive cycle of female mosquitoes and obtaining the required blood-fed status for the subsequent experiments. Throughout the entire duration of the experiments, these controlled laboratory conditions were maintained to minimize any external factors that could potentially influence the outcome of the tests (Munstermann, 1997).

2.2.2a *Aedes aegypti* L.

Family : Culicidae
Subfamily: Culicinae
Tribe: Aedini
Genus: *Aedes*
Subgenus: *Stegomyia*
Species: *aegypti*

Eggs: Female mosquitoes could lay between 100 and 200 eggs on average per batch when given a complete blood meal. Female mosquitoes can lay roughly five batches

of eggs during their entire lifespan. Female mosquitoes prefer moist surfaces like water-filled tree holes, temporarily flooded areas, and artificial containers to lay eggs (Clements, 1999). Compared to the eggs of other container breeders, *Ae. aegypti* eggs are smoother-textured, cigar-shaped, and about one millimetre long. These eggs are laid individually on the walls of oviposition sites, just above the water level. Eggs undergo a quick colour change from white to shiny black after being laid. Embryonic development of *Ae. aegypti* eggs are usually completed in two days in warm, humid conditions, but it may take up to 5 days in colder climates. Once fully developed, the eggs can withstand prolonged desiccation for up to a year (Bova et al., 2016). This feature makes managing *Ae. aegypti* population extremely difficult as the eggs can travel long distances in dry containers, allowing for reinfestation even months after adult mosquitoes and larvae have been removed from a particular area (Nelson, 1986; Clemons et al.,2010; Foster and Walker, 2019).

Larvae: The larvae and pupae of *Ae. aegypti* are fully aquatic, undergoing complete metamorphosis. During the larval stage, dedicated to feeding and growth, they utilise fan-like mouth brushes to consume submerged objects and organic matter on container surfaces. *Ae. aegypti* larvae share standard features with other mosquito larvae, possessing ovoid heads, thoraxes, and 9-segmented abdomens. Their abdomen's posterior and anal segments have four-lobed gills for osmotic regulation and a siphon or air tube for respiration at the water surface. Distinguishing characteristics include shorter siphons compared to other culicine mosquitoes and the absence of a siphon as in anophelines. *Anopheles* larvae lie almost parallel to the surface of breeding water, *Culex* mosquito larvae rest at a particular angle, while *Ae. aegypti* larvae hang vertically down from the water surface. The duration of larval development varies depending on temperature, food availability, and larval density. Under optimal conditions, the hatching to pupation can be as short as five days but typically lasts 7 to 14 days. The first three instars progress rapidly, while the fourth instar takes longer to develop and significantly increases in size and weight. In challenging conditions of low temperatures or limited food, the fourth stage may extend for several weeks before pupation. Additionally, male larvae and pupae tend to develop more rapidly than females (Nelson, 1986; Farajollahi and Price, 2013).

Pupae: Mosquito pupae transform from larval to adult stages and do not feed. Unlike other holometabolous insects, mosquito pupae react remarkably to external stimuli like vibrations. Their buoyancy helps the adult mosquito emerge by allowing them to float on the water surface when not moving. The pupal stage usually lasts for two or three days. A pair of trumpet-shaped breathing tubes seen at the base of the thorax allow mosquito pupae to access air above the water surface. They bear swimming paddles at the tip of the abdomen that are used for propulsion. The short, non-flared trumpets of *Aedes* pupae and the single hair on the tip of each swimming paddle set them apart from pupae of other genera. *Ae. aegypti* pupae have short, well-developed setae on the lower side of the second to sixth abdominal segments, distinguishing them from other *Aedes* species (Nelson, 1986).

Adult: *Ae. aegypti* is a holometabolous insect species that undergoes complete metamorphosis, progressing through all the four stages (egg, larva, pupa, and adult). The duration of the adult phase of *Ae. aegypti*'s life cycle varies from two weeks to a month, influenced by prevailing environmental conditions. Notably, *Ae. aegypti* manifests itself in three polytypic forms: domestic, sylvan, and peri domestic. The domestic variant predominantly breeds within urban habitats, often near or within human dwellings. Conversely, the sylvan form primarily inhabits rural areas, explicitly opting for tree holes commonly found within forests. Lastly, the peri-domestic form thrives in areas that have undergone environmental modifications, such as coconut groves and farms (Tabachnick et al., 1979; Nelson, 1986; Zettel and Kaufman, 2009). The adult stage of *Ae. aegypti* is recognized as the reproductive phase. Similar to other flying insects, including mosquito species, the adult stage plays a crucial role in dispersion. However, in the case of *Ae. aegypti*, passive dispersion through transporting eggs and larvae in containers is believed to be more significant than active dispersal through adult flight. Adult *Aedes* mosquitoes, including *Ae. aegypti*, can be differentiated from *Anopheles* mosquitoes by their petite palps and resting position, which is more parallel or horizontal to the resting surface. Additionally, *Aedes* mosquitoes can be differentially identified from most other Culicinae species by their pointed abdomen and lack of spiracular bristles. *Ae. aegypti* is characterized by its dark colouration with white bands at the bases of the tarsal leg segments and a distinct "lyre"-shaped pattern on the mesonotum. Although

the lyre marking may fade with age, the characteristic white scales on the clypeus and palpi usually remain, aiding in identifying *Ae. aegypti* (Nelson, 1986).

2.2.3 Screening of Plants

A preliminary screening involved the preparation of a 1mg/ml (1000ppm) concentration of plant extract using three different solvents with varying polarities. A Soxhlet apparatus was used to perform extractions with different solvents during the initial screening of plants. Solvents and essential components were allowed to pass through a filter paper thimble containing twenty grams of dried plant material placed in the Soxhlet extractor. 250 ml of the selected solvent was kept in a round-bottomed flask that was attached to an isomantle along with a condenser to the Soxhlet extractor. Organic solvents with increasing polarity, such as ethyl acetate, methanol, and hexane, were heated independently to their respective boiling points for six to twelve hours. This allowed the plant extracts containing bioactive components to enter the condenser. When the solvent reached a certain level in the siphon, it was allowed to cycle back to the flask, and this process was repeated until all of the plant constituents were extracted. *Ae. aegypti* larvae were used to evaluate the larvicidal efficacy of the remaining evaporated extracts (Aivazi and Vijayan, 2009). Test concentrations were made by adding 249 ml of water to 1 ml stock solution of each plant extract in a 500 ml beaker. One control was set up for each bioassay by different solvent extracts, where 1 ml of solvent, instead of plant extract, was added to 249 ml of water. Subsequently, 25 healthy early fourth instar *Ae. aegypti* larvae were introduced into each individual beaker. Six replicates were arranged for each extract. For calculating the mortality rate, *Ae. aegypti* larvae were considered dead if they did not exhibit outward movement for respiration. After pooling the dead larvae from six test replicates, the percentage of larval mortality for each concentration was calculated. The plant that showed maximum efficiency, *C. bonplandianus* Baill, was selected for further analysis.

2.2.3a *Croton bonplandianus* Baill.

Kingdom: Plantae
Order: Malpighiales
Family: Euphorbiaceae
Genus: *Croton*
Species: *bonplandianus*

C. bonplandianus Baill. is a wild croton species classified as an undesirable weed in the Euphorbiaceae family. It originated from South America and was first observed in India in the late 1890s. It has since proliferated extensively, commonly appearing along roadsides, railways, abandoned grounds, and paddy or sugarcane fields, particularly on sandy or sandy clay soils. However, this species is rarely found in areas densely populated with shrubs and trees, as it thrives in locations with unrestricted airflow (Sisodia and Siddiqui, 2010). The leaves and flowers bear a striking resemblance to Tulsi, also known as Ban Tulsi or Jungle Tulsi. This plant is considered a small herb, typically reaching a height of 1-2 feet. The leaves are arranged alternately and have a lance-shaped structure with a serrated edge. The flowers, found in racemes measuring approximately 3 to 8 cm long, are small and white. Each flower comprises five sepals, five petals, and several prominent stamens extending outward. The fruit is an oblong capsule with a rough surface, measuring around 5 mm in size. The flowering season of *C. bonplandianus Baill* occurs from September to November (Islam et al., 2010; Ghosh et al., 2018).

2.2.4 Extraction of Selected Plant for Larval Bioassay

The plant species selected for the current study was collected from the Palakkal Kol situated in Thrissur, Kerala, India. The preliminary criteria for the selection were the aromatic nature of the plant species. It was identified as *C. bonplandianus*, which is coming under the family Euphorbiaceae. For the larval bioassay, the leaves of the plant were collected, cleaned with tap water, and was subjected to shade drying. Fine powder of the dried leaves was made by crushing it in a hand mixer grinder. The extractions were done in a Dionex ASE 150 accelerated solvent extractor at 100^0C and 1700 PSI for 20 minutes using hexane as the solvent. The collected extracts were then subjected to HS-2005V-N Rotary Flash evaporator under reduced pressure at 40^0C to remove the solvent (Kraujalis et al., 2013; Kettle et al., 2016). The rest of the extract was lyophilized for further drying procedures. These procedures were carried out at Central Instruments Laboratory, Kerala Veterinary and Animal Science University, Mannuthy, Kerala. Stock solutions were prepared by dissolving a specific quantity of extract in acetone to prepare the stock solution.

2.2.5 Susceptibility of Phyto- extract

The procedure outlined by the WHO (WHO, 2005a) was followed to conduct the larval bioassay, with necessary modifications to suit the specific requirements of the study. The test concentrations were prepared by adding 1 ml of the plant extract stock solution to the experimental beakers. This volume was then made up with dechlorinated tap water to achieve a final volume of 250 ml. A separate control was maintained with 1 ml of acetone blended with 249 ml of water for each experiment. In each experimental setup, twenty-five *Ae. aegypti* larvae were introduced into the test concentrations and the control group, ensuring that six replicates were maintained for each condition. The experimental setup remained undisturbed throughout the 24-hour test duration. No food was provided to the larvae during this time in both the control and test groups. The larvae showing no response to gentle nudging with a fine needle were considered dead. The entire experiment was conducted under constant environmental circumstances, with a room temperature of 27±2°C and a humidity level of 75±5%. The observed mortality percentage was corrected for control mortality, if present, using Abbott's formula and reported as adjusted mortality (Abbott, 1925).

2.2.6 Statistical Analysis

Lethal values of the tested plant extracts, including LC_{25}, LC_{50}, and LC_{90}, were computed using SPSS version 26 to discuss the outcomes of the larval bioassay. A one-way ANOVA analysis was also performed using the observed larval bioassay values.

2.2.7 Isolation and Identification of Bioactive Compound

The phyto-extract was initially evaluated using Gas Chromatography-Mass Spectrometry (GCMS) (Figure 2.2.1) to identify the chemical compounds present, which informed the selection of appropriate separation techniques. Column chromatography (Figure 2.4) and thin layer chromatography (TLC) (Figure 2.3)were employed to isolate the bioactive compound. TLC was performed using a mobile phase composed of chloroform and hexane in a 1:1 ratio for primary separation. The TLC analysis separated the plant extract into five fractions: FRA, FRB, FRC, FRD, and FRE. Each fraction was then tested for larvicidal activity against *Ae. aegypti* larvae. FRD exhibited remarkable larvicidal activity among these fractions,

while the others were discarded. To further isolate the bioactive compound within fraction FRD, column chromatography was employed using a solvent mixture of hexane, chloroform, and ethyl acetate in a ratio of 1:1:5. This process yielded a total of 18 fractions, labelled as FD1, FD2, FD3, FD4, FD5, FD6, FD7, FD8, FD9, FD10, FD11, FD12, FD13, FD14, FD15, FD16, FD17, and FD18. Each fraction was then analysed for its larvicidal activity against *Ae. aegypti* larvae, revealing that FD7 exhibited the highest larvicidal activity. Next, a modified column chromatography procedure, as described by Bajpai et al. (2016), was employed to fractionate fraction FD7 using varying proportions of hexane, ethyl acetate, and methanol. The mobile phase volume was made up to 50 ml for all the fractions. The initial separation began with using 100% hexane as the solvent to obtain the first fraction, with a volume of 50 ml. The subsequent fraction (Fraction 2) involved a solvent mixture of hexane and ethyl acetate in a ratio of 10:1, and Fraction 3 employed a hexane-to-ethyl acetate ratio of 5:1.The separation continued with Fraction 4, which employed a hexane to ethyl acetate ratio of 1:1, Fraction 5 involved a solvent mixture of hexane and ethyl acetate in a ratio of 1:5, and Fraction 6 employed a hexane-to-ethyl acetate ratio of 1:10.Fraction 7 utilised 100% ethyl acetate as the solvent, Fraction 8 involved a solvent mixture of ethyl acetate and methanol in a ratio of 10:1, Fraction 9 employed an ethyl acetate to methanol ratio of 5:1, and Fraction 10 utilised a solvent mixture of ethyl acetate and methanol in a ratio of 1:1. Fraction 11 involved an ethyl acetate to methanol ratio of 1:5, Fraction 12 employed an ethyl acetate to methanol ratio of 1:10, and the final fraction, Fraction 13, utilised 100% methanol as the solvent. This process generated 13 fractions, named FR1, FR2, FR3, FR4, FR5, FR6, FR7, FR8, FR9, FR10, FR11, FR12, and FR13. All fractions were tested for larvicidal activity, and fraction FR4, eluted at a solvent concentration of hexane and ethyl acetate in a 1:1 ratio, exhibited significant larvicidal activity. The other fractions were discarded. Subsequently, the selected fraction, FR4, underwent additional TLC using chloroform and ethyl acetate in a 2:1 ratio. This process resulted in the isolation of a single band with an RF value of 0.5.

The fraction FR4 was purified and applied to GCMS (Figure 2.2.2) analysis to gain a comprehensive understanding of the compound present in the selected fraction. The purified compound in FR4 was subjected to NMR spectrum analysis. 1H-NMR spectra (Figure 2.5.1 and 2.5.2) and 13C-NMR (Figure 2.5.3 and 2.5.4) spectra were

recorded on VNMRS-400 "Agilent-NMR" spectrophotometer using Dimethyl sulfoxide (DMSO) as solvent at Central Instrumentation and Research Facility, Institution of Excellence, University of Mysore, Mysuru, India. The isolated compound was named CB1.

The isolated compound was identified using GC/MS analysis carried out at Care Keralam PVT. Ltd., Kinfra Park, Koratty, Thrissur, Kerala, India. The analysis was performed using a GC system consisting of a Model 7890 A instrument equipped with a triple-axis detector (5975C) and a DB 5MS column measuring 30 m in length and 0.250 mm in diameter, with a thickness of 0.25mm. During the analysis, the injecting temperature was carefully maintained at 280°C while a pressure of 7.0699 was applied. Helium gas served as the carrier gas for the analysis, and the ionization temperature was set to 80eV.

Analytical grade ethyl acetate, methanol, and hexane were employed for column chromatography and TLC. Silica gel 60 with a mesh size of 230-400 was used for column preparation, while analytical grade n-hexane was used for column packing. All chemicals used were of analytical grade and were obtained from Merck Co. (Germany).

2.2.8 Antimicrobial Activity Study

The antimicrobial susceptibility of the test samples was evaluated using the agar well diffusion method, as described by Murray et al. (1995) with modifications by Olurinola (1996). The testing was conducted on solid media, specifically agar-agar, which was poured into sterile petri plates.

The nutrient agar medium used in this study consisted of the following components: peptone (0.5g), yeast extract (0.3g), NaCl (0.5g), agar (3g), and distilled water (100ml). The pH of the medium was adjusted to 7.2. This composition provided a suitable growth medium for the bacterial strains under investigation.

The bacterial strains selected for antimicrobial susceptibility testing were as follows:

1. *Escherichia coli* (*E. coli*)
2. *Staphylococcus aureus*
3. *Klebsiella pneumoniae*
4. *Proteus vulgaris*

These strains were chosen due to their clinical significance and relevance to antimicrobial research.

2.2.8.1 Pure culture

The streak plate technique was employed to obtain pure cultures of clinically significant microbial pathogens. The initial inoculum from the culture was transferred to 50 ml of nutrient broth. The nutrient broth was incubated at room temperature (30°C) to facilitate the growth and multiplication of the pathogens for further study and analysis.

2.2.8.2 Antimicrobial Test

Nutrient agar medium was prepared and then sterilized using autoclaving to perform the antibacterial sensitivity studies. The sterilized medium was then transferred into sterile petri dishes of 4mm depth, in sterile conditions and solidified at room temperature (30°C). Once solidified, the test microbial organism was transferred onto the surface of the nutrient agar medium using a sterile swab. This step allowed the consistent growth of microorganisms, enabling the assessment of antibacterial activities. Three wells of 7mm diameter were made on agar medium in each Petri dish using a cup borer. In each well, 50µl of the extract samples, an antibiotic (chloramphenicol), and a control (hexane) were individually introduced. Then, the petri dishes were incubated for 24 hours at 37°C. After the incubation period, the zone of inhibition produced by the different organisms on the various plates was measured. This measurement indicated the antibacterial activity exhibited by the test samples against the tested microorganisms.

2.3 Results

In this study, a total of 30 plant species were subjected to screening against *Ae. aegypti* larvae (Figure 2.1.1- 2.1.30), and a fixed screening concentration of 1mg/ml was used for all the tested species. The bioassay results using different plant species and plant parts against various solvent extracts are summarized in Table 2.1. The percentage of mortality at a concentration of 1mg/ml (1000ppm) was evaluated to assess the bioactivity of each combination. Among the tested plant species, *Cosmos sulphureus* Cav displayed moderate toxicity with a mortality rate ranging from 10 ± 0.5% for the hexane extract to 3.33 ± 0.75% for the ethyl acetate extract and 32.66 ±

1.6% for the methanol extract. *Duranta erecta* L. (leaf) showed mortality rates of 15.33 ± 0.89% for the hexane extract, 0.66 ± 0.4% for the ethyl acetate extract, and 4 ± 0.89% for the methanol extract. *Duranta erecta* (seed) exhibited a mortality rate of 52 ± 0.57% for the hexane extract, 39.33 ± 1.32% for the ethyl acetate extract, and 18.66 ± 1.21% for the methanol extract. *Terminalia paniculata* demonstrated a mortality rate of 22 ± 0.95% for the hexane extract, 6.66 ± 1.03% for the ethyl acetate extract, and 47.33 ± 0.98% for the methanol extract. *Cyanthillium cinereum* (leaf) showed mortality rates of 18.66 ± 0.74% for the hexane extract and 40.66 ± 1.16% for the ethyl acetate extract. *Selaginella bryopteris* (leaf) exhibited a mortality rate of 1.33 ± 0.47% for the hexane extract, 17.33 ± 0.51% for the ethyl acetate extract, and 18 ± 1.22% for the methanol extract. *Datura stramonium* (leaf) displayed a mortality rate of 39 ± 1.95% for the hexane extract, 6 ± 0.83% for the ethyl acetate extract, and 17.33 ± 0.81% for the methanol extract, while *Datura stramonium* (seed) showed a mortality rate of 18 ± 0.76% for the hexane extract, 28 ± 1.09% for the ethyl acetate extract, and 38.66 ± 1.21% for the methanol extract. The mortality rate for *Mussaenda erythrophylla* (leaf) was 33.33 ± 1.49% in hexane extract, 5.33 ± 0.81% in ethyl acetate extract, and 7.33 ± 1.32% in methanol extract.

A few plant species exhibited either no mortality or limited mortality. For example, *Bougainvillea glabra* (leaf) showed 0% mortality for the hexane extract, 3.33 ± 1.16% for the ethyl acetate extract, and 47.33 ± 1.47% for the methanol extract respectively. The flower of *Bougainvillea glabra* showed no mortality when exposed to hexane extract, 7.33 ± 0.75% when exposed to ethyl acetate extract, and 24.66 ± 0.98% when exposed to methanol extract. The mortality rates for *Solanum torvum* (leaf) and *Solanum torvum* (seed) were 0.66 ± 0.37% and 58.66 ± 1.21%, and 18.66 ± 0.94%, 40.66 ± 0.4%, and 1.33 ± 0.51%, respectively, for the ethyl acetate and methanol extracts. The mortality rate of *Grangea maderaspatana* (leaf) extracts was found to be 14.66 ± 0.47% for hexane, 16.66 ± 0.75% for ethyl acetate, and 21.33 ± 1.5% for methanol. The mortality rate of *Tridax procumbens* (leaf) was found to be 1.33 ± 0.74% in the hexane extract and 19.33 ± 1.47% in the ethyl acetate extract. The mortality rate for the *Vitex negundo* (leaf) extract was 2.0 ± 0.83% for the ethyl acetate extract, 28.66 ± 0.89% for the hexane extract, and 32.0 ± 2.19% for the methanol extract. The hexane extract of *Lantana camara* (leaf) showed no mortality, the ethyl acetate extract showed 40.66 ± 0.98%, and the

methanol extract showed 8 ± 167%. The mortality rate for *Ixora coccinea* (leaf) extracts in hexane, ethyl acetate, and methanol was 11.33 ± 037%, 28.66 ± 0.75%, and 28.66 ± 1.83%, respectively. For the hexane extract, *Crotalaria retusa* (leaf) showed 0% mortality, the ethyl acetate extract showed 1.33 ± 0.51%, and the methanol extract showed 41.33 ± 2.25%. The mortality rate for *Cassia fistula* (leaf) was 12.66 ± 1.06% in the hexane extract, 43.33 ± 1.6% in the ethyl acetate extract, and 4 ± 1.54% in the methanol extract. The mortality rate for *Clitoria ternatea* (leaf) extracts was 19.33 ± 0.89% in hexane, 11.33 ± 0.98% in ethyl acetate, and 40.66 ± 1.94% in methanol. The hexane extract of *Hygrophila auriculata* showed a mortality rate of 36.66 ± 0.68%, while the ethyl acetate extract showed a mortality rate of 0.66 ± 0.4%. The hexane extract of *Abrus precatorius* (leaf) showed no mortality, the ethyl acetate extract showed 44.66 ± 1.83%, and the methanol extract showed 20 ± 1.41%. The hexane extract of *Adenanthera pavonina* (leaf) showed no mortality, while the ethyl acetate and methanol extracts showed 30.66 ± 1.75% and 42.66 ± 2.16% of mortality, respectively. The mortality rate for *Hemigraphis alternata* (leaf) extracts was 61.33 ± 1.1%, 20.66 ± 1.83%, and 19.33 ± 1.47% for hexane, ethyl acetate, and methanol, respectively. For hexane, ethyl acetate, and methanol extracts, the mortality rate for *Nymphaea nouchali* (flower) was 1.33 ± 0.47%, 35.33 ± 2.71%, and 50.66 ± 2.73%, respectively. The hexane extract showed no mortality in *Eichhornia crassipes* (leaf), while the ethyl acetate extract showed 38 ± 2.58%. A mortality rate of 8 ± 0.57% for the hexane extract, 42 ± 0.54% for the ethyl acetate extract, and 12.66 ± 2.71% for the methanol extract was observed in *Biophytum sensitivum* (whole plant). The mortality rate for *Holarrhena antidysenterica* (leaf) extracts was 10.66 ± 0.74% for hexane, 4.66 ± 1.47% for ethyl acetate, and 15.33 ± 2.63% for methanol. The hexane extract of *Trichosanthes dioica* (leaf) showed no mortality, while the ethyl acetate extract showed 41.33 ± 1.63% and the methanol extract showed 11.33 ± 0.75%. The mortality rate of *Acacia auriculiformis* (leaf) was 56 ± 0.81% in hexane extract, 16.66 ± 1.94% in ethyl acetate extract, and 4.66 ± 1.83% in methanol extract. The mortality rate for *Millettia pinnata* (leaf) extracts in hexane, ethyl acetate, and methanol was 64.66 ± 0.89%, 12.66 34 ± 2.56%, and 1.33 ± 0.81%, respectively. The mortality rate for *Millettia pinnata* (seed coat) was 14 ± 1.04% in the methanol extract, 27.33 ± 0.4% in the ethyl acetate extract, and 72 ± 0.81% in the hexane extract. The mortality rate for *Eclipta prostrata* (leaf) extracts in hexane, ethyl acetate, and methanol was 77.33 ± 1.24%, 7.33 ± 1.47%, and 19.33

± 2.48%, respectively. *C. bonplandianus* (leaf) showed a 100% mortality rate for all three solvent extracts, making it a choice for analysis to elucidate the bioactive compounds responsible for the larvicidal efficacy.

Three different solvents of varying polarity, namely ethyl acetate, methanol, and hexane, were utilised for the extraction process of *C. bonplandianus*. The extracts were tested at varying concentrations, ranging from 10 ppm to 105 ppm for ethyl acetate, 20 ppm to 70 ppm for methanol, and 10 ppm to 60 ppm for hexane. As shown in Table 2.2, the results unveiled the percentage of mortality observed for each concentration of the tested extracts. For ethyl acetate, at 30 ppm, the mortality observed was 10.67%, which progressively increased to 24.00% at 45 ppm, 33.33% at 60 ppm, 49.33% at 75 ppm, 65.33% at 95 ppm, and reached 86.67% at 105 ppm. The methanol extract exhibited mortality rates of 5.33% at 20 ppm, 20.00% at 30 ppm, 30.67% at 40 ppm, 45.33% at 50 ppm, 52.00% at 60 ppm, and 76.00% at 70 ppm. Similarly, the hexane extract showed mortality rates of 12% at 10 ppm, 36% at 20 ppm, 52% at 30 ppm, 64% at 40 ppm, 84% at 50 ppm, and 92% at 60 ppm.

In the next phase of the study, the larvicidal efficacy of the Hexane extract of *C. bonplandianus* was evaluated. Multiple parameters were recorded and analysed, including mean mortality, percentage, and lethal concentration values. These assessments were crucial in gaining a deeper understanding of the potency of the extract and its potential as a viable larvicidal agent against *Ae. aegypti*. The detailed results and data from these investigations have been compiled and presented in Table 2.3. The table displays the concentration (in ppm), log concentration, probit kill %, and per cent kill % for each tested concentration. At a concentration of 10 ppm, the log concentration was 1, resulting in a probit kill % of 3.82 and a per cent mortality % of 12%. As the concentration increased to 20 ppm, the log concentration became 1.30, leading to a probit mortality % of 4.64 and a per cent mortality % of 36%. Subsequently, at 30 ppm, the log concentration reached 1.48, resulting in a probit mortality % of 5.05 and a per cent mortality % of 52%. As the concentration further increased to 40 ppm, the log concentration became 1.60, a probit mortality % of 5.36 and a per cent mortality % of 64%. Similarly, at 50 ppm, the log concentration was 1.70, resulting in a probit mortality % of 5.99 and a per cent mortality % of 84%. Finally, at 60 ppm, the log concentration reached 1.78, leading to a probit mortality % of 6.41 and a per cent mortality % of 92%.

Table 2.4 presents the lethal concentrations (LC) of the hexane extract of *C. bonplandianus* against early fourth instar *Ae. aegypti* larvae. The table includes the LC_{25}, LC_{50}, and LC_{90} values along with their respective Lower Confidence Limit (LCL) and Upper Confidence Limit (UCL), as well as the p-value for the statistical significance of the results. The LC_{25} value, representing the concentration of the plant extract required to cause 25% mortality in the tested larvae, was determined to be 16.0 ppm, with the LCL and UCL being 11.3 ppm and 19.8 ppm, respectively. Similarly, the LC_{50} value, indicating the concentration causing 50% mortality, was found to be 26.3 ppm, with the LCL and UCL being 21.6 ppm and 31.0 ppm, respectively. Furthermore, the LC_{90} value, representing the concentration required for 90% mortality, was measured as 67.5 ppm, with the LCL and UCL being 53.1 ppm and 101.7 ppm, respectively. The obtained p-value for the statistical analysis was found to be less than 0.001, indicating a highly significant and reliable outcome.

After the initial screening of the selected plant extracts, the Hexane extract of *C. bonplandianus* demonstrated significant potential as it exhibited notable larvicidal efficacy against *Ae. aegypti* larvae. Therefore, it was selected for subsequent susceptibility and synergistic studies and for the isolation of bioactive compounds. The compound isolated was identified as Eicosane, denoted as CB1, with the chemical formula $C_{20}H_{42}$ (Figure 2.6).

In the study, various organisms, including *E. coli*, *S. aureus*, *K. pneumoniae* and *P. vulgaris*, were tested for their susceptibility to different agents. The zones of inhibition, representing the extent of growth inhibition around each organism, were measured. When exposed to antibiotics, *E. coli* exhibited a zone of inhibition measuring 3.6 cm, *S. aureus* showed a zone of 3.5 cm, and *K. pneumoniae* had a zone of 2.5 cm. *P. vulgaris* displayed a zone of 3.4 cm in the presence of antibiotics. When subjected to control, all organisms displayed no inhibition zones, indicating no growth inhibition. Notably, the introduction of CB1 resulted in some level of inhibition, with zones measuring 3 cm for *E. coli*, 2.0 cm for *S. aureus*, and 2.1 cm for *K. pneumoniae*. In contrast, no inhibition was observed for *P. vulgaris*. These findings indicated that CB1 possessed a potential inhibitory effect on three of the tested organisms (Table 2.5).

Table 2.1: List of plant specimens collected and screened for activity against fourth instar *Ae. aegypti* larvae

SL NO.	Plant Species	Plant Family	Plant Part used	Percentage of mortality at 1mg/ml(1000ppm) Mean ±SD		
				Hexane extract	Ethyl acetate extract	Methanol extract
1	*Cosmos sulphureus* Cav	Asteraceae	Leaf	10 ± 0.5	3.33 ± 0.75	32.66 ± 1.6
2a	*Duranta erecta* L.	Verbenaceae	Leaf	15.33 ± 0.89	0.66 ± 0.4	4 ± 0.89
2b	*Duranta erecta* L.	Verbenaceae	seed	52 ± 0.57	39.33 ± 1.32	18.66 ± 1.21
3	*Terminalia paniculata* Roth	Combretaceae	Leaf	22 ± 0.95	6.66 ± 1.03	47.33 ± 0.98
4	*Cyanthillium cinereum* (L.)	Asteraceae	Leaf	18.66 ± 0.74	40.66 ± 1.16	0
5	*Selaginella bryopteris*(L.)	Selaginellaceae	Leaf	1.33 ± 0.47	17.33 ± 0.51	18 ± 1.22
6a	*Datura stramonium* L.	Solanaceae	leaf	39 ± 1.95	6 ± 0.83	17.33 ± 0.81
6b	*Datura stramonium* L.	Solanaceae	seed	18 ± 0.76	28 ± 1.09	38.66 ± 1.21
7	*Mussaenda erythrophylla* Schumach. and Thonn. (1827)	Rubiaceae	leaf	33.33 ± 1.49	5.33 ± 0.81	7.33 ± 1.32
8a	*Bougainvillea glabra* Choisy	Nyctaginaceae	leaf	0	3.33 ± 1.16	47.33 ± 1.47
8b	*Bougainvillea glabra* Choisy	Nyctaginaceae	Flower	0	7.33 ± 0.75	24.66 ± 0.98
9a	*Solanum torvum* Sw.	Solanaceae	leaf	0.66 ± 0.37	0	58.66 ± 1.21
9b	*Solanum torvum* Sw.	Solanaceae	seed	18.66 ± 0.94	40.66 ± 0.4	1.33 ± 0.51
10	*Grangea maderaspatana* (L.)	Asteraceae	leaf	14.66 ± 0.47	16.66 ± 0.75	21.33 ± 1.5
11	*Tridax procumbens* L.	Asteraceae	leaf	1.33 ± 0.74	19.33 ± 1.47	0
12	*Vitex negundo* L.	Lamiaceae	leaf	28.66 ± 0.89	2 ± 0.83	32 ± 2.19
13	*Lantana camara* L.	Verbenaceae	leaf	0	40.66 ± 0.98	8 ± 1.67
14	*Ixora coccinea* L.	Rubiaceae	leaf	11.33 ± 0.37	28.66 ± 0.75	28.66 ± 1.83
15	*Crotalaria retusa* L.	Fabaceae	leaf	0	1.33 ± 0.51	41.33 ± 2.25
26	*Cassia fistula* L.	Fabaceae	leaf	12.66 ± 1.06	43.33 ± 1.6	4 ± 1.54
17	*Clitoria ternatea* L.	Fabaceae	leaf	19.33 ± 0.89	11.33 ± 0.98	40.66 ± 1.94
18	*Hygrophila auriculata*	Acanthaceae	Whole plant	36.66 ± 0.68	0.66 ± 0.4	0
19	*Abrus precatorius* L.	Fabaceae	leaf	0	44.66 ± 1.83	20.0 ± 1.41
20	*Adenanthera pavonina* L.	Fabaceae	leaf	0	30.66 ± 1.75	42.66 ± 2.16
21	*Hemigraphis alternata* (Burm. f.)	Acanthaceae	leaf	61.33 ± 1.1	20.66 ± 1.83	19.33 ± 1.47
22	*Nymphaea nouchali* Burm. f.	Nymphaeaceae	flower	1.33 ± 0.47	35.33 ± 2.71	50.66 ± 2.73
23	*Eichhornia crassipes* (Mart.)	Pontederiaceae	leaf	0	38 ± 2.58	0
24	*Biophytum sensitivum* (L.)	Oxalidaceae	Whole plant	8 ± 0.57	42 ± 0.54	12.66 ± 2.71
25	*Holarrhena antidysenterica*	Apocynaceae	leaf	10.66 ± 0.74	4.66 ± 1.47	15.33 ± 2.63
26	*Trichosanthes dioica* Roxb.	Cucurbitaceae	leaf	0	41.33 ± 1.63	11.33 ± 0.75
27	*Acacia auriculiformis* A. Cunn. ex Benth.	Fabaceae	leaf	56 ± 0.81	16.66 ± 1.94	4.66 ± 1.83
28a	*Millettia pinnata* (L.)	Fabaceae	leaf	64.66 ± 0.89	12.66 ± 2.56	1.33 ± 0.81
28b	*Millettia pinnata* (L.)	Fabaceae	Seed coat	72 ± 0.81	27.33 ± 0.4	14 ± 1.04
29	***Croton bonplandianus* Baill.**	**Euphorbiaceae**	**leaf**	**100**	**100**	**100**
30	*Eclipta prostrata* (L.)	Asteraceae	leaf	77.33 ± 1.24	7.33 ± 1.47	19.33 ± 2.48

Table 2.2: Evaluation of *C. bonplandianus* extracts in three different solvents with varying polarity against fourth instar *Ae. aegypti* larvae

Solvent	Test Concentrations in ppm	Percentage mortality observed
Ethyl acetate	30	10.67
	45	24.00
	60	33.33
	75	49.33
	95	65.33
	105	86.67
Methanol	20	5.33
	30	20.00
	40	30.67
	50	45.33
	60	52.00
	70	76.00
Hexane	**10**	**12**
	20	**36**
	30	**52**
	40	**64**
	50	**84**
	60	**92**

Table 2.3: Percentage and probit mortality of hexane extract of *C. bonplandianus* against fourth instar *Ae. aegypti* larvae

Sl. No.	Concentration (ppm)	Log Concentration	Probit Mortality %	Percent Mortality %
1	10	1	3.82	12%
2	20	1.30	4.64	36%
3	30	1.48	5.05	52%
4	40	1.60	5.36	64%
5	50	1.70	5.99	84%
6	60	1.78	6.41	92%

Table 2.4: Lethal concentrations of hexane extract of *C. bonplandianus* against fourth instar *Ae. aegypti* larvae

Test material	LC_{25} (LCL-UCL)	LC_{50} (LCL-UCL)	LC_{90} (LCL-UCL)	p-value
Plant extract	16.0 (11.3 -19.8)	26.3 (21.6 -31.0)	67.5 (53.1 -101.7)	$p<0.001$

Statistical significance $p<0.05$

Table 2.5: Zone of inhibition of antimicrobial activity of CB1

Organism used	Zone of inhibition (cm)		
	Zone by antibiotic (cm)	Zone by control (cm)	Zone by CB1 (cm)
E. coli	3.6	Nil	3
S. aureus	3.5	Nil	2.0
K. pneumoniae	2.5	Nil	2.1
P. vulgaris	3.4	Nil	Nil

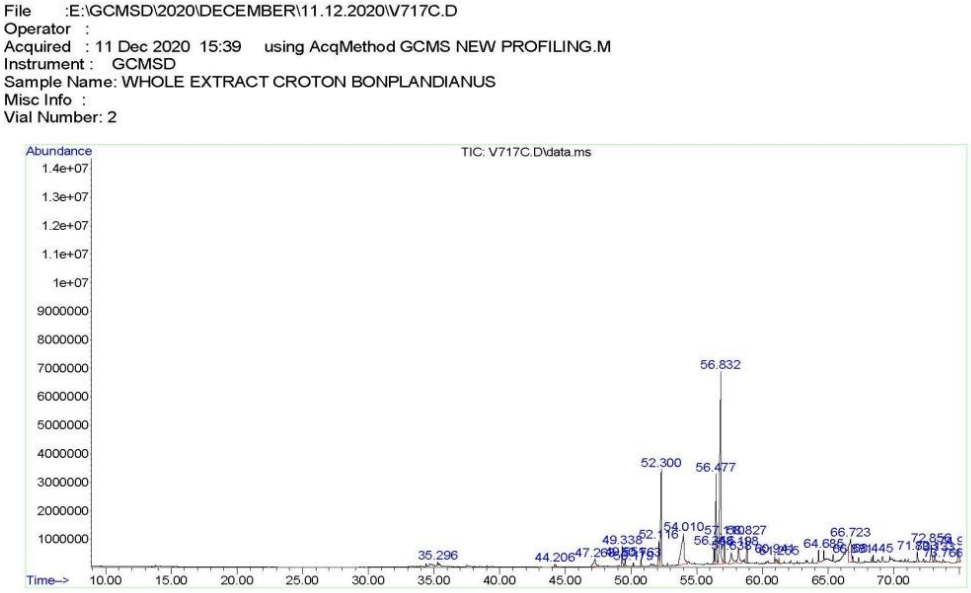

Figure 2.2.1 GCMS chromatogram of Whole plant extract of *C. bonplandianus*

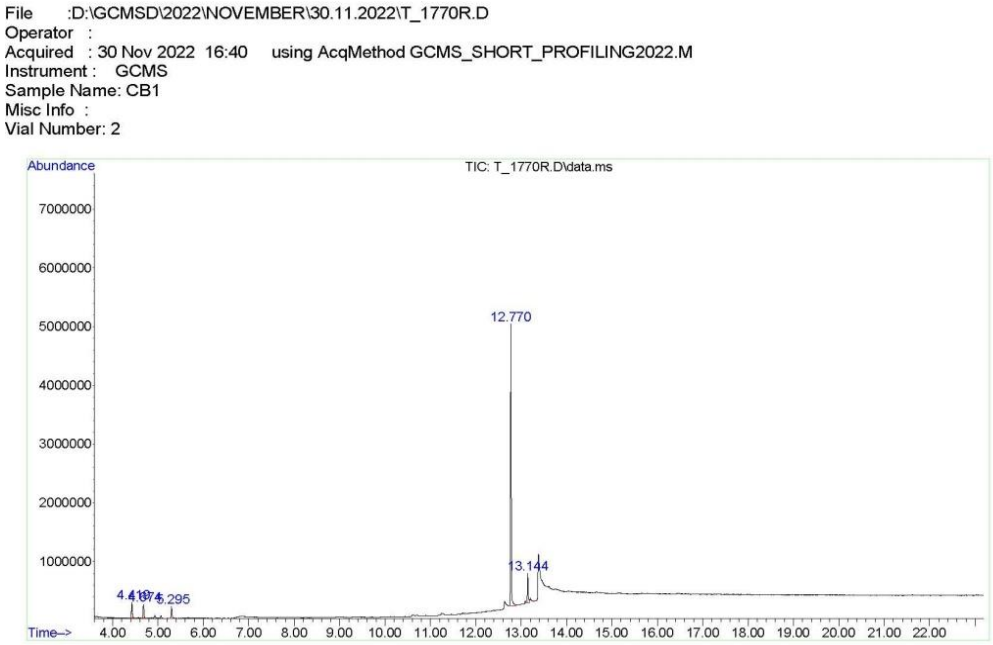

Figure 2.2.2 GCMS chromatogram of isolated compound CB1 of *C. bonplandianus*

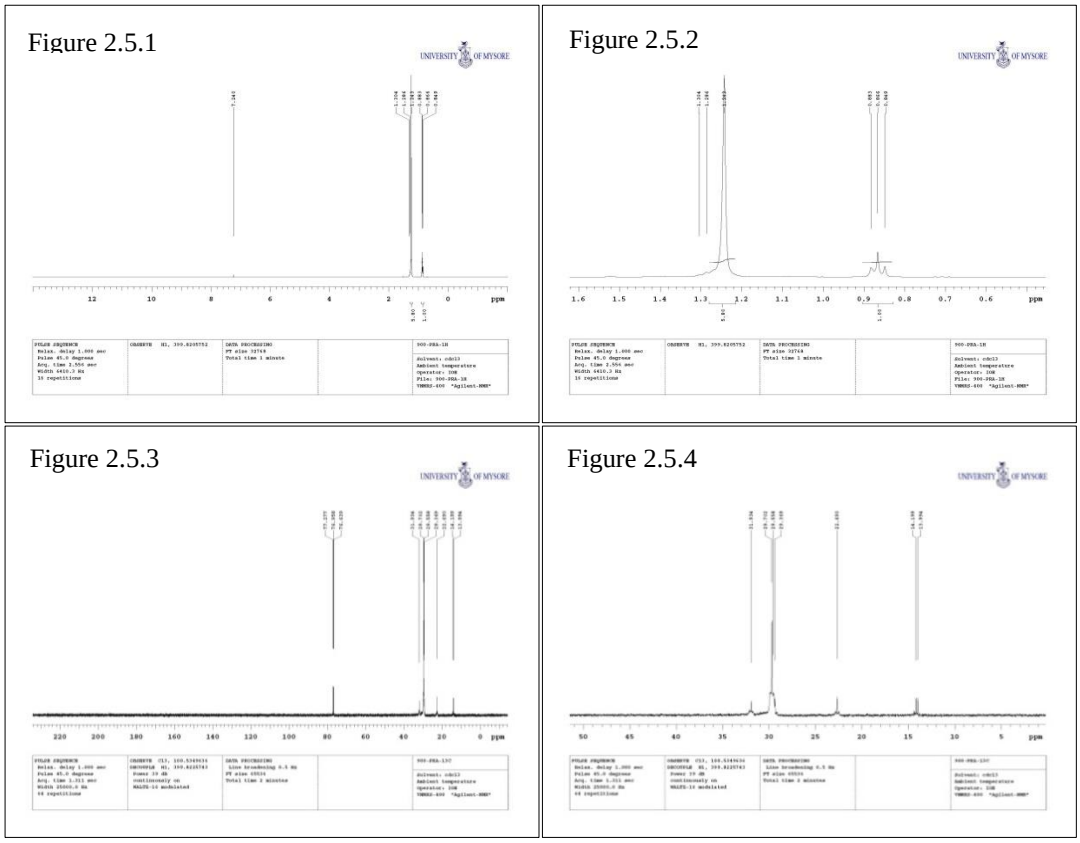

Figure 2.5.1- 2.5.4 NMR spectra of the isolated bioactive compound CB1

Figure 2.6 Structure of bioactive plant isolate (CB1) (Eicosane, $C_{20}H_{42}$)

2.4 Discussion

With a history of evolution spanning over 400 million years, plants have developed a powerful chemical defence system of secondary metabolites to protect themselves against destructive pests (Tehri and Singh, 2015). Exploring floral biodiversity and adopting safer insecticides with phytochemical composition represents a straightforward and sustainable approach to mosquito control, offering a promising alternative within potential biocontrol programs. Plant-derived pesticides, containing a rich mixture of chemical compounds, act cooperatively, affecting both physiological and behavioural processes in mosquitoes. This stands in contrast to conventional insecticides, which rely on individual active ingredients. As a result, the risk of pests developing resistance to plant-derived compounds is significantly

reduced, making them a more viable and enduring solution for vector control. This holistic approach to mosquito control involves understanding the intricate interactions between plant-based compounds and the targeted mosquito species. By embracing this paradigm, researchers and public health professionals can effectively develop appropriate and region-specific strategies to fight against mosquito-borne diseases. Emphasizing the utilisation of bio-insecticides derived from plants, with their diverse and complex chemical compositions, can set the foundation for environmentally friendly and sustainable mosquito control initiatives. Such approaches align with reducing mosquito populations and limiting disease transmission (Kalyanasundaram and Babu, 1982; Das et al., 2007; Ghosh et al., 2012).

In the present study, an evaluation was conducted on the larvicidal efficacy of several locally available plant species against *Ae. aegypti*. The results showed the substantial larvicidal effectiveness found in many of these plant species when subjected to extraction using solvents of different polarities. These findings highlighted the abundant variety of natural resources available for effective mosquito control strategies. In biological pest management programs, more than 2000 botanical species have been utilised, out of which 344 species' secondary metabolites or products have been scientifically proven to possess significant anti-mosquito properties (Remia and Logaswamy, 2010).These organic chemicals, including terpenoids, phenolics, and alkaloids, have demonstrated remarkable effectiveness as larvicides, adulticides, antifeedants, oviposition inhibitors, growth and moulting hormone disruptors, anti-moulting hormones, juvenile hormone analogues, and repellents, interfering with essential biological processes in targeted vector mosquitoes (Rattan, 2010). It has been proposed that plant extracts containing a diverse array of chemicals exhibit higher heterogeneity compared to synthetic pesticides, which in turn hinders the development of resistance (Rattan and Sharma, 2011). Laboratory studies have shown that *Plutella xylostella* (L.), a destructive pest, rapidly develops resistance to various synthetic pesticides. However, the intriguing mode of action and complex constituents of neem (*Az. indica*) have proven effective in preventing neem resistance even after 42 generations of selection with the same compound (Schmutterer, 1990). Pesticides derived from plants employ a multifaceted approach involving both behavioural and physiological

activities, which collectively prevent the emergence of resistance (Rattan, 2010). In a study, Kishore et al., 2011 investigated the impact of larvicidal activity of phytochemicals against mosquito larvae, focusing on their distinctive chemical characteristics. The study explored a diverse array of secondary compounds derived from plants, including essential oils, alkanes, alkenes, alkynes, terpenes, lactones, fatty acids, alkaloids, isoflavonoids, steroids, dipterocarps, and lignans, to ascertain their potential as mosquito larvicides.

Furthermore, the researchers extensively documented the lethal potential of various bioactive toxic components towards a wide range of mosquito species and successfully isolated these compounds from diverse plants. Some notable plant-isolated compounds include neoduline and nepseudin (Breytenbach and Rall, 1980), dioncophylline-A (Francois et al., 1996), geranial (Kelm et al., 1997), pipernonaline (Lee, 2000), octacosane (Rajkumar and Jebanesan, 2004), azadirachtin (Nathan et al., 2005), α-terpinene (Jantan et al., 2005), germacrene D (Ravi Kiran and Devi, 2007), hugorosenone (Baraza et al., 2008), β-sitosterol (Rahuman and Venkatesan, 2008), stemocurtisine, stemocurtisinol, and oxyprotostemonine (Mungkornasawakul et al., 2009), plumbagin compound (Maniafu et al., 2009), and methyl-p-hydroxybenzoate (Kannathasan et al., 2011). These compounds, extracted from various plants, have demonstrated significant and efficient lethal activity against mosquitoes, presenting promising prospects for potential mosquito control strategies.

Within this analysis, among the different plants and plant parts tested, a member of the Euphorbiaceae family, *C. bnoplandianus*, showed significant efficiency in mosquito control, serving as a larvicide against *Ae. aegypti*, the test organism. When applied at a concentration of 1mg/ml, it achieved a 100% mortality rate. A more detailed examination of this extract indicated that the hexane extract exhibited exceptional efficiency compared to other solvents. The hexane extract of *C. bonplandianus* exhibited lethal concentrations against fourth instar larvae of *Ae. aegypti*, with LC_{25} (LCL-UCL) measuring 16.0 (11.3-19.8), LC_{50} (LCL-UCL) at 26.3 (21.6-31.0), and LC_{90} (LCL-UCL) recorded as 67.5 (53.1-101.7). These results yielded a statistically significant P value of <0.001.

The results of this study were harmonious with the outcomes observed by Nazar et al. in 2009, where they observed strong larvicidal effects of C. *bonplandianus* stem

extracts against *Cx. quinquefasciatus* mosquitoes. In a study by Jeeshna et al., 2010, the investigation focused on the larvicidal activity of leaf extracts from *C. bonplandianus* at various concentrations. The results revealed its effectiveness in controlling the mosquito species *Ae. aegypti*, regarding larvicidal activity. Patel et al. (2000) proposed that the substantial biomass of the weed *C. bonplandianus*, abundantly found in the wastelands of southern India, holds the potential to serve as a valuable bioresource for the commercial production of mosquito repellent targeting *Ae. aegypti*. Furthermore, Bagavan and Rahuman (2011) conducted a study in Vellore District, Tamil Nadu, India, evaluating the larvicidal activity of *C. bonplandianus* extracts against different mosquito species *An. vagus*, *Ar. subalbatus*, and *Cx. Vishnui*. Their study revealed that the leaf ethyl acetate extract of *C. bonplandianus* exhibited high larval mortality against all the tested species. These findings are consistent with the present research, suggesting the potential of *C. bonplandianus* as a potent larvicidal agent. Moreover, a detailed review conducted by Ghosh et al. in 2018 further explored the pharmacology, traditional uses, and phytochemical properties of *C. bonplandianus*, reaffirming its remarkable larvicidal impact on the *Ae. aegypti* vector mosquito. These collective outcomes indicated *C. bonplandianus* could be used as an essential natural resource for developing eco-friendly larvicidal agents for mosquito control, positioning it as a promising resource for further research and exploration in vector-borne disease management.

The search for effective chemicals from the plant kingdom has been a subject of intensive exploration. Botanicals are naturally occurring secondary metabolites produced by plants, which function as defence mechanisms against various adverse factors. These compounds help plants withstand continuous selection pressure. Numerous studies have reported insecticidal activities among the various groups of phytochemicals, including essential oils, terpenoids, alkaloids, steroids, and phenolics. The insecticidal capability of plant extracts can vary based on plant variety, species of mosquito tested, geographical variations, and the plant part used for extraction. The extraction methodology and the polarity of the solvents used during the process also influence the insecticidal properties. A diverse range of plants, including large trees, shrubs, and herbs were selected for extracting natural compounds with mosquitocidal properties. Campbell et al. (1933) reported early findings on plant alkaloids, specifically nicotine, anabasine, methyl anabasine, and

Iupinine, isolated from the Russian weed, *Anabasis aphylla*. These alkaloids were found to exhibit larvicidal activity against the larvae of mosquito species, including *Cx. pipiens*, *Cx. territans*, and *Cx. quinquefasciatus*. Phytochemicals were extracted from various parts of the plants, such as fruits, leaves, stems, barks, and roots (Shaalan et al., 2005b; Ghosh et al., 2012).

During this research, the bioactive compound primarily responsible for the larvicidal effectiveness of a weed species, *C. bonplandianus* was isolated using a series of distinct chromatographic separation techniques. These phytochemical investigations conducted in this study unveiled the presence of an alkane compound, eicosane (CB1), which contributed to the larvicidal activity of *C. bonplandianus* against *Ae. aegypti* mosquitoes. The antimicrobial assessment of CB1 against clinically significant microbial strains revealed its capability to inhibit the proliferation of three bacterial strains: *S. aureus, E. coli*, and *K. pneumoniae*. This lined up with the previous research by Mohammed et al., 2016. They assessed the larvicidal action of essential oil from *Coccinia grandis* leaves on three different vector mosquito species and found that the isolated eicosane compound exhibited remarkable lethal activity against all tested species. The authors suggested that the potent larvicidal activity of *Co. grandis* essential oil could be attributed to the main component, eicosane, or its synergistic interactions with other minor components in the extract. Moreover, the literature review revealed additional evidence supporting the diverse biological activities of eicosane. Studies by Akpuaka et al., 2013, examining the biological activities of compound isolates from n-hexane extracts of *Az. indica* leaves reported that eicosane displayed excellent antifungal, antibacterial, antitumor, and cytotoxic effects. Furthermore, Mathur et al., 2014, investigated the in vitro multiplication and production of steroidal sapogenins from *Moringa oleifera*, observed that eicosane exhibited both antibacterial and larvicidal potential. These findings suggested the adaptability of eicosane as a bioactive compound with potential applications in various fields, including mosquito control and antimicrobial treatments.

2.5 Conclusion

This chapter examined various plants screened for their larvicidal efficacy against the dengue fever vector, *Ae. aegypti*. The assessment involved the utilisation of three distinct solvents with varying polarities, hexane, methanol, and ethyl acetate, to extract essential components from these plant specimens. The determination of their effectiveness as larvicides relied on the derivation of lethal concentration values through susceptibility tests performed on early fourth-instar larvae of *Ae. aegypti*. Among the plants studied, *C. bonplandianus* exhibited the highest mortality rate when subjected to extraction using hexane as the solvent. Consequently, this plant was selected for further investigation. After this selection, various chromatographic techniques were employed to elucidate the bioactive compound that demonstrated the most promising efficacy from the selected plant species, which was identified as eicosane (CB1). The antimicrobial properties of the bioactive compound were examined to determine its potential effects on the microbial fauna within the environment upon dispersion. The results of these investigations revealed the remarkable larvicidal efficacy of the compound against the *Ae. aegypti* vector and its substantial capacity to control certain microbial organisms within the ecosystem. This plant-based approach could offer an essential instrument for reducing vector mosquito populations and the transmission of diseases like dengue while minimizing adverse effects on ecosystems and human health.

CHAPTER III

Susceptibility Assessment of Four Different Conventional Insecticides and Isolated Plant Bioactive Compound on Fourth Instar Larvae of *Ae. aegypti*

3.1 Introduction

Mosquitoes have long been recognized as the principal vectors responsible for transmitting a multitude of diseases that retain substantial risks to both human and animal health. Among these, dengue fever stands out as one of the most alarming and hazardous due to its high mortality rate and the escalating frequency of its occurrence (Morra et al., 2018). In recent times, the global burden of dengue fever has shown a concerning increase, prompting severe public health concerns. Current reports suggest that nearly 390 million people are infected with the dengue virus annually, with approximately 90 million individuals experiencing severe manifestations of the disease (Brady et al., 2012; Bhatt et al., 2013). The prevalence of dengue fever remains highly unpredictable, leading to its spread into new areas and an uncontrollable surge in the number of cases. The transmission of the dengue fever virus to humans primarily occurs through mosquitoes belonging to the genus *Aedes*, with *Ae. aegypti* being recognized as the most significant dengue vector. This vector's widespread presence and efficient transmission capability contribute to the rapid and persistent spread of dengue fever, making it a major public health concern worldwide (Paul et al., 1965; Carey et al., 1996; Chaturvedi and Nagar, 2008; Mutheneni et al., 2017). Effective mosquito control methods are fundamental in diminishing vector-borne disease transmission and reducing morbidity. The basis of disease prevention hinges on effective mosquito control strategies. Among these methods, larval control, aimed at mosquito larvae, and adult control, which focuses on adult mosquitoes, emerged as crucial techniques (Sharma et al., 2016; Paul et al., 2020).

Since the 1920s, insecticides have become integral to pest management strategies due to their increasing effectiveness. The initial emergence of synthetic organic insecticides began with the invention of Dinitro-o-cresol, and it marked the foundation of a series of analogous compounds (Cremlyn, 1978). By the late 1930s, several innovative discoveries had identified new synthetic insecticides with enormous potential for widespread use, highlighting the necessity of a chemical approach to pest control. These insecticides have gradually become the preferred

method of controlling insect pests. Chlorinated hydrocarbons such as DDT (1939), lindane (1942), chlordane (1945), toxaphene (1947), aldrin/dieldrin (1949), and endosulfan (1956) were notable pesticides. Carbamate insecticides included agents like carbaryl (1957), aldicarb (1965), carbofuran (1965), pirimicarb (1969), and alanycarb (1984). Organophosphates (Ops) introduced compounds like TEPP (1938), parathion (1946), malathion (1952), diazinon (1953), chlorpyriphos (1965), and chlorethoxyphos (1986). Pyrethroids (Pys) entered the list with allethrin (1949), resmethrin (1967), permethrin (1973), deltamethrin (1974), cypermethrin (1974), esfenvalerate (1984), silafluofen (1990), and lambda-cyhalothrin (1990), illustrating the dynamic landscape of insect control approaches (Casida and Quistad, 1998; Prato et al., 2012).

Organophosphates form a group of insecticides with varied chemical structures, united by the presence of a phosphate (or thio- or dithio-phosphate) component and an organic constituent. Initially developed as hazardous biowarfare agents in the 1940s, these compounds, including modern derivatives like sarin, were intended for pest control but pose significant disposal challenges today. While some OPs, such as Parathion, were designed as alternatives to DDT, they exhibited higher acute toxicity, leading to human fatalities. These pesticides inhibit cholinesterase and interfere with neurotransmitter degradation. Their effects extend beyond insects, affecting wildlife and humans as well (Prato et al., 2012).

During the transition from the 1960s to the 1970s, significant changes occurred in the landscape of insecticide usage, primarily driven by the discovery of photostable pyrethroids. These synthetic pyrethroids emerged as a new class of insecticides in the 1980s, characterized by their neurotoxic properties that target the voltage-sensitive sodium channels in the nervous systems of insects. Despite their structural dissimilarity from organochlorides, organophosphates, and carbamates, PYs still exert their effects on nerve cells. PYs were found to have a higher impact on insects' sodium channels than mammals, as demonstrated in the study by Vais et al. in 2001. PYs trace their origins back to the 1800s, derived from the natural pyrethrum in Chrysanthemum flowers. Over the past two decades, their utilisation has experienced a significant upsurge (Narahashi, 1976; Lund and Narahashi, 1983). PYs can be categorized into three groups: Type I, Type II, or intermediate, based on their chemical structures. These synthetic pyrethroids mimic the desirable properties

of natural pyrethrins and can act as both stomach and contact poisons. Various PYs exhibit distinct structural attributes that influence their lethality towards insects and, in some cases, mammals. As more hazardous insecticides like OPs and carbamates have declined in use, synthetic pyrethroids have gained prominence in recent years (Thatheyus and Selvam, 2013).

Despite the success of these insecticides in effectively managing mosquito populations over several years, heavy reliance on these limited active ingredients and their widespread use has led to the development of resistance against these compounds. While most synthetic insecticides currently in use remain effective against their intended target species, concerns persist regarding resistance, which can potentially spark the resurgence of vector-borne infections (Sarkar et al., 2009). Consequently, biomagnification poses substantial risks to a diverse array of non-target species and the surrounding environment (Gold et al., 2001). Chemical pesticides inflict significant harm as their residues persist in the ecosystem over prolonged durations, posing risks to life and aiding in the development of insect resistance (Pushpanathan et al., 2008). Studies examining resistance patterns have revealed that, *Ae. aegypti* has acquired resistance to various insecticides, including carbamates, organochlorides, organophosphates, and pyrethroids. The emergence of resistance and growing environmental concerns has motivated researchers to redirect their efforts toward phytochemicals as a strategic approach achieving effective vector control while circumventing these challenges (Vontas et al., 2012).

Botanical insecticides emerge as a natural and environmentally conscious substitute for synthetic or chemical pesticides in vector control strategies. These insecticides, derived from plants, provide a compelling strategy to counter the undesirable consequences often associated with synthetic counterparts (Hikal et al., 2017). Incredibly diverse secondary metabolites found in plants have been shown to be effective against mosquito species of significant medical and veterinary importance as well as against other arthropod nuisance pests and vectors (Isman, 2006; Isman and Grieneisen, 2014; Benelli et al., 2018). A brief exploration of the available literature uncovers numerous laboratory and applied studies (Roth et al., 1998; Momin and Nair, 2002; Supabphol and Tangjitjareonkun, 2014; Karthi et al., 2020) that have investigated the biological activity of various plant components against different pathogens and arthropods. These investigations have unveiled various

botanical insecticides, including different bioactive agents, such as fungicides, nematicides, acaricides, insecticides, and growth inhibitors. Some isolated active compounds from various plant species included (5E)-ocimenone from *Tagetes minuta* (Maradufu et al., 1978), rotenone from *D. elliptica* (Ameen et al., 1983), azadirachtin from *Az. indica* (Schmutterer, 1981), capillin from *Artemisia nilagirica* (Banerji et al., 1990), quassin from *Quassia amara* (Evans and Raj, 1991), neolignans from *Piper decurrens* (Chauret et al., 1996), arborine, a novel bioactive compound related to quinazolone alkaloid, from *Glycosmis pentaphylla* (Muthukrishnan et al., 1999), and goniothalamin from *Bryonopsis laciniosa* (Kabir et al., 2003).

Studies have indicated that plant-derived larvicides present a secure, easily accessible, and economically viable option compared to chemical insecticides for eradicating mosquitoes in their larval or adult forms (Elumalai et al., 2017). In the context of resistance, the potential for developing resistance in the intended species remains unlikely owing to the multidimensional mechanisms of action exhibited by plant-derived insecticides. These mechanisms comprise the inhibition of acetylcholinesterase and modulation of GABA and octopamine receptors, as reported in studies by Enan (2005) and Price and Berry (2006). Furthermore, despite certain conflicting findings, these insecticides have demonstrated a degree of safety for vertebrates and generally exhibit low toxicity levels toward non-target aquatic invertebrates, as suggested by Pavela and Benelli (2016).

3.2 Methodology

3.2.1 Mosquito Colony Maintenance

The maintenance of the *Ae. aegypti* colony followed the methods explained in Chapter II (Munstermann, 1997).

3.2.2 Plant Isolate Bioassay

The bioactive compound CB1 was isolated using Thin-Layer Chromatography and Column chromatography, as described in Chapter II. To prepare the stock solution of the plant isolate, a specific quantity of the CB1 was dissolved in acetone. The stock solution was then used to prime the test concentrations. For this purpose, 1 ml of the identified concentration of the plant isolate was added to a volumetric flask,

and dechlorinated tap water was added to make up a total volume of 250 ml. The experiment also included a control group consisting of 1 ml of acetone mixed with 249 ml of water. Further dilutions of the stock solution were made using dechlorinated tap water to create various test concentration levels. For the larval bioassay, twenty-five *Ae aegypti* larvae were introduced into each test concentration and control. Six replicates were maintained for both the control and the test to ensure the reliability and accuracy of the results. During the 24-hour experiment, the larvae were not provided additional food and the whole setup was kept undisturbed. At the end of the 24 hours, the larvae were examined, and those showing no response to gentle nudging with a fine needle were considered dead. The entire experiment was conducted under controlled room temperature, maintaining a constant temperature of 27±2°C and humidity at 75±5%. WHO (2005a) procedure for larval bioassay was followed.

3.2.3 Insecticide Bioassay

Four conventional insecticides, Lambda-cyhalothrin, Cypermethrin, Temephos, and Malathion, were chosen for investigation. Insecticide stock solutions were composed by dissolving 1 milligram of individual insecticide in 10 ml of acetone. A series of different concentrations were prepared from this stock solution for each insecticide, which was then used in insecticide susceptibility studies. The standard WHO procedure was followed for determining larval susceptibility (Brown, 1986). The bioassays were carried out against the fourth instar larvae of *Ae. aegypti*.

3.2.4 Statistical Analysis

Statistical software SPSS version 26 was utilised to calculate the lethal values of the tested insecticides. LC_{25}, LC_{50}, and LC_{90} values were computed to examine and interpret the outcomes obtained from the larval bioassay with the experimental insecticides. Additionally, one-way ANOVA analysis was conducted using the observed values from the larval bioassay to analyse the data further and draw relevant conclusions.

3.3 Result

In this study, four conventional insecticides were chosen, comprising two from the organophosphate family and two from the pyrethroid group. The specific

insecticides included in the study were malathion, temephos, cypermethrin, and lambda-cyhalothrin. These selections were made to represent different classes of insecticides commonly used in mosquito control programs and to assess their effectiveness against the target mosquito species.

The susceptibility assay results indicated the insecticidal activity of malathion, an organophosphate insecticide widely used for mosquito control. The varying concentrations of malathion used for the analysis were 2.1×10^{-3} ppm, 2.7×10^{-3}ppm, 3.3×10^{-3}ppm, 3.9×10^{-3}ppm, 4.5×10^{-3} ppm, and 5.1×10^{-3} ppm. In a 24-hour bioassay, the percentage mortalities observed were 12%, 24%, 36%, 56%, 76%, and 92%, respectively. Probit mortalities were also recorded, corresponding to 3.82, 4.25, 4.64, 5.15, 5.71, and 6.41. The LC_{25}, LC_{50}, and LC_{90} values for malathion were found to be 2.7×10^{-3}ppm (with a confidence interval of 2.4×10^{-3}-3.0×10^{-3}ppm), 3.5×10^{-3} ppm (with a confidence interval of 3.2×10^{-3}-3.8×10^{-3}ppm), and 5.6×10^{-3} ppm (with a confidence interval of 4.9×10^{-3}-6.9×10^{-3}ppm), respectively (Table 3.1 and Table 3.6).

The insecticidal potential of temephos, another organophosphate, was assessed using test solutions with concentrations of 5.0×10^{-4} ppm, 1.2×10^{-3}ppm, 1.9×10^{-3} ppm, 2.6×10^{-3}ppm, 3.3×10^{-3}ppm, and 4.0×10^{-3}ppm. The corresponding percentage mortalities were 8%, 24%, 44%, 60%, 72%, and 92%, while the probit mortalities were recorded as 3.59, 4.25, 4.85, 5.25, 5.58, and 6.41. The LC_{25}, LC_{50}, and LC_{90} values for temephos were determined as 1.1×10^{-3}ppm (with a confidence interval of 8×10^{-4}-1.4×10^{-3}ppm), 1.9×10^{-3} ppm (with a confidence interval of 1.6×10^{-3}-2.3×10^{-3} ppm), and 5.3×10^{-3}ppm (with a confidence interval of 4.0×10^{-3}-8.7×10^{-3}ppm), respectively (Table 3.2 and Table 3.6).

The larvicidal potential of synthetic pyrethroids, cypermethrin and lambda-cyhalothrin, was investigated for varying concentrations of 4.0×10^{-5}ppm, 1.0×10^{-4} ppm, 1.6×10^{-4}ppm, 2.2×10^{-4}ppm, 2.8×10^{-4} ppm, and 3.4×10^{-4}ppm for cypermethrin. The corresponding percentage mortalities were 16%, 32%, 48%, 64%, 80%, and 88%, and probit mortalities were calculated as 4.01, 4.53, 4.95, 5.36, 5.84, and 6.18, respectively (Table 3.3). For lambda-cyhalothrin, test concentrations of 2.0×10^{-5} ppm, 4.0×10^{-5}ppm, 6.0×10^{-5}ppm, 8.0×10^{-5}ppm, 1.0×10^{-4}ppm, and 1.2×10^{-4} ppm exhibited percentage mortalities of 16%, 36%, 52%, 64%, 84%, and 96%, with probit mortalities as 4.01, 4.64, 5.05, 5.36, 5.99, and 6.75, respectively (Table 3.4).

The susceptibility values for cypermethrin were noted as 7.0×10^{-5}ppm (with a confidence interval of 4.0×10^{-5}-9.0×10^{-5}ppm) for LC_{25}, 1.4×10^{-4}ppm (with a confidence interval of 1.1×10^{-4}-1.7×10^{-4}ppm) for LC_{50}, and 5.0×10^{-4}ppm (with a confidence interval of 3.5×10^{-4}-9.3×10^{-4}ppm) for LC_{90}. For lambda-cyhalothrin, the susceptibility values were determined as 3.0×10^{-5}ppm (with a confidence interval of 2.0×10^{-5} -4.0×10^{-5}ppm) for LC_{25}, 5.0×10^{-5}ppm (with a confidence interval of 4.0×10^{-5} -6.0×10^{-5}ppm) for LC_{50}, and 1.3×10^{-4}ppm (with a confidence interval of 1.0×10^{-4}-2.0×10^{-4}ppm) for LC_{90} (Table 3.6).

The results demonstrated varying effectiveness among the tested chemical insecticides against *Ae. aegypti* larvae. Based on the research findings, it was evident that lambda-cyhalothrin emerged as the most effective chemical insecticide in controlling *Ae. aegypti* larvae, exceeding the efficacy of cypermethrin, temephos, and malathion in that order. While cypermethrin, temephos, and malathion also showed potential in mosquito control programs, each with its specific advantages and applications. This potency is crucial in mosquito control efforts, especially in regions where *Ae. aegypti* poses a significant threat as a vector for various diseases. The effectiveness of lambda-cyhalothrin as a synthetic pyrethroid highlights its potential in combating mosquito-borne diseases and mitigating the risk of transmission. Cypermethrin, although slightly less potent than lambda-cyhalothrin, also exhibited considerable larvicidal activity. It holds promise as an alternative insecticide for mosquito control programs, offering an additional option in situations where lambda-cyhalothrin may not be readily available or suitable. Temephos and malathion, belonging to the organophosphate family, displayed lower larvicidal efficacy than synthetic pyrethroids. Nevertheless, they can still serve as viable options in integrated pest management strategies, especially when resistance to pyrethroids is a concern.

The larval bioassay utilised various concentrations of the isolated compound, CB1, derived from *C. bonplandianum*. The concentrations tested were 1.8, 3.6, 5.4, 7.2, 9.0, and 10.8 ppm. The results revealed that as the concentration increased, the percentage of mortalities of the tested mosquito larvae also increased, with observed values of 12%, 36%, 52%, 64%, 84%, and 92% for each respective concentration. The observed probit mortalities were 3.82, 4.64, 5.05, 5.36, 5.99, and 6.41. The susceptibility of CB1, against the larvae was evident, as it demonstrated mortality at

even lower concentrations (Table 3.5). The LC_{25}, LC_{50}, and LC_{90} values were determined to be 2.9 (2.0-3.6) ppm, 4.7 (3.9-5.6) ppm, and 12.2 (9.6-18.3) ppm, respectively, indicating the concentration at which 25%, 50%, and 90% of the larvae were affected. These findings highlighted the potential larvicidal activity of CB1 and its effectiveness in controlling mosquito larvae (Table 3.6).

Table 3.1: Percentage and probit mortality of Malathion against fourth instar *Ae. aegypti* larvae

Sl. No.	Concentration (ppm)	Log Concentration	Probit Mortality %	Percent Mortality %
1	2.1×10^{-3}	-2.68	3.82	12%
2	2.7×10^{-3}	-2.57	4.25	24%
3	3.3×10^{-3}	-2.48	4.64	36%
4	3.9×10^{-3}	-2.41	5.15	56%
5	4.5×10^{-3}	-2.35	5.71	76%
6	5.1×10^{-3}	-2.30	6.41	92%

Table 3.2: Percentage and probit mortality of Temephos against fourth instar *Ae. aegypti* larvae

Sl. No.	Concentration (ppm)	Log Concentration	Probit Mortality %	Percent Mortality %
1	5.0×10^{-4}	-3.30	3.59	8%
2	1.2×10^{-3}	-2.92	4.25	24%
3	1.9×10^{-3}	-2.72	4.85	44%
4	2.6×10^{-3}	-2.59	5.25	60%
5	3.3×10^{-3}	-2.48	5.58	72%
6	4.0×10^{-3}	-2.40	6.41	92%

Table 3.3: Percentage and probit mortality of Cypermethrin against fourth instar *Ae. aegypti* larvae

Sl. No.	Concentration (ppm)	Log Concentration	Probit Mortality %	Percent Mortality %
1	4.0×10^{-5}	-4.40	4.01	16%
2	1.0×10^{-4}	-4.00	4.53	32%
3	1.6×10^{-4}	-3.80	4.95	48%
4	2.2×10^{-4}	-3.66	5.36	64%
5	2.8×10^{-4}	-3.55	5.84	80%
6	3.4×10^{-4}	-3.47	6.18	88%

Table 3.4: Percentage and probit mortality of Lambda-cyhalothrin against fourth instar *Ae. aegypti* larvae

Sl. No.	Concentration (ppm)	Log Concentration	Probit Mortality %	Percent Mortality %
1	2.0×10^{-5}	-4.70	4.01	16%
2	4.0×10^{-5}	-4.40	4.64	36%
3	6.0×10^{-5}	-4.22	5.05	52%
4	8.0×10^{-5}	-4.09	5.36	64%
5	1.0×10^{-4}	-4.00	5.99	84%
6	1.2×10^{-4}	-3.92	6.75	96%

Table 3.5: Percentage and probit mortality CB1 against fourth instar *Ae. aegypti* larvae

Sl. No.	Concentration (ppm)	Log Concentration	Probit Mortality %	Percent Mortality %
1	1.8	0.26	3.82	12%
2	3.6	0.56	4.64	36%
3	5.4	0.73	5.05	52%
4	7.2	0.86	5.36	64%
5	9.0	0.95	5.99	84%
6	10.8	1.03	6.41	92%

Table 3.6: Mosquito larvicidal effect of CB1, Malathion, Temephos, Cypermethrin, and Lambda- cyhalothrin against fourth instar *Ae. aegypti* larvae

Test Material	LC_{25} (LCL-UCL) ppm	LC_{50} (LCL-UCL) ppm	LC_{90} (LCL-UCL) ppm	p-value
Plant isolate (CB1)	2.9 (2.0-3.6)	4.7 (3.9-5.6)	12.2 (9.6-18.3)	<0.01
Malathion	2.7×10^{-3} (2.4×10^{-3}-3.0×10^{-3})	3.5×10^{-3} (3.2×10^{-3}-3.8×10^{-3})	5.6×10^{-3} (4.9×10^{-3}-6.9×10^{-3})	<0.01
Temephos	1.1×10^{-3} (8.0×10^{-4}-1.4×10^{-3})	1.9×10^{-3} (1.6×10^{-3}-2.3×10^{-3})	5.3×10^{-3} (4.0×10^{-3} - 8.7×10^{-3})	<0.01
Cypermethrin	7.0×10^{-5} (4.0×10^{-5}-9.0×10^{-5})	1.4×10^{-4} (1.1×10^{-4}-1.7×10^{-4})	5.0×10^{-4} (3.5×10^{-4}-9.3×10^{-4})	<0.01
Lambda-cyhalothrin	3.0×10^{-5} (2.0×10^{-5}-4.0×10^{-5})	5.0×10^{-5} (4.0×10^{-5}-6.0×10^{-5})	1.3×10^{-4} (1.0×10^{-4}-2.0×10^{-4})	<0.01

Statistical significance p-value<0.05

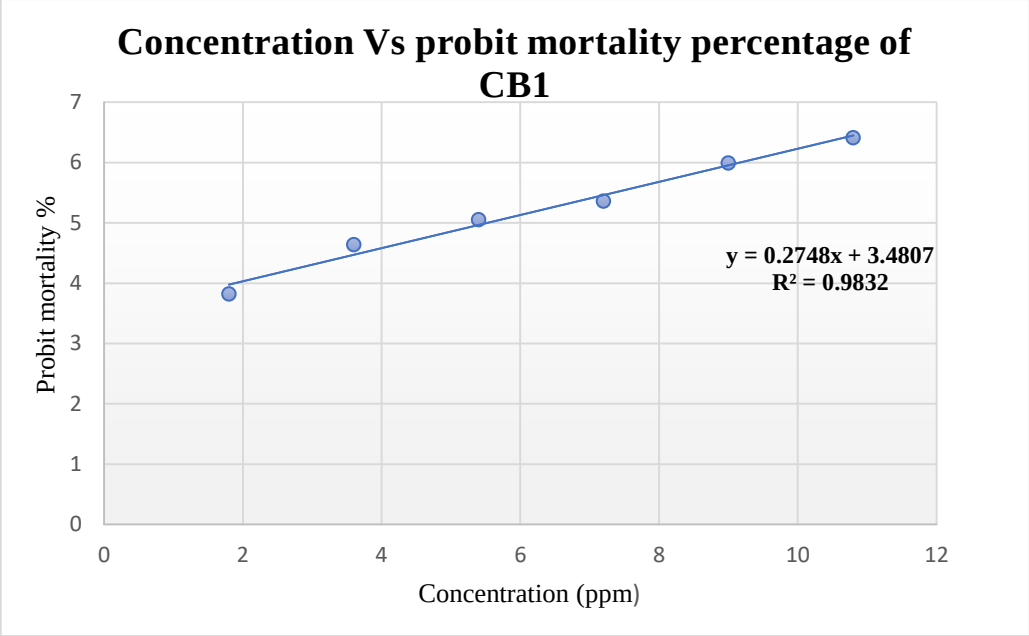

Figure 3.1 Concentration vs probit mortality percentage of CB1 against *Ae. aegypti* larvae

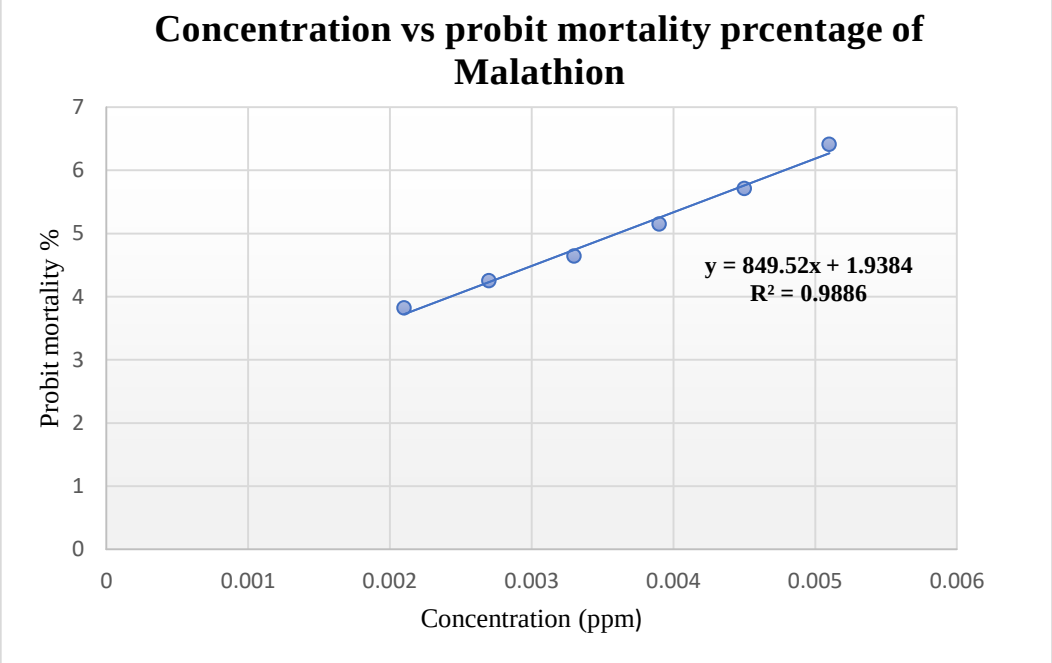

Figure 3.2 Concentration vs probit mortality percentage of Malathion against *Ae. aegypti* larvae

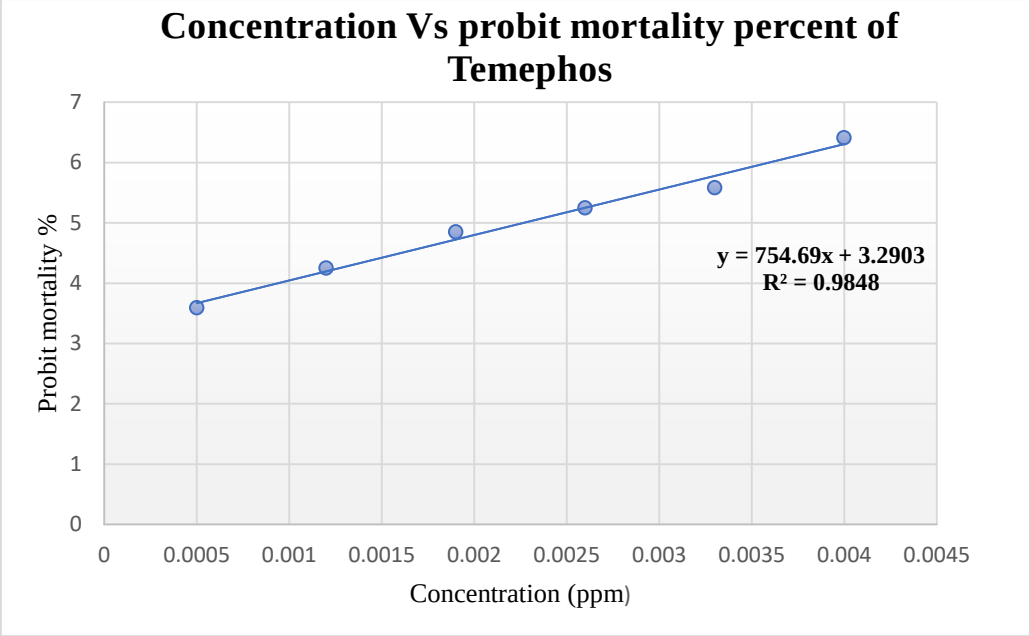

Figure 3.3 Concentration vs probit mortality percentage of Temephos against *Ae. aegypti* larvae

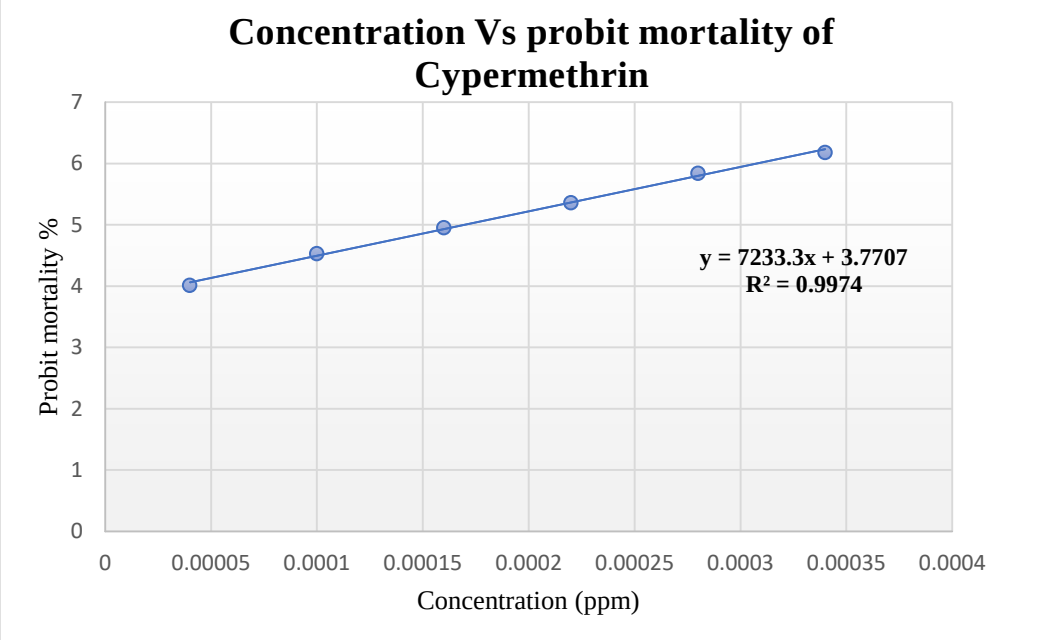

Figure 3.4 Concentration vs probit mortality percentage of Cypermethrin against *Ae. aegypti* larvae

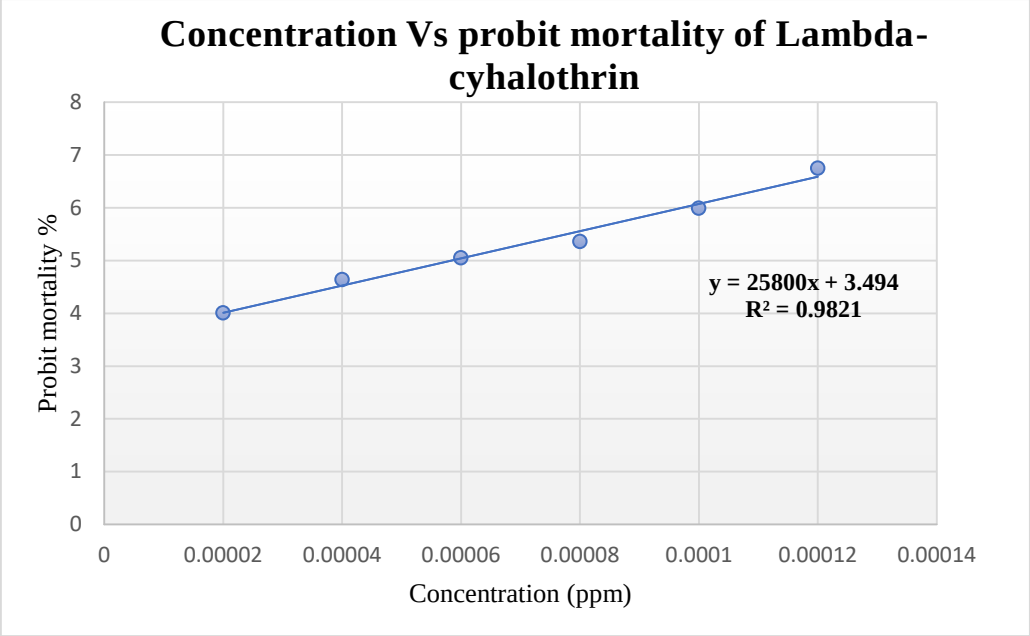

Figure 3.5 Concentration vs probit mortality percentage of Lambda- cyhalothrin against *Ae. aegypti* larvae

3.4 Discussion

The *Ae. aegypti* mosquito plays a critical role as the primary vector for transmitting dengue fever (Smith, 1956; Hammon, 1966; Rudnick, 1967; Gubler et al., 1979; Rohani et al., 2001) chikungunya and zika virus (Bodenmann and Genton, 2006; Pialoux et al., 2007; Díaz-González et al., 2015; Lounibos and Kramer, 2016). This invasive mosquito species has successfully spread across tropical to temperate regions worldwide. Its adaptability to breed in artificial containers has facilitated its passive dispersal over the past decades through significant transportation routes (Vezzani and Carbajo, 2008). *Ae. aegypti vector* mosquitoes are commonly found coexisting in artificial containers in rural, urban, and suburban communities within subtropical and tropical regions. Their ability to thrive in such varied environments has contributed to their widespread presence. Controlling and managing of *Ae. aegypti vector* populations are crucial to preventing disease outbreaks and safeguarding public health in affected regions (Rohani et al., 2001; Gomes et al., 2005; Jirakanjanakit et al., 2007).

Chemical treatment remains a fundamental and essential approach in pest management due to its fast-acting nature and broad coverage in treating infested areas. WHO has endorsed several chemical insecticides for managing mosquito

populations in mosquito control strategies worldwide (Meier et al., 2022). These insecticides fall under different categories, Organochlorines, Organophosphates, Carbamates, and Pyrethroids (WHO, 2006; Marcombe et al., 2009).

Malathion is classified as an organophosphate insecticide, effective in killing insects while posing lesser harm to mammals. Since the 1970s, this second-generation insecticide has been widely utilised for efficient mosquito vector control, especially after the phasing out of DDT and dieldrin in the United States of America. It finds prevalent applications in agricultural pest management and is also incorporated into vector control strategies for public health purposes (Prato et al., 2012; Cox, 2003). The primary mechanism of organophosphate poisoning involves inhibiting acetylcholinesterase in the nervous system, increasing acetylcholine levels (Ecobichon and Joy, 1993). Consequently, a single presynaptic stimulus can produce multiple postsynaptic impulses, resulting in hyperexcitability. Exposure to malathion and other organophosphorus substances at work has been linked to significantly impaired neutrophil chemotaxis, according to Hermanowicz and Kossman (1984). Additionally, the incidence of upper respiratory infections among these individuals rose as sensitivity to organophosphorus chemicals grew over time. In vitro, macrophage and lymphocyte immunological responses can also be impacted by organophosphorus chemicals (Karalliedde and Senanayake, 1989; Pruett et al., 1994).

In the current susceptibility assay, the observed results unveiled a considerable positive association between the mortality rate of *Ae. aegypti* larvae and the all the tested concentrations of malathion. This correlation signifies the presence of susceptibility to the insecticide, as evidenced by an LC_{50} value of 3.5×10^{-3}ppm. In the 24-hour bioassay, higher concentrations of malathion were associated with increased mortality percentages, supporting the findings. This result aligned with previous research conducted by Huong et al. (2004), where they investigated the susceptibility of *Ae. aegypti* to insecticides across multiple locations in Vietnam and found the species susceptible to malathion, suggesting its potential effectiveness in mosquito control within those regions. Similarly, Ponlawat et al. (2005) examined *Ae. aegypti*'s susceptibility to insecticides across Thailand and reported that the species was susceptible to malathion. In a study by Hidayati et al. (2011), the first generation of *Ae. aegypti* mosquitoes, which had no prior exposure to malathion,

showed susceptibility to the insecticide. However, subsequent generations of the species displayed increasing resistance with continuous exposure to malathion. The present study, in line with findings by Samal and Kumar (2018), confirms *Ae. aegypti*'s sensitivity to malathion, strengthening the existing evidence of the species' vulnerability to this organophosphate insecticide.

WHO considers temephos insecticide to be a highly acceptable and effective mosquito larvicide, capable of controlling most mosquito vectors, even when used in drinking water. One of its key advantages is its minimal toxicity, posing little acute risk to humans. The mechanism of action of temephos involves the irreversible inhibition of acetylcholinesterase, an essential enzyme seen in metazoans, which is responsible for neurotransmission, particularly at nerve endings, that results in the disruption of acetylcholine-mediated processes (Fournier et al., 1992; Silman and Sussman, 2005). Temephos has been extensively used worldwide after its initial applications in the early 1970s for targeting *Ae. aegypti* larvae (Maestre-Serrano et al., 2014). However, the continuous usage of this insecticide has led to the surfacing resistance in *Ae. aegypti* populations in various regions.

The current study undertaken discovered that the test species showed sensitivity to temephos, as specified by an LC_{50} value of 1.9×10^{-3}ppm. In a 2003 study conducted in Thailand, Paeporn et al. investigated the potential development of resistance in *Ae. aegypti* to the organophosphate insecticide temephos. The research spanned nineteen generations of *Ae. aegypti* mosquitoes which were exposed to temephos to assess LC_{50} values. Interestingly, the initial generation exhibited remarkable susceptibility to the insecticide, with mortality occurring even at lower concentrations. In contrast, the 19^{th} generation demonstrated an elevated tolerance to the pesticide, indicating the emergence of resistance. In Brazil, Luna et al. (2004) performed bioassays to evaluate the susceptibility of *Ae. aegypti* to the temephos insecticide, determining its response to the chemical. The findings from the research study revealed that, *Ae. aegypti* demonstrated susceptibility to temephos. As per another research conducted in Venezuela to evaluate *Ae. aegypti*'s susceptibility to temephos revealed that the larval populations from western Venezuela, collected during the study period, showed susceptibility to the insecticide. The study observed low resistance ratios and the absence of enzyme overexpression in the tested populations (*Alvarez et al., 2014*). In their study, Muthusamy and Shivakumar

(2015b) investigated the susceptibility status of *Ae. aegypti* to temephos in three districts of Tamil Nadu, India. *Ae. aegypti* larvae were collected from their natural habitats in three distinct areas, Salem, Namakkal, and Dharmapuri, for the study. The findings indicated that all three strains of *Ae. aegypti* tested showed susceptibility to temephos. Fatimah and Hasmiwati (2020) conducted an experiment to evaluate the lethal concentrations of *Ae. aegypti* larvae when exposed to a range of temephos concentrations, and results from the experiment indicated that the species sustained susceptibility to temephos. Considering the results obtained in the current investigation and a detailed review of previous research, it is apparent that *Ae. aegypti* consistently demonstrates susceptibility to temephos.

Pyrethroids available in the market are the result of efforts to create synthetic analogues of naturally occurring pyrethrins with improved environmental stability (Elliott, 1989). These pyrethroids were discovered when DDT use faced resistance and limitations, and the rising problem of resistance to organophosphate and carbamate insecticides needed to be addressed. Some of the widely used synthetic pyrethroids include permethrin, cypermethrin, deltamethrin, letherin, furethrin, fenevelerate, alpha-cyperamethrin, and lambda-cyhalothrin. Even today, pyrethroids remain a critical class of insecticides, widely employed in mosquito control, especially for treating bed nets and as adulticides. On a global scale, pyrethroid holds a substantial share of around 20% in the insecticide market (Scott et al., 2015; Abubakar et al., 2020). In 1993, Dorta et al. carried out a detailed study focusing on the susceptibility of six mosquito vector species to pyrethroids and organophosphorus insecticides to assess the relative effectiveness of these two insecticides in controlling the targeted vector populations. The results of their investigation demonstrated that synthetic pyrethroids displayed significantly higher insecticidal efficiency when compared to organophosphates, indicating the potential superiority of pyrethroids as a vector control strategy. These findings are consistent with the results obtained in the current study. The results of the study confirmed that, *Ae. aegypti*, the target mosquito species, displayed a high level of susceptibility to the pyrethroid insecticides that were tested. When exposed to cypermethrin, the LC_{50} value was measured at an exceptionally low concentration of 1.4×10^{-4} ppm, indicating that this chemical was highly effective at causing mortality in the mosquito larvae. Lambda-cyhalothrin, another pyrethroid insecticide, exhibited even

greater potency, with an even lower LC_{50} value of 5.0×10^{-5} ppm. These outcomes validated the vulnerability of *Ae. aegypti* to pyrethroids, suggesting that these chemicals could be effective in mosquito control efforts, particularly in areas where this species is a significant vector of diseases.

Cypermethrin, a highly potent synthetic pyrethroid insecticide, was reportedly developed in 1974 and later commercialized in 1977. It has proven to be operative against a broad spectrum of insect pests in various domains, including agriculture, public health, and animal husbandry. The chemical structure of cypermethrin comprises the alpha-cyano-3-phenoxybenzyl ester of chrysanthemic acid's dichloro analogue, known as 2,2-dimethyl-3-(2,2-dichlorovinyl) cyclopropanecarboxylic acid. The molecule possesses three chiral centres, with two located on the cyclopropane ring and one on the alpha cyano carbon. The isomers of cypermethrin are categorized into four cis- and four trans-isomers, with the cis-group exhibiting greater potency as an insecticide. The cis-to-trans isomer ratio typically ranges from 50:50 to 40:60. Cypermethrin is a combination of all eight isomers, and in most cases, it primarily refers to the racemic mixture with a ratio of 50:50. It possesses a remarkably low vapour pressure and water solubility but exhibits high solubility in a diverse array of organic solvents (WHO, 1989; Velisek et al., 2006; Ullah et al., 2018). Luna et al. (2004) conducted a study in Brazil to evaluate the susceptibility of *Ae. aegypti* to cypermethrin, and the assessment revealed a survival rate of 35% and a fatality rate of 65% for cypermethrin. In another research study investigating the association between insecticide use and changes in *Ae. aegypti* susceptibility, the susceptibility status of nine vector populations from the Northeast region of Brazil was compared. The results showed that the sample populations collected from Campinas and Marília exhibited an average mortality rate in response to cypermethrin (Macoris et al., 2007). The assessment of pyrethroids efficacy in *Ae. aegypti* larvae from the semi-arid zone of Jaipur demonstrated that the species exhibited the highest susceptibility towards cypermethrin compared to other tested pyrethroids (Meena and Kachhwaha, 2016). Similarly, in the investigation of Pyrethroid resistance in adult mosquitoes, specifically *Ae. aegypti* from Jaipur City, Rajasthan, it was observed that the tested species exhibited a significant mortality rate when exposed to cypermethrin insecticide (Meena, 2017). In a research work conducted by Piedra et al. (2022) at The Zoological Garden of Havana, Cuba, the

insecticide resistance in *Ae. aegypti* was characterized, and the results of the investigation suggested that the tested species exhibited susceptibility to pyrethroid insecticides, with cypermethrin showing maximum effectiveness. These findings provide evidence of the potential susceptibility of *Ae. aegypti* to cypermethrin, as reported in the current study.

Lambda-cyhalothrin is composed of a ratio of 1:1 combination of two different stereoisomers:(S)-cyano-3-phenoxybenzyl-(Z)-(1R,3R)-3-cyhalothrin(2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropanecarboxylate and (R)-cyano-3-phenoxybenzyl-(Z)-cyclopropanecarboxylate(1S,3S)-3-(2-chloro-3,3,3trifluoroprop-1-enyl)-2,2-dimethylcyclopropanecarboxylate. This specific chemical composition of lambda-cyhalothrin was first documented by Robson and Crosby in 1984, and it was then immediately introduced to Central America in 1985 by ICI Agrochemicals. This pyrethroid compound has since become widely used in various regions to effectively control insects, particularly those that act as disease vectors, making it a valuable tool in pest management and public health initiatives. Its formulation has successfully prevented vector mosquitoes, cockroaches, ticks, and flies, thereby reducing the transmission of harmful diseases (Anadon et al., 2006; He et al., 2008). In research conducted at International Airports in Thiruvananthapuram and Cochin, located in southern India, the susceptibility of *Ae. albopictus* and *Ae. aegypti* in their aquatic and adult stages was examined, and the findings demonstrated that adult *Ae. aegypti* and *Ae. albopictus* exhibited susceptibility to lambda-cyhalothrin (Sharma et al., 2004). Lawler et al. (2007) examined to assess the duration of insecticidal activity of the pyrethroid lambda-cyhalothrin on predatory insects in rice fields and three types of mosquito larvae. These included a pyrethroid-sensitive strain of *Cx. tarsalis*, a pyrethroid-resistant strain of *Cx. pipiens*, and non-resistant *Cx. pipiens*. The results indicated that lambda-cyhalothrin was highly effective in controlling the majority of the susceptible mosquito poopulations. In 2007, Rodríguez et al. published comprehensive data on the dose mortalities of lambda-cyhalothrin involving *Ae. aegypti* collected from four different regions in Latin America. The strain obtained from Havana City exhibited an LC_{50} value of 2.7×10^{-2}ppm, while Cuba displayed 5.90×10^{-3}ppm. Similarly, Jamaica and Panamá had LC_{50} values of 5.50×10^{-3}ppm and 4.80×10^{-4}ppm, respectively, confirming their susceptibility to the tested insecticide. To assess the susceptibility of the *Ae. aegypti* strain discovered in

El Salvador to lambda-cyhalothrin, Lazcano et al. (2009) undertook a study using an *Ae. aegypti* strain obtained from the municipality of Soyapango, El Salvador. The investigation results revealed that the adult mosquitoes belonging to this strain displayed a significant degree of susceptibility to lambda-cyhalothrin. Another study aimed to determine diagnostic doses of insecticides for the Rockefeller susceptible strain of *Ae. aegypti*, Rodríguez et al. (2017) utilised the CDC bottle bioassay to monitor insecticide resistance in the Cuban vector control program. The findings of the study revealed that despite being temephos-resistant, the strain remained susceptible to lambda-cyhalothrin. A study was conducted at The Zoological Garden of Havana, Cuba, to characterise insecticide resistance in *Ae. aegypti*. The investigation sought to assess the population's susceptibility level and identify potential insecticide resistance mechanisms. The findings revealed that adult mosquitoes in the *Ae. aegypti* population remained susceptible to the pyrethroid insecticide lambda-cyhalothrin (Piedra et al., 2022). The collective evidence from several studies, including investigations assessing insecticide susceptibility in *Ae. aegypti*, consistently supported that, this vector species remains highly susceptible to lambda-cyhalothrin as observed in the current study.

Phytochemicals derived from various botanical sources have proven to be versatile and beneficial in various applications, ranging from medicinal uses to insecticides. Even though synthetic organic pesticides have demonstrated remarkable efficacy against targeted pests like mosquitoes, they also pose risks to a wide range of non-target organisms, including humans (Matsumura, 1975). Moreover, many crucial mosquito species responsible for transmitting diseases have developed physiological resistance to several conventional chemical pesticides (Brown, 1986). As a result, there is a growing need for environmentally friendly, biodegradable, and highly specific mosquito insecticides that make minimal harm to non-target organisms.

In the present study conducted, the isolated compound eicosane (CB1) from *C. bonplandianum* exhibited larvicidal activity against mosquito larvae. It was observed that as the concentration of CB1 increased, the mortality rate of the larvae also increased, and the compound showed susceptibility to the tested mosquito larvae, even at lower concentrations. The lethal concentration values were determined to assess the concentration at which specific percentages of larvae were affected, and these results marked the potential of CB1 as an effective agent for

controlling mosquito larvae. Numerous plant species from diverse botanical families have been examined for their larvicidal effects on *Ae. aegypti*. Patterson et al. (1975) studied more than three hundred wild-growing plants in North Dakota and western Minnesota, evaluating their effects on *Ae. aegypti* larvae. The research found that the impact varied not only among different plant species but also among different parts of the same plant species. Marcard et al. (1986) reported that the extracts obtained from different plant parts of *Ajuga remota* and *A. reptans* exhibited varying levels of efficiency against *Ae. aegypti*. The root extract exhibited the highest activity, followed by leaves, shoots, and flowers of the plant. Within the Annonaceae family, *Annona crassiflora* demonstrated efficient larvicidal activity in its root wood and root bark. Additionally, *A. glabra* seeds and *A. muricata* roots also found to be exhibit efficacy against *Ae. aegypti* mosquito larvae. From the Leguminoseae plant family, *Pterodon polygalaeflorus* seeds demonstrated larvicidal efficacy (Macêdo et al., 1997). In another study, *Rhizophora mucronate* from the Rhizophoraceae family exhibited larvicidal activity in its bark, pith, and stem wood (Kabaru and Gichia, 2001).

Similarly, when tested, the seeds of *Cassia tora* from the Caesulpinaceae family and *Cassia obtusifolia* from the Leguminosae plant family demonstrated larvicidal properties in two independent studies (Jang et al., 2002; Yang et al., 2003). In a study, Cheng et al. (2003) investigated the larvicidal efficacy of different plant species and their components as potential mosquito vector control agents. Among the tested plants, *Cryptomeria japonica* essential oils extracted from bark and leaf exhibited solid larvicidal efficacy. Azadiractin, the main chemical found in neem, was recognized to be efficient in preventing mosquito larvae proliferation. When tested, the phyto-extracts from *M. koenigii*, *Coriandrum sativum*, and *Trigonella foenum graceum* were effective against *Ae. aegypti* mosquito larvae. *Apium graveolens* seeds from Umbelliferae and *Curcuma aromatica* rhizome from Zingiberaceae displayed larvicidal efficacy as reported by separate research investigations (Choochote et al., 2004; Choochate et al., 2005). The stem wood of *Cybistax antisyphilitica* from the Bignoniaceae family exhibited larvicidal potential (Rodrigues et al., 2005), while the fruit of *Momordica charantia* from Cucurbitaceae demonstrated larvicidal activity as well against mosquito vectors (Singh et al., 2006). In 2005, Chansang et al. tested and identified the larvicidal

efficacy of the unripe and ripe fruit of *Piper retrofractum* from Piperaceae against vector mosquitoes. In 2006, Chaithong et al. reported significant larvicidal activity exhibited by the fruit exocarp of *P. longum*, *P. ribesoides*, and *P. sarmentosum* from Piperaceae and in 2008, Chowdhury et al. revealed the larvicidal potential of the leaves of *Solanum villosum* from Solanaceae. Similarly, the *Coccinia indica*, *Cucumus sativus*, and *Momordica charantia* leaves from Cucurbitaceae displayed larvicidal activity in a study conducted by Rahuman and Venkatesan (2008). In separate studies conducted by Sivagnaname and Kalyanasundaram (2004) and Mgbemena in 2010, the leaves of *Atlantia monophylla* from the Rutaceae family, as well as the leaves of *Ocimum gratissimum* from the Meliaceae family and *Citrus citratus* from the Rutaceae family, respectively, demonstrated larvicidal efficacy. These findings indicated the significant potential of diverse plant sources in efficiently managing the mosquito larvae over time, supporting the outcomes of the current study.

3.5 Conclusion

This chapter evaluated the larvicidal efficacy of four conventional insecticides frequently employed in mosquito control programs. These insecticides belonged to two distinct classes, entailing organophosphates and pyrethroids. The results from the susceptibility assay demonstrated the insecticidal activity of malathion, temephos, cypermethrin, and lambda-cyhalothrin, each at varying concentrations. Lambda-cyhalothrin showed maximum activity among the tested insecticides, suggesting it could be a sustainable substitute for mosquito control initiatives. It was followed by cypermethrin, which also showed considerable larvicidal activity but was less than lambda-cyhalothrin. However, temephos and malathion, which are members of the organophosphate family, demonstrated reduced larvicidal effectiveness compared to synthetic pyrethroids. The plant isolate CB1, derived from *C. bonplandianum*, also exhibited larvicidal activity against *Ae. aegypti* larvae. The results clearly indicated its potential effectiveness in controlling mosquito larvae, unfolding its significance as a natural botanical resource in vector mosquito control. Excessive reliance on chemical insecticides can lead to adverse consequences, including developing insecticide-resistant mosquito strains and potentially harming non-target species and ecosystems. It is essential to strike a balance between effective mosquito control and minimizing the negative impact on

ecosystems through judicious and responsible use of these chemicals. The findings of this chapter revealed the susceptibility status of four frequently used chemical insecticides against *Ae. aegypti*. Furthermore, this study advocated a promising plant-based compound for effective mosquito control.

CHAPTER IV

The Effect of Synergistic Interaction between Plant Isolate and Four Conventional Insecticides on Fourth Instar Larvae of *Ae. aegypti*

4.1 Introduction

Efforts to control mosquitoes encounter significant hurdles that impede their efficacy in addressing the menace of mosquito-borne diseases. Chief among these challenges is the emergence of insecticide resistance within mosquito populations. Over time, mosquitoes have developed adaptive mechanisms to withstand the lethal impact of commonly employed insecticides, diminishing the effectiveness of once-potent chemicals. This resistance represents a formidable barrier to the success of vector control initiatives, necessitating an ongoing quest for novel insecticides and innovative strategies. Equally, environmental factors are a central aspect of the complexities surrounding mosquito control. Abundant chemical insecticides employed in vector management can exert detrimental effects on non-target organisms and ecosystems, potentially precipitating ecological imbalances. Moreover, the persistent presence of pesticide residues in the environment exacerbates environmental concerns, imposing the urgency of uniting sustainable and environmentally harmless approaches as an imperative response (Nauen and Denholm, 2005; Sharma et al., 2013; Pavela, 2014).

This scenario has spurred a pressing need to swiftly explore novel alternatives that align with both environmental and health considerations, offering adequate vector protection. Among the contemporary strategies geared towards moderating pest populations, using pesticides derived from plant extracts emerges as a particularly assuring avenue (Chockalingam et al., 1990). In present-day approaches against vector mosquito proliferation, allelochemicals originating from plant extracts are increasingly being explored as potential substitutes or supplements to synthetic insecticide methods (Attia et al., 2013). These chemical messengers facilitate communication between plants and herbivores (Regnault-Roger, 1997). Certain plants have even developed sufficient chemical and physical defences against various insect groups (Ryan and Byrne, 1988). Thus, plant extracts have emerged as influential pest behaviour and physiological factors in current pest control approaches. These extracts possess the ability to repel pests, impede their feeding,

disrupt growth, affect moulting and respiration, and reduce reproductive capabilities. The combination of diverse bioactive compounds within these extracts has demonstrated the potential to delay the development of resistance. Crucially, these extracts align with environmental concerns, as they are biodegradable and have minimal effects on non-target organisms and ecosystems (Isman and Machial, 2006; Isman and Akhtar, 2007; Pavela and Vrchotová, 2013).

Even though plant-based insecticides have many advantages, there are some disadvantages as well that should be acknowledged. One of their main disadvantages is that they are more expensive to produce and use than synthetic chemical substitutes. The processing of plant products, which includes their extraction and formulation, increases the cost of production. The lower potency and rapid breakdown tendency causing reduced persistence demands a repeated and large-scale application of botanicals compared to synthetic insecticides. Also, the efficacy of plant-based insecticides can vary across different pest species and environmental conditions. These conditions may make it necessary to modify application rates or combine different plant extracts to target various pest species effectively. Such a need for repeated applications can escalate labour and operational costs, particularly in situations where continuous and consistent pest control is needed (Damalas and Koutroubas, 2020).

Synergists represent a direct and uncomplicated approach to counteracting the metabolic resistance of the pest species, as they can directly obstruct the mechanisms responsible for resistance. Since the initial illustration of insecticide synergism more than several decades ago (Haller et al., 1942; Raffa and Priester, 1985), their practical implementation in combating pests has held significant potential. This prospect of a synergistic blend of biological and chemical insecticides as a viable solution for managing insect pests is endorsed by Koppenhöfer and Fuzy (2003) and Morales-Rodriguez and Peck (2009). The synergistic method proposes that if the combined substances demonstrate synergistic effects, they could provide equivalent control at reduced concentrations compared to their separate application. Reducing expenses and minimizing toxicity to mammals and non-target organisms are the direct benefits of this strategy. The fundamental idea behind this tactical approach is that it is rare that the target organism could

develop resistance to both ingredients in the mixture simultaneously if resistance to one of the two insecticides occurs infrequently and independently (Curtis, 1985).

Synergists have been used commercially for almost fifty years and have greatly improved the efficacy of insecticides, particularly regarding resistance issues. These natural or artificial materials that improve the effectiveness and potency of insecticides currently on the market are considered safe. Most synergists work by blocking the metabolic pathways that break down pesticide molecules, which impacts enzymes and detoxification processes. Synergists play a crucial role in resistance management because they inhibit enzymes, making insects more susceptible to chemicals. As a result, synergists are thought to be immediately effective in preventing metabolic resistance or delaying its onset. However, the full potential of these substances for managing resistance is yet to be realized. As control agents, synergists have the capacity to convert resistant populations into susceptible ones, thereby preventing the occurrence of resistance (Bernard and Philogène, 1993; Picollo et al., 2000; Jensen et al., 2006; Pasay et al., 2009; Sarwar and Salman, 2015; Sarwar, 2016).

The synergy, where the combined impact of two substances exceeds the total of their individual effects, appears to be more frequent in situations where synthetic insecticides are mixed with phytochemicals, compared to instances where different phytochemicals exhibit synergistic interactions. This indicates that the synergy between synthetic insecticides and plant-derived compounds frequently results in enhanced pest control effects. The effectiveness of certain phytochemicals can exhibit a range of responses depending on the specific synthetic insecticides with which they are paired. This variability results in the complexity of interactions within these mixtures, increasing the need for careful consideration when designing and implementing such combinations for pest management strategies. With an expanding comprehension of these interactions, utilizing the possibilities of synergy between synthetic and natural compounds offers the prospect of creating more efficient and sustainable approaches to insect pest control (Thangam and Kathiresan, 1990; Mansour et al., 2000; Shaalan et al., 2005a).

Biochemical assessments have emerged as the preferred method for understanding insecticide resistance mechanisms among insects. With the advent of sophisticated and highly sensitive biochemical assays, it is now feasible to analyse insecticide

resistance mechanisms with reasonable precision. Within the context of metabolic-based insecticide resistance mechanisms, non-specific esterases, Glutathione S-transferases (GSTs), and P450-mediated monooxygenases (MFOs) are recognised for their involvement in detoxifying organophosphate, pyrethroid, and carbamate insecticides (Hemingway and Ranson, 2000). Among insects, significant research has focused on Acetylcholinesterase (AChE) concerning insecticide resistance, primarily because this enzyme is the primary target for organophosphate and carbamate insecticides. The insensitivity of AChE to these insecticides represents a significant contributing factor to resistance. Consequently, these enzymes are established as reliable markers for assessing the impact of toxic compounds on a wide range of test organisms (Smirle et al., 2010; Muthusamy et al., 2014).

4.2 Methodology

4.2.1 Mosquito Colony Maintenance

The maintenance of the *Ae. aegypti* colony adhered to the methods elucidated in Chapter II (Munstermann, 1997).

4.2.2 Plant Isolate Bioassay and Insecticide Bioassay

The methodologies for conducting the plant isolate bioassay (WHO, 2005a) and insecticide bioassay (Brown, 1986) were followed as described in Chapter III.

4.2.3 Synergistic Assay of Phyto-Extracts and Insecticides

Two separate test protocols were set up, each focusing on the interaction between the insecticide and plant extract to unveil the Co-toxicity coefficient (CTC) and Synergistic factor (SF) (Kalyanasundaram and Das 1985). These tests were designed to provide deeper insights into the possible cumulative effects of these substances on *Ae. aegypti* larvae. Analysing the Co-toxicity coefficient aimed to ascertain the level to which the combined mixture influenced mortality rates compared to expected outcomes. Similarly, the assessment of the Synergistic factor aimed to understand the extent of synergism or antagonism between the insecticide and plant isolate against *Ae. aegypti* larvae. The synergistic combinations were prepared by combining CB1 with different insecticides. Malathion and CB1 combination were denoted as SC1, temephos and CB1 as SC2, cypermethrin and CB1 as SC3 and lambda-cyhalothrin and CB1 as SC4.

4.2.3.1 Co-toxicity Coefficient (CTC)

To calculate the CTC, a test concentration was meticulously prepared by mixing the insecticide and CB1 in a consistent ratio of 1:1. Specifically, the concentrations of insecticide and CB1 that had been determined as LC_{25} were selected for this experiment. Each solution, measuring 500 µl, was mixed and then diluted to a total volume of 250 ml using tap water. As a control, a mixture of 500 µl of alcohol and acetone in the same proportion and volume was added to tap water.

$$CTC = 100X\frac{(\text{Observed \% mortality} - \text{Expected \% mortality})}{\text{Expected \% mortality}}$$

A positive CTC value greater than 20 (CTC >20) indicated synergism, while a negative value below 0 (CTC <0) suggested antagonism. Values falling between 0 and 20 (0 < CTC < 20) indicated an additive effect. The expected mortality was calculated by combining the percentage of mortalities at the LC_{25} concentration of the individual test materials. The observed mortality was recorded 24 hours after exposure to the mixtures, following the methodology established by Sun and Johnson in 1960.

4.2.3.2 Synergistic Factor (SF)

The test solution was created using a constant LC_{25} concentration of CB1, achieved by diluting the 100% concentration. Additionally, varying concentrations of individual insecticide test solutions were prepared independently and introduced into the test solutions. Synergistic factors were calculated following the approach outlined by Kalyanasundaram and Das in 1985.

$$SF = \frac{(\text{LC50 or LC90 of the insecticide alone})}{(\text{LC50 or LC90 of the insecticide with plant extract})}$$

A synergistic factor value greater than one indicates synergism, while a value lower than one indicates antagonism.

4.2.3.3 Statistical Analysis

Larval mortality counts were corrected using Abbott's formula (Abbott, 1925) to account for mortality in the control group. The adjusted mortality data were then subjected to probit mortality regression analysis based on log dosage (Finney, 1971). To examine the significant effects of the plant-isolated compound both on its own

and in combination with various insecticides, ANOVA was performed using SPSS software version 26.

4.2.4 Quantitative Enzymatic Assay

To assess alterations in the enzymatic activity of detoxifying enzymes in *Ae. aegypti*, two distinct quantitative enzymatic assays were carried out. In the initial assay, 30 third instar larvae were exposed to a 10-fold diluted LC_{50} value of CB1 and SC4. Enzymatic assays for the specified detoxifying enzymes were performed on the dead larvae from both test groups after 24, 48, and 72 hours, aiming to reveal the influence of these compounds on the detoxification mechanisms of *Ae. aegypti*.

The second experiment was designed to determine whether any form of resistance had developed within the tested species against SC4 over successive generations. This investigation was conducted using both a susceptible line (F0) and a selected line (F5) exposed to selection pressure for five consecutive generations.

All the test batches were replicated three times to ensure accuracy.

4.2.4.1 Selection Experiment

In the research methodology, a susceptible laboratory-reared population of *Ae. aegypti* was exposed to selection pressure using the synergistic compound SC4. SC4 was chosen for the selection experiment and subsequent analysis of resistance development because it demonstrated the highest larvicidal efficacy among all the tested combinations against fourth instar *Ae. aegypti larvae*. This selected synergistic combination was administered to the fourth instar *Ae. aegypti* larvae over five consecutive generations. The mosquito strain that had been colonised for 25 generations in the CDRL laboratory was designated as the F0 generation. The F0 strain utilised had no prior exposure to insecticides.

The standard method outlined by the WHO (Brown, 1986) was employed for bioassay experiments. 1 ml of the synergistic combination was thoroughly mixed with 249 ml of dechlorinated water in 500 ml glass beakers. Six concentrations of the compound were applied to the *Ae. aegypti* larvae in ascending series. Dose determination was based on preliminary experiments. Control groups were maintained alongside the experiments. Each experiment used 25 fourth instar larvae, and after 24 hours of continuous exposure at 55-60% relative humidity, a

temperature of 27±2°C, and a 14 L:12 D photoperiod, the mortality rate was recorded. The surviving synergistic compound-exposed larvae were used for selection experiments in each generation from F0 to F5. The larvae that had survived each experiment were washed and set aside for rearing. Surviving larvae from the experiment (LC_{50}) were used to produce the progeny of the succeeding generations.

Probit regression analysis was utilised to compute the LC_{50} and LC_{90} values of the respective compounds against *Ae. aegypti* larvae. Control mortality between 5% and 20% was corrected using Abbott's formula (Abbott, 1925).

4.2.4.2 Resistance Ratio (RR)

$$RR = \frac{(\text{LC50 of the insecticide treated mosquito strain})}{(\text{LC50 of the susceptible strain})}$$

RR is frequently computed and serve as valuable tools for tracking the progression of insecticide resistance within a population in the field. To calculate an RR, the LC_{50} value of the insecticide-treated strain was divided by the LC_{50} value of a susceptible strain. The field population was categorized as susceptible as the RR fell below 5. RR values between 5 and 10 indicated moderate resistance among the mosquitoes, and RR values exceeding 10 denoted a high resistance level in the mosquito population (WHO, 2016a).

4.2.4.3 Preparation of Sample Solution

In this study, thirty early fourth instar larvae of *Ae. aegypti* were individually homogenized using 200 µl of distilled water in separate 1.5 ml capacity Eppendorf tubes. The homogenates were prepared at a temperature maintained at 4ºC and were subsequently subjected to centrifugation at 5,000 rpm for 10 minutes. After centrifugation, approximately 20 µl of the supernatant from each sample was transferred to another Eppendorf tube. The sample volume was then madeup to a final volume of 100 µl using a 0.1 M potassium phosphate (KH_2PO_4) buffer solution with a pH of 7.2. This entire procedure was repeated for the preparation of samples required for all conducted experiments.

4.2.4.4 Total Protein Quantification

The protein content of both susceptible and SC4-selected lines of *Ae. aegypti* was assessed using Lowry's method (Lowry et al.,1951).

i) Folin-Ciocalteau's Reagent: Equal volumes of distilled water and Folin-Ciocalteau's reagent were meticulously mixed just before the experiment.

ii) Lowry's Reagent: A solution containing 2% of copper sulphate, 2% of sodium potassium tartarate, and 4% of sodium carbonate dissolved in a 1:1:98 ratio was prepared.

A volume of 10 µl of mosquito homogenate was diluted with distilled water to make a final volume of 1 ml. To this, 5 ml of reagent (ii) was added and left for 15 minutes. Subsequently, 0.5 ml of reagent (i) was added. The Optical density was measured at 660 nm after 20-30 minutes of incubation of the test sample. Bovine serum albumin (BSA) calibration curve was prepared and used as a reference standard calibration curve. The total protein concentration of the test sample was determined by correlating the optical density values with the standard protein curve.

4.2.4.5 Acetylcholinesterase Assay

Acetylcholinesterase (AChE) activity was determined by the method outlined by Kranthi (2005). For this, a reaction mixture comprising 2.86 ml of 0.1M sodium phosphate buffer (pH 8.0) and 100 µl of the enzyme extract was prepared. It was then incubated for 5-minutes at room temperature. After that, 10 µl of the DTNB (0.01 M) solution and 30 µl of 0.10M acetylthiocholine iodide were added to this. At 412 nm, the change in absorbance was monitored for 30 minutes using a spectrophotometer (Shimadzu UV-1900i UV-VIS spectrophotometer, Japan). The enzymatic activity of the AChE was quantified in nmoles of AChE/min/mg protein unit.

4.2.4.6 Glutathione-S-Transferase Assay

The specific enzymatic activity of Glutathione S-transferase (GST) was assessed using a modified procedure by Habig et al. (1974). For this, 50µl of 50mM CDNB and 150µl of 50mM reduced glutathione and 0.77ml of phosphate buffer (100mM, pH 6.5) were mixed carefully. Then, 30µl of the enzyme extract was added to this mixture. The mixture was gently stirred and then incubated at 25°C for 2-3 minutes. Using a spectrophotometer, the change in absorbance of the sample was observed at 340 nm for 10 minutes. Specific enzymatic activity was quantified according to the CDNB-GSH conjugate formed and expressed as µmoles/min/mg protein.

4.2.4.7 Esterase Assay

A modified method outlined by Kranthi (2005) was employed to determine the specific enzymatic activity of esterases. The reaction mixture was prepared by mixing 0.1ml of enzyme extract (10µl enzyme solution mixed with 90µl sodium phosphate buffer, 0.04M, pH 6.8), and 0.5 ml of a 0.3mM α/β-naphthyl acetate substrate solution. This was then kept at room temperature in the dark for incubation for 20 minutes. The mixture was shaken in definite intervals during incubation. Then, a mixture of 0.1 ml of 1% Fast blue BB salt in sodium phosphate buffer (pH 6.8, 0.04 M) and 5% Sodium dodecyl sulphate (SDS) staining solution was added to the sample. It was then kept at room temperature for another 20-minute incubation. Using 0.1 ml of sodium phosphate buffer, 0.5 ml of substrate solution, and 0.1 ml of staining solution, a blank was prepared. At 590 nm, the absorbance of α-naphthol and at 540 nm, the absorbance of β-naphthol was observed and recorded using a spectrophotometer. The optical density (OD) values were compared with standard curves prepared from known concentrations of the respective products, α/β-naphthol. The specific enzymatic activity was calculated by nanomoles of product formed per minute per milligram of protein unit.

4.2.4.8 Monooxygenase (Cytochrome P450) Assay

A modified method described by Khan et al. (2020) was followed to determine the specific enzymatic activity of monooxygenase (cytochrome P450). At first, 0.3g of the sample was homogenized in a mixture of 900µl containing 1.0mM dithiothreitol (DTT), 1.0mM PMSF, 1.0mM EDTA, and 10% glycerol (0.1M, pH 7.5) on ice. After that, the homogenate was centrifuged at 4^{o}C and 10,000 RPM for 15 minutes. The reaction mixture included 125µl of enzyme solution, 365µl of sodium phosphate buffer (0.1M, pH 7.5), 10µl of NADPH (10 mM), and 5µl of 7-ethoxycoumarin-Odethylase (40mM). The mixture was incubated at 30°C for 15 minutes after vigorous mixing.150µl of 15% trichloroacetic acid (TCA) was added to terminate the reaction, and the entire mixture was centrifuged at 10,000 RPM for two minutes. The resulting supernatant was collected, and 40µl of 1.6 mM glycine-NaOH buffer (pH 10.5) was added. The optical density of the experiment (OD) was measured at 650 nm. The enzymatic activity of cytochrome P450 was determined using a standard curve of absorbance for known concentrations of cytochrome C and

expressed as nmoles equivalent units of Cytochrome P450 per minute per milligram of protein.

4.2.4.9 Statistical Analysis

All statistical analysises were done using R statistical software, R version 4.3.0 (R Core team 2023). A mixed-design ANOVA was done using the anova_test function in the rstatix package version 0.7.2 (Kassambara, 2023) to analyse the effect of SC4 and CB1 on specific enzymatic activity of detoxifying enzymes of *Ae. aegypti* larvae. A Two-sample t-test was employed to compare the specific enzymatic activity of SC4 selected F5 generation with the susceptible strain F0 of *Ae. aegypti (*Fox and Weisberg, 2019).

4.3 Result

4.3.1 Co-toxicity Coefficient Evaluation

The CTC of *Ae. aegypti* against the combination of the plant isolate CB1 and various insecticides, malathion (SC1), temephos (SC2), cypermethrin (SC3), and lambda-cyhalothrin (SC4), was determined. When the plant extract was combined with malathion at a concentration of LC_{25}+ LC_{25} (1:1), the observed mortality was 62%, while the expected mortality was 50%. This resulted in a co-toxicity coefficient of 24. For the combination of the plant extract with temephos at the same concentration, the observed mortality was 67.32%, compared to the expected mortality of 50%, yielding a co-toxicity coefficient of 34.64. Similarly, when combined with cypermethrin, the observed mortality was 70.68%, higher than the expected mortality of 50%, resulting in a co-toxicity coefficient of 41.36. In the case of the combination of the plant extract with lambda-cyhalothrin, the observed mortality stood at 80%, which was notably higher than the expected mortality of 50%, resulting in a co-toxicity coefficient of 60. These results demonstrated different levels of co-toxicity and synergistic effects between the plant extract and the selected insecticides against *Ae. aegypti*. All the tested combinations displayed CTC values greater than 20, indicating a synergistic impact on the tested organism. The SC1 exhibited the lowest CTC value among these combinations, and the SC4 showed the highest value (Table 4.1).

4.3.2 Synergistic Effect Evaluation

SC4 showed the highest synergistic effect, with LC_{25}, LC_{50}, and LC_{90} values of 3.0×10^{-6}, 6.0×10^{-6}, and 2.8×10^{-5}, respectively. Following this, SC3 exhibited a synergistic impact with 1.6×10^{-5} (LC_{25}), 4.5×10^{-5} (LC_{50}), and 3.1×10^{-4} (LC_{90}). SC2 displayed LC_{25}, LC_{50}, and LC_{90} values of 6.0×10^{-4}, 1.0×10^{-3}, and 3.0×10^{-3}, respectively. Among the tested combinations, SC1 exhibited the lowest synergistic effect, with 1.5×10^{-3} (LC_{25}), 2.1×10^{-3} (LC_{50}), and 3.7×10^{-3} (LC_{90}) (Table 4.2).

SC1 showed a synergistic factor of 1.75, indicating moderate synergism against *Ae. aegypti*. Similarly, SC2 displayed a synergistic factor of 1.9. SC3 exhibited a synergistic factor 3.11, reflecting a more pronounced synergistic effect. SC4 displayed the highest synergistic factor of 8.33, suggesting a strong and significant synergistic interaction between these two components. These findings indicated the potential of these synergistic combinations for the efficient control of *Ae. aegypti* populations (Table 4.3).

4.3.3 Quantitative Enzyme Assays

In the study of detoxifying enzyme activity in *Ae. aegypti* larvae exposed to SC4 and CB1, specific enzymatic activities and Total protein concentrations were observed over time intervals of 24 hours, 48 hours, and 72 hours starting with a non-insecticide treated T0. The selected synergistic combination represented a 10-fold diluted concentration of the LC_{50} previously determined during susceptibility testing, and the obtained results were compared with those of the non-treated T0. A mixed-design analysis of variance (ANOVA) was applied to examine the effects of the time period and compounds SC4 and CB1 on total protein and the detoxifying enzymes of *Ae. aegypti* larvae. There were no extreme outliers, as calculated by the box plot method. All the data were normally distributed, as evaluated by Shapiro-Wilk's test of normality ($p > 0.05$). There was homogeneity of variances for all the data ($p > 0.05$) as evaluated by Levene's test of homogeneity of variances. A Bonferroni adjustment was applied for the simple main effect of compounds at each time period, simple pairwise comparisons of compounds at each time period, simple main effect of time periods with different compounds, and simple pairwise comparisons of time periods for each compound.

4.3.3.1 Quantitative Enzyme Assay at Different Time Period

4.3.3.1.1a Specific Activity of GST in SC4 Treated Test Groups

GST activity of *Ae. aegypti* larvae was measured in µmoles/min/mg protein and demonstrated variations over time compared to the baseline T0 activity. The initial reading in T0 for both SC4 and CB1 was $1.36\times10^{-1}\pm3.80\times10^{-3}$ µmoles/min/mg protein. For the SC4 group, there was a notable decrease, with activity recorded at $4.91\times10^{-2}\pm3.40\times10^{-3}$ µmoles/min/mg protein at 24 hours, further dropping to $3.95\times10^{-2}\pm 5.30\times10^{-3}$ µmoles/min/mg protein at 48 hours and reaching its lowest level at $3.09 \times 10^{-2}\pm6.40 \times10^{-3}$ µmoles/min/mg protein at 72 hours (Table 4.5).

4.3.3.1.1b Specific Activity of GST in CB1 Treated Test Groups

Initially at T0, the enzyme's activity measured $1.36\times10^{-1}\pm3.80\times10^{-3}$µmoles/min/mg protein. At the 24-hour, a decline in activity was observed with $1.16\times10^{-1}\pm1.45\times10^{-2}$ µmoles/min/mg protein. This decreasing trend persisted at 48 hours, with GST activity further decreasing to $7.36\times10^{-2}\pm1.69\times10^{-2}$µmoles/min/mg protein. At 72-hours the enzyme activity reached at $5.20\times10^{-2}\pm1.93\times10^{-2}$ µmoles/min/mg protein (Table 4.6).

4.3.3.1.1c Statistical Analysis of Specific Activity of GST in SC4 and CB1 Treated Test Groups

The Mixed- Design ANOVA of GST analysis showed significant difference between compounds $F (1, 4) = 6.71\times10^{1}$, $p < 0.05$) and Time Periods ($F (3, 12) = 7.60\times10^{1}$, $p < 0.05$) and a significant interaction between compounds and time period ($F (3, 12) = 8.62\times10^{0}$, $p < 0.05$) (Table 4.7). The simple main effect of compounds (CB1 vs SC4) was significant at T24 ($p.adj = 4.00\times10^{-3}$) but not significant at T48 ($p.adj = 1.16\times10^{-1}$) and T72 ($p = 5.88\times10^{-1}$) (Table 4.8). The simple pairwise of compounds (CB1 vs SC4) was significant at T24 and T48 ($p.adj< 0.05$) (Table 4.9). The simple main effect of Time periods was significant for SC4 and CB1 ($p.adj < 0.05$) (Table 4.10). Simple pairwise comparisons of GST between time periods were significantly different between T0 and T24, T0 and T48, and T0 and T72 and T24 and T72 for SC4 and CB1 ($p < 0.05$) except between T0 and T24 of CB1 ($P>0.05$) (Table 4.11).

4.3.3.1.2a Specific Activity of AChE in SC4 Treated Test Groups

AChE activity quantified in nMoles/min/mg protein displayed fluctuations at different time intervals compared to the initial activity. At T0, AChE specific enzymatic activity was $1.80\times10^{-2}\pm8.1\times10^{-3}$nmoles/min/mg protein. However, with time, the activity reduced to $5.20\times10^{-3}\pm3.81\times10^{-2}$ nmoles/min/mg protein. Then 24 hours later, further declining to $4.40\times10^{-3}\pm7.40\times10^{-3}$ nmoles/min/mg protein at 48 hours and reaching $2.70\times10^{-3}\pm1.79\times10^{-2}$ nmoles/min/mg protein at 72 hours (Table 4.5).

4.3.3.1.2b Specific Activity of AChE in CB1 Treated Test Groups

Specific enzymatic activity of AChE exhibited varying levels across different time intervals. At T0, the specific enzyme activity was observed at $1.80\times10^{-2}\pm 8.10\times10^{-3}$ nmoles/min/mg protein. The values were then decreased to $1.14\times10^{-2}\pm2.83\times10^{-2}$ nmoles/min/mg protein at 24 hours, $9.30\times10^{-3}\pm3.51\times10^{-2}$ nmoles/min/mg protein at 48 hours, and $8.9\times10^{-3}\pm1.20\times10^{-2}$ nmoles/min/mg protein at 72 hours (Table 4.6).

4.3.3.1.2c Statistical Analysis of Specific Activity of AChE in SC4 and CB1 Treated Test Groups

In the mixed -design ANOVA for AChE, the difference between compounds did not show significance ($F (1, 4) = 6.79\times10^{0}$, $p = 6.00\times10^{-2}$), while the time periods showed a significant difference ($F (3, 12) = 1.95\times10^{1}$, $p < 0.05$). Also, a statistically non-significant interaction between Compounds and time period was observed ($F (3, 12) = 1.36\times10^{0}$, $p =3.01\times10^{-1}$) (Table 4.7). The simple main effect of compounds (CB1 vs SC4) was found significant for T72 ($p.adj = 3.40\times10^{-1}$) in the case of AChE and found to be not significant at T24 ($p.adj = 2.80\times10^{-2}$) and T48 ($p.adj = 3.12\times10^{-1}$) (Table 4.8). The simple pairwise of compounds (CB1 vs SC4) was significant at T72 ($p.adj< 0.05$) (Table 4.9). The simple main effect of Time periods was significant for SC4 ($p.adj < 0.05$) and not for CB1 ($p.adj = 8.0\times10^{-1}$) (Table 4.10). Simple pairwise comparisons of AChE between time periods were significantly different between T0 and T24, T0 and T48, and T0 and T 72 for SC4 ($p < 0.05$) except between CB1 T0 and T24, T0 and T48 and T0 and T72 ($P>0.05$) (Table 4.11).

4.3.3.1.3a Specific Activity of α-esterase and β-esterase in SC4 Treated Test Groups

The enzyme activity of α-esterase and β-esterase, quantified in nmoles/min/mg protein, exhibited fluctuations during the experiment. Initially, α-esterase activity at T0 was $1.21\times10^{-1}\pm1.30\times10^{-3}$ nmoles/min/mg protein, while β-esterase activity was $1.65\times10^{-1}\pm4.2\times10^{-3}$ nmoles/min/mg protein. Subsequently, both activities were reduced. At 24 hours, α-esterase activity remained relatively stable at 1.17×10^{-1} $\pm2.40\times10^{-3}$ nmoles/min/mg protein, while β-esterase activity slightly decreased to $1.30\times10^{-1}\pm1.70\times10^{-3}$ nmoles/min/mg protein. At 48 hours, both α-esterase and β-esterase activities were reduced with values of $9.89\times10^{-2}\pm1.60\times10^{-3}$ nmoles/min/mg protein and $1.25\times10^{-1}\pm7.00\times10^{-3}$ nmoles/min/mg protein, respectively. By 72 hours, α-esterase activity further decreased to $6.88\times10^{-2}\pm1.60\times10^{-3}$ nmoles/min/mg protein, while β-esterase activity measured $1.14\times10^{-1}\pm1.30\times10^{-3}$ nmoles/min/mg protein (Table 4.5).

4.3.3.1.3b Specific Activity of α-esterase and β-esterase in CB1 Treated Test Groups

The specific enzymatic α-esterase activity was measured as $1.21\times10^{-1}\pm1.30\times10^{-3}$ nmoles/min/mg protein at T0, while β-esterase activity was recorded as 1.65×10^{-1} $\pm4.20\times10^{-3}$ nmoles/min/mg protein. At 24 hours, α-esterase activity slightly decreased to $1.18\times10^{-1}\pm1.2\times10^{-3}$ nmoles/min/mg protein, and β-esterase activity decreased to $1.42\times10^{-1}\pm1.4\times10^{-3}$ nmoles/min/mg protein. At 48 and 72 hours, α-esterase activity decreased to $1.01\times10^{-1}\pm7.10\times10^{-3}$ nmoles/min/mg protein and $8.04\times10^{-2}\pm2.30\times10^{-3}$ nmoles/min/mg protein, respectively, and β-esterase activity decreased to$1.31\times10^{-1}\pm1.65\times10^{-2}$ nmoles/min/mg protein and $1.22\times10^{-1}\pm4.20\times10^{-3}$ nmoles/min/mg protein (Table 4.6).

4.3.3.1.3c Statistical Analysis of Specific Activity of α-esterase and β-esterase in SC4 and CB1 Treated Test Groups

In the mixed- design ANOVA of the Esterases, there was a significant difference between compounds with α ($F(1, 4) = 9.98\times10^{0}$, $p < 0.05$) and β esterases ($F(1, 4) = 1.07\times10^{1}$, $p < 0.05$). Time period also showed significant difference for both esterases ($F(3, 12) = 2.93\times10^{2}$, $p < 0.05$) and β ($F(3, 12) = 4.34\times10^{1}$, $p < 0.05$). But the interaction between Compounds and Time period was not significant for both α ($F(3, 12) = 4.56\times10^{0}$, $p = 8.40\times10^{-2}$), and β esterases ($F(3, 12) = 6.60\times10^{-1}$,

p =5.92×10^{-1}) (Table 4.7). The simple main effect of compounds (CB1 vs SC4) was found significant only for T72(p.adj =1.20×10^{-2}) in the case of α- esterase and T24 (p.adj =2.76×10^{-3}) in the case of β esterase (Table 4.8). The simple pairwise of compounds (CB1 vs SC4) was significant at T72 (p.adj< 0.05) for α esterase and T24 and T72 (p.adj< 0.05) for β esterase (Table 4.9). For α- esterase, the simple main effect of the Time period was significant for SC4 and CB1 ($p < 0.05$) and for β-esterase, it was significant for SC4 and CB1 ($p < 0.05$) (Table 4.10).Simple pairwise comparisons of α- esterase between time periods were significantly different between T0 andT48, and T0 and T 72 for SC4 and CB1 ($p < 0.05$) except between T0 and T24 of SC4 and T0 and T24 of CB1 (P>0.05) and of β-esterase, it was significantly different between T0 and T24, T0 and T48, and T0 and T 72 for SC4 and CB1 ($p < 0.05$) (Table 4.11).

4.3.3.1.4a Specific Activity of Monooxygenase (Cytochrome p450) in SC4 Treated Test Groups

Monooxygenase activity, expressed in nmoles/min/mg protein, exhibited variations over time. An initial Monooxygenase activity of $1.24\times10^{-1}\pm1.6\times10^{-3}$ nmoles/min/mg protein was observed at T0, which decreased to $1.01\times10^{-1}\pm7.00\times10^{-4}$ nmoles/min/mg protein at 24 hours, further dropping to $8.56\times10^{-2}\pm2.70\times10^{-3}$ nmoles/min/mg protein at 48 hours, and $7.01\times10^{-2}\pm2.0\times10^{-3}$ nmoles/min/mg protein at 72 hours (Table 4.5).

4.3.3.1.4b Specific Activity of Monooxygenase in CB1 Treated Test Groups

At T0, Cytochrome P450 activity was initially noted at $1.24\times10^{-1}\pm1.6\times10^{-3}$ nmoles/min/mg protein. At 24 hours, the activity decreased to $1.21\times10^{-1}\pm1.40\times10^{-3}$ nmoles/min/mg protein, followed by a further decline to $1.08\times10^{-1}\pm1.60\times10^{-3}$ nmoles/min/mg protein at 48 hours. At 72 hours, the activity was observed to be $7.60\times10^{-2}\pm2.60\times10^{-3}$ nmoles/min/mg protein (Table 4.6).

4.3.3.1.4c Statistical Analysis of Specific Activity of Monooxygenase in SC4 and CB1 Treated Test Groups

For the enzyme monooxygenase, the difference between compounds ($F (1, 4) = 1.37\times10^{2}$, $p < 0.05$) and Time period ($F (3, 12) = 1.12\times10^{3}$, $p < 0.05$) showed significance in mixed- design ANOVA. The interaction between Compounds and Time period was also observed to be significant ($F (3, 12) = 6.87\times10^{1}$, $p < 0.05$) (Table 4.7). The simple main effect of compounds (CB1 vs SC4) was found

significant at T24 (p.adj =9.96×10^{-5}) and T48 (p.adj =9.12×10^{-4}), but not significant at T72 (p.adj=1.44×10^{-1}) (Table 4.8). The simple pairwise of compounds (CB1 vs SC4) was significant at T24, T48 and T72 (p.adj< 0.05) (Table 4.9). The simple main effect of Time periods was significant for SC4 and CB1 ($p < 0.05$) (Table 4.10). Simple pairwise comparisons of Monooxygenase between Time periods were significantly different between T0 and T24, T0 and T48, and T0 and T 72 for SC4 and CB1 ($p < 0.05$) except between T0 and T24 of CB1 ($P>0.05$) (Table 4.11).

4.3.3.1.5a Total protein concentration in SC4 Treated Test Groups

Total protein concentration, measured in mg/ml, when observed, decreased notably over time compared to the initial concentration. A total protein concentration value of $1.39\times10^{-1}\pm8.00\times10^{-4}$ mg/ml was observed at T0, which decreased to $1.18\times10^{-1}\pm1.60\times10^{-3}$ mg/ml at 24 hours, further reducing to $9.95\times10^{-2}\pm1.2\times10^{-3}$ mg/ml at 48 hours and $8.24\times10^{-2}\pm1.7\times10^{-3}$ mg/ml at 72 hours (Table 4.5).

4.3.3.1.5b Total protein concentration in CB1 Treated Test Groups

Total protein concentration, measured in milligrams per millilitre (mg/ml), displayed significant fluctuations over the experimental timeline. At T0, the total protein concentration was determined to be $1.39\times10^{-1}\pm8.00\times10^{-4}$ mg/ml. At 24 hours, the concentration decreased to $1.20\times10^{-1}\pm3.00\times10^{-4}$ mg/ml, followed by a further reduction to $1.05\times10^{-1}\pm6.00\times10^{-4}$ mg/ml at 48 hours and $9.35\times10^{-2}\pm1.60\times10^{-3}$ mg/ml at 72 hours (Table 4.6).

4.3.3.1.5c Statistical Analysis of Total protein concentration in SC4 and CB1 Treated Test Groups

Mixed-design ANOVA of Total protein concentration showed a significant difference between compounds ($F (1, 4) = 1.63\times10^{1}$, $p < 0.05$) and Time Periods ($F (3, 12) = 1.98\times10^{3}$, $p < 0.05$). Also, there was a significant statistical interaction between Compounds and the Time period in evaluating the total protein levels, ($F (3, 12) = 2.44\times10^{1}$, $p < 0.05$) (Table 4.7). The simple main effect of compounds (CB1 vs SC4) was significant at T48 (p.adj = 8.00×10^{-3}) and T72 (p.adj = 4.00×10^{-3}) but not at T24 (p.adj = 2.88×10^{-1}) (Table 4.8). The simple pairwise of compounds (CB1 vs SC4) was significant at T24, T48 and T72 (p.adj< 0.05) (Table 4.9). The simple main effect of Time periods was significant for SC4 and CB1 (p.adj < 0.05) (Table 4.10). Simple pairwise comparisons of total proteins between time periods

were significantly different between T0 and T24, T0 andT48, and T0 and T 72 for SC4 and CB1 ($p < 0.05$) (Table 4.11).

4.3.3.2 Quantitative Enzyme Assay of Susceptible F0 and SC4 selected F5 strain.

The specific enzymatic activity of essential enzymes and total protein concentration were compared between the susceptible F0 and the SC4 selected F5 strain of mosquitoes. In the F0 strain, GST activity was measured at $1.36\times10^{-1}\pm3.80\times10^{-3}$ µmoles/min/mg protein, whereas the F5 Strain showed a reduced enzymatic activity of $4.28\times10^{-2}\pm8.7\times10^{-3}$ µmoles/min/mg protein. Similarly, AChE activity in the F0 strain was $1.80\times10^{-2}\pm8.1\times10^{-3}$ nmoles/min/mg protein, but in the F5 Strain, it decreased to $3.70\times10^{-3}\pm2.44\times10^{-2}$ nmoles/min/mg protein. α-esterase activity was $1.21\times10^{-1}\pm1.30\times10^{-3}$ nmoles/min/mg protein in the F0 strain and the enzymatic recduced to $8.97\times10^{-2}\pm1.30\times10^{-3}$ nmoles/min/mg protein in the F5 strain. For β-esterase, the F0 strain exhibited an activity of $1.65\times10^{-1}\pm 4.2\times10^{-3}$ nmoles/min/mg protein, whereas the F5 strain had $1.18\times10^{-1}\pm4.2\times10^{-3}$ nmoles/min/mg protein. Monooxygenase activity in the F0 strain was $1.24\times10^{-1}\pm1.60\times10^{-3}$ nmoles/min/mg protein, which reduced to $7.87\times10^{-2}\pm2.7\times10^{-3}$ nmoles/min/mg protein in the F5 strain. Additionally, total protein concentration was $1.39\times10^{-1}\pm8.00\times10^{-4}$ mg/ml in the susceptible F0 strain and $9.19\times10^{-2}\pm1.50\times10^{-3}$ mg/ml in the F5 Strain (Table 4.12).

Table 4.1: Co-toxicity coefficient of synergistic combinations SC1, SC2, SC3 and SC4 against *Ae. aegypti* larvae

Test substance	Concentration in ppm	Observed percent mortality	Expected percent mortality	Co-toxicity coefficient
SC1(Malathion + CB1)	LC_{25}+ LC_{25} (1:1)	62	50	24
SC2(Temephos+ CB1)	LC_{25}+ LC_{25} (1:1)	67.32	50	34.64
SC3(Cypermethrin+ CB1)	LC_{25}+ LC_{25} (1:1)	70.68	50	41.36
SC4 (Lambda-cyhalothrin+ CB1)	LC_{25}+ LC_{25} (1:1)	80	50	60

Table 4.2: Synergistic effect of synergistic combinations SC1, SC2, SC3 and SC4 against *Ae. aegypti* larvae

TEST MATERIAL	LC_{25} (LCL-UCL)	LC_{50} (LCL-UCL)	LC_{90} (LCL-UCL)	p-value
SC1(Malathion + CB1)	1.5×10^{-3} (1.3×10^{-3}-1.7×10^{-3})	2.1×10^{-3} (1.8×10^{-3}-2.3×10^{-3})	3.7×10^{-3} (3.2×10^{-3}-4.8×10^{-3})	<0.01
SC2(Temephos+ CB1)	6×10^{-4} (3×10^{-4}-7.0×10^{-4})	1.0×10^{-3} (8×10^{-4}-1.2×10^{-3})	3.0×10^{-3} (2.3×10^{-3}-4.7×10^{-3})	<0.01
SC3(Cypermethrin+ CB1)	1.6×10^{-5} (7.0×10^{-6}-2.6×10^{-5})	4.5×10^{-5} (2.8×10^{-5}-6.2×10^{-5})	3.1×10^{-4} (1.9×10^{-4}-7.8×10^{-4})	<0.01
SC4 (Lambda-cyhalothrin+ CB1)	3.0×10^{-6} (2×10^{-6}-4×10^{-6})	6.0×10^{-6} (5.0×10^{-6}-8.0×10^{-6})	2.8×10^{-5} (1.9×10^{-5}-5.5×10^{-5})	<0.01

Statistical significance $p<0.05$

Table 4.3: Synergistic factor of tested synergistic combinations SC1, SC2, SC3 and SC4 against *Ae. aegypti* larvae

Test Material		Synergistic Factor
SC1	Malathion + Plant Isolate	1.75
SC2	Temephos+ Plant Isolate	1.9
SC3	Cypermethrin+ Plant Isolate	3.11
SC4	Lambda Cyhalothrin+ Plant Isolate	8.33

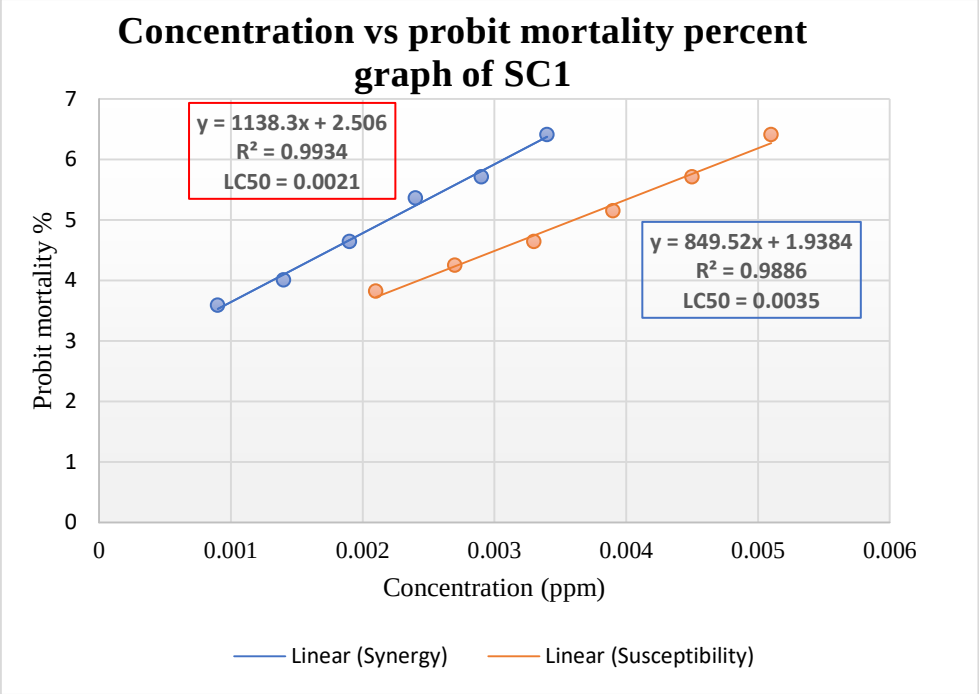

Figure 4.1 Comparison of larvicidal synergistic effect of SC1 and the larvicidal activity of Malathion alone against *Ae. aegypti* larvae

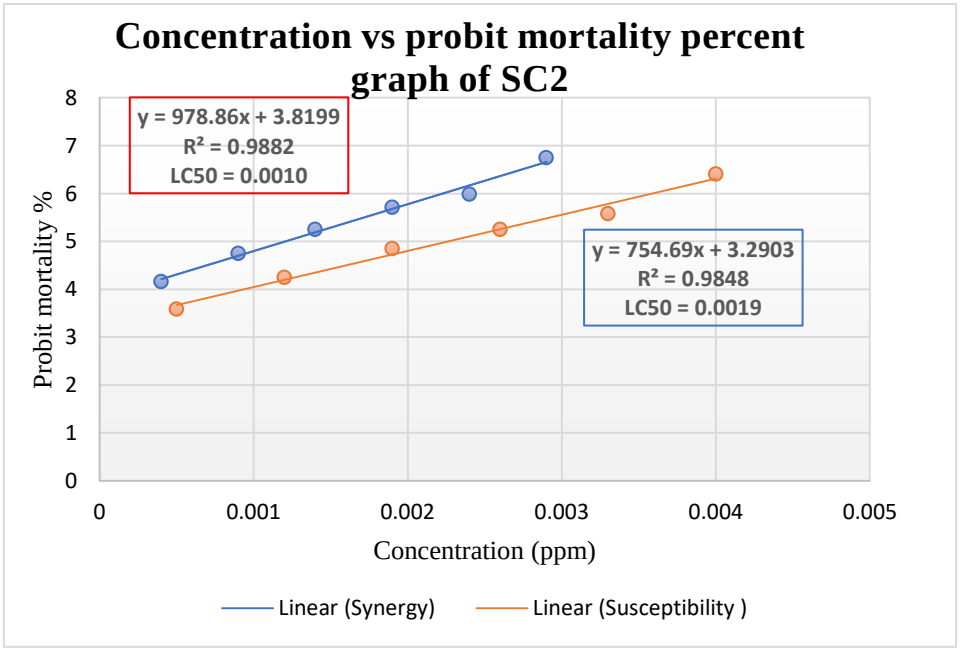

Figure 4.2 Comparison of larvicidal synergistic effect of SC2 and the larvicidal activity of Temephos alone against *Ae. aegypti* larvae

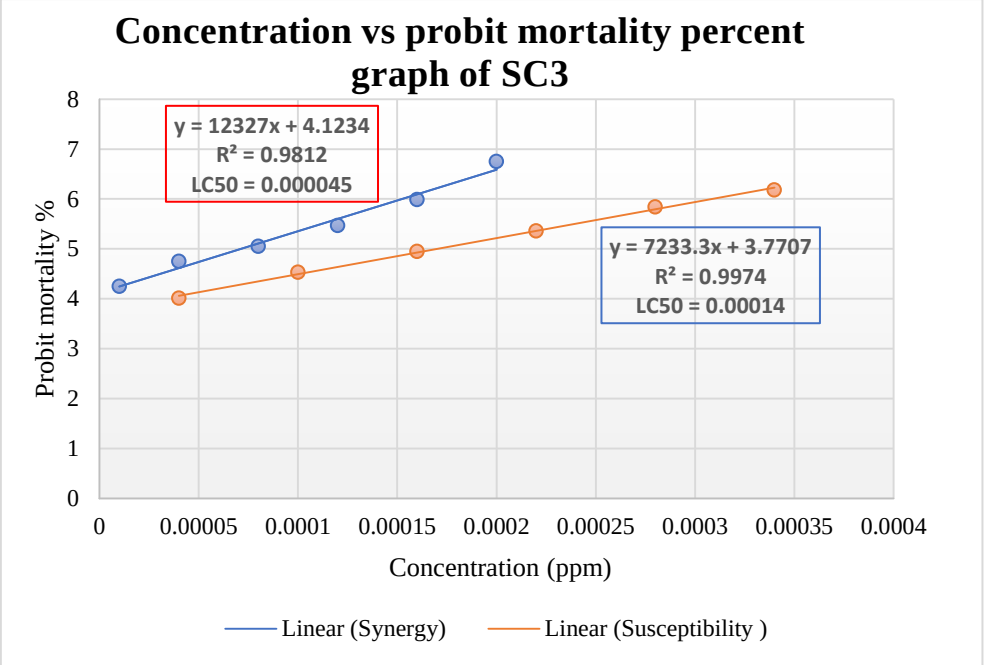

Figure 4.3 Comparison of larvicidal synergistic effect of SC3 and the larvicidal activity of Cypermethrin alone against *Ae. aegypti* larvae

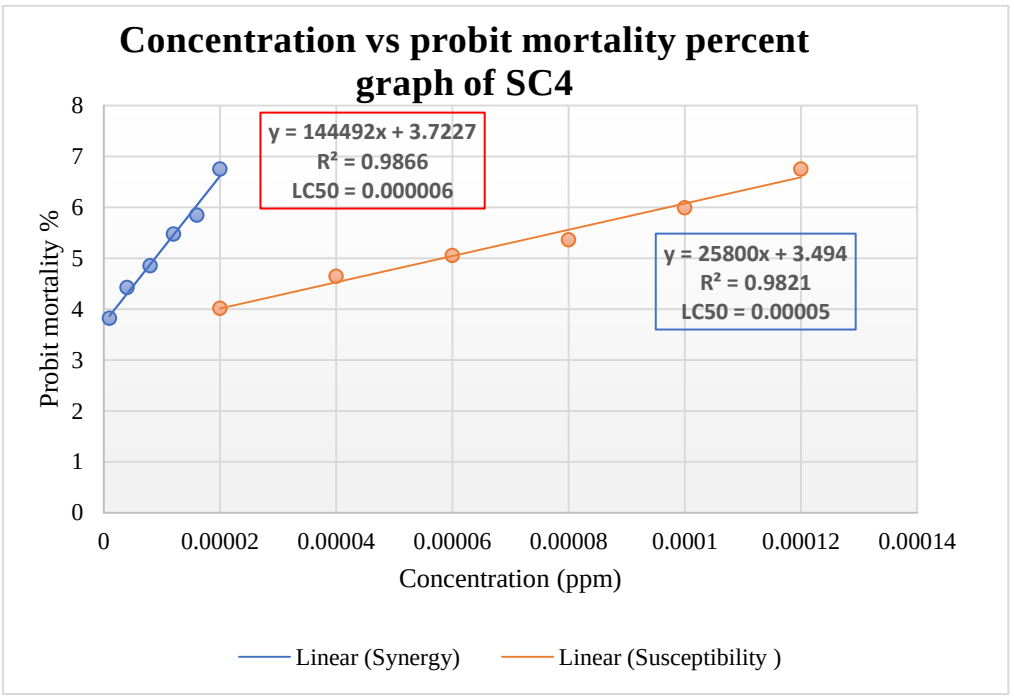

Figure 4.4 Comparison of larvicidal synergistic effect of SC4 and the larvicidal activity of Lambda- cyhalothrin alone against *Ae. aegypti* larvae

Table 4.4: Results of selection trials using SC4 on *Ae. aegypti* larvae

Generation	LC_{25} (LCL-UCL)	LC_{50} (LCL-UCL)	LC_{90} (LCL-UCL)	Heterogeneity (df)	P Value	RR value (LC_{50})
F0	3.00×10^{-6} (1.75×10^{-6}-4.16×10^{-6})	6.49×10^{-6} (4.78×10^{-6} - 8.42×10^{-6})	2.82×10^{-5} (1.93×10^{-5} - 5.38×10^{-5})	4	P< 0.05	-
F1	3.80×10^{-6} (2.44×10^{-6}-5.01×10^{-6})	7.32×10^{-6} (5.66×10^{-6} - 9.16×10^{-6})	2.55×10^{-5} (1.85×10^{-5} - 4.35×10^{-5})	4	P< 0.05	1.13
F2	4.18×10^{-6} (2.79×10^{-6} - 5.40×10^{-6})	7.64×10^{-6} (6.02×10^{-6} - 9.38×10^{-6})	2.40×10^{-5} (1.80×10^{-5} - 3.87×10^{-5})	4	P< 0.05	1.17
F3	4.65×10^{-6} (3.20×10^{-6}-5.89×10^{-6})	8.10×10^{-6} (6.50×10^{-6} - 9.79×10^{-6})	2.32×10^{-5} (1.79×10^{-5} - 3.59×10^{-5})	4	P< 0.05	1.24
F4	5.06×10^{-6} (3.57×10^{-6}-6.32×10^{-6})	8.53×10^{-6} (6.93×10^{-6}-1.02×10^{-5})	2.23×10^{-5} (1.79×10^{-5} - 3.44×10^{-5})	4	P< 0.05	1.31
F5	5.37×10^{-6} (3.88×10^{-6}-6.60×10^{-6})	8.66×10^{-6} (7.12×10^{-6}-1.021×10^{-5})	2.146×10^{-5} (1.717×10^{-5}-3.07×10^{-5})	4	P< 0.05	1.33

Statistical significance ($P < 0.05$); RR- Resistance Ratio

Table 4.5: Differential activity of detoxifying enzymes in susceptible and SC4 treated *Ae. aegypti* larvae (10 times dilution of LC_{50} value)

Samples	GST (µmoles/min/mg protein) (Mean± SD)	AChE (nmoles/min/mg protein) (Mean± SD)	Carboxyl esterase		Monooxygenase (Cytochrome p450) (nmoles/min/mg protein) (Mean±SD)	Total protein (mg/ml) (Mean± SD)
			α- esterase (nmoles/min/mg protein) (Mean±SD)	β- esterase (nmoles/min/mg protein) (Mean±SD)		
T0	1.36×10^{-1} $\pm3.8\times10^{-3}$	1.80×10^{-2} $\pm8.1\times10^{-3}$	1.21×10^{-1} $\pm1.30\times10^{-3}$	1.65×10^{-1} $\pm4.2\times10^{-3}$	1.24×10^{-1} $\pm1.60\times10^{-3}$	1.39×10^{-1} $\pm8.00\times10^{-4}$
T24	4.91×10^{-2} $\pm3.4\times10^{-3}$	5.2×10^{-3} $\pm3.81\times10^{-2}$	1.17×10^{-1} $\pm2.40\times10^{-3}$	1.30×10^{-1} $\pm1.70\times10^{-3}$	1.01×10^{-1} $\pm7.00\times10^{-4}$	1.18×10^{-1} $\pm1.60\times10^{-3}$
T48	3.95×10^{-2} $\pm5.3\times10^{-3}$	$4.40\times10^{-3}\pm$ 7.40×10^{-3}	9.89×10^{-2} $\pm1.60\times10^{-3}$	1.25×10^{-1} $\pm7.00\times10^{-3}$	8.56×10^{-2} $\pm2.70\times10^{-3}$	9.95×10^{-2} $\pm1.2\times10^{-3}$
T72	3.09×10^{-2} $\pm6.4\times10^{-3}$	$2.70\times10^{-3}\pm$ 1.79×10^{-2}	6.88×10^{-2} $\pm2.00\times10^{-3}$	1.14×10^{-1} $\pm1.30\times10^{-3}$	7.01×10^{-2} $\pm2.0\times10^{-3}$	8.24×10^{-2} $\pm1.7\times10^{-3}$

Table 4.6: Differential activity of detoxifying enzymes in susceptible and CB1 treated strains of *Ae. aegypti* larvae (10 times dilution of LC_{50} value)

Samples	GST (µmoles/min/mg protein) (Mean±SD)	AChE (nmoles/min/mg protein) (Mean±SD)	Carboxyl esterase		Monooxygenase (Cytochrome p450) (nmoles/min/mg protein) (Mean±SD)	Total protein (mg/ml) (Mean± SD)
			α- esterase (nmoles/min/mg protein) (Mean± SD)	β- esterase (nmoles/min/mg protein) (Mean± SD)		
T0	1.36×10^{-1} $\pm3.8\times10^{-3}$	1.80×10^{-2} $\pm 8.10\times10^{-3}$	1.21×10^{-1} $\pm1.30\times10^{-3}$	1.65×10^{-1} $\pm4.20\times10^{-3}$	1.24×10^{-1} $\pm1.6\times10^{-3}$	1.39×10^{-1} $\pm8.00\times10^{-4}$
T24	1.16×10^{-1} $\pm1.45\times10^{-2}$	1.14×10^{-2} $\pm2.83\times10^{-2}$	1.18×10^{-1} $\pm1.2\times10^{-3}$	1.42×10^{-1} $\pm1.4\times10^{-3}$	1.21×10^{-1} $\pm1.40\times10^{-3}$	1.20×10^{-1} $\pm3.00\times10^{-4}$
T48	7.36×10^{-2} $\pm1.69\times10^{-2}$	9.30×10^{-3} $\pm3.51\times10^{-2}$	1.01×10^{-1} $\pm7.10\times10^{-3}$	1.31×10^{-1} $\pm1.65\times10^{-2}$	$1.08\times10^{-1}\pm$ 1.60×10^{-3}	1.05×10^{-1} $\pm6.00\times10^{-4}$
T72	5.20×10^{-2} $\pm1.93\times10^{-2}$	8.9×10^{-3} $\pm1.20\times10^{-2}$	8.04×10^{-2} $\pm2.30\times10^{-3}$	1.22×10^{-1} $\pm4.20\times10^{-3}$	7.60×10^{-2} $\pm2.60\times10^{-3}$	9.35×10^{-2} $\pm1.60\times10^{-3}$

Table 4.7: Results of Mixed-Design analysis (ANOVA) of Total protein, GST, AChE, α- esterase, β- esterase and Monooxygenase (Cytochrome p450)

Total protein			
Effect	DFn	F value	p-value
Compound	1	1.63×10^{1}	4.19×10^{-4}
Timeperiod	3	1.98×10^{3}	1.95×10^{-16}
Compound: Time.period	3	2.44×10^{1}	2.13×10^{-5}
GST			
Compound	1	6.71×10^{1}	1.00×10^{-3}
Time period	3	7.60×10^{1}	4.48×10^{-8}
Compound: Time.period	3	8.62×10^{0}	3.00×10^{-3}
AChE			
Compound	1	6.79×10^{0}	6.00×10^{-2}
Time period	3	1.95×10^{1}	6.62×10^{-5}
Compound: Time.period	3	1.36×10^{0}	3.01×10^{-1}
α- esterase			
Compound	1	9.98×10^{0}	3.40×10^{-2}
Time period	3	2.93×10^{2}	1.14×10^{-5}
Compound: Time.period	3	4.56×10^{0}	8.40×10^{-2}
β- esterase			
Compound	1	1.07×10^{1}	3.10×10^{-2}
Time period	3	4.34×10^{1}	1.02×10^{-6}
Compound: Time.period	3	6.60×10^{-1}	5.92×10^{-1}
Monooxygenase (Cytochrome p450)			
Compound	1	1.37×10^{2}	3.03×10^{-4}
Time period	3	1.12×10^{3}	6.00×10^{-15}
Compound: Time.period	3	6.87×10^{1}	7.95×10^{-8}

Statistical significance p-value<0.05; Time Period: T0, T24, T48, T72; Compounds: SC4, CB1

Table 4.8: Post hoc test of simple main effect of compounds at each time period

Time Period	Effect	Df	F value	p-value	p.adj value
Total Protein					
T0	Compounds	1	0	1	1
T24	Compounds	1	5.90×10^{0}	7.20×10^{-2}	2.88×10^{-1}
T48	Compounds	1	5.53×10^{1}	2.00×10^{-3}	8.00×10^{-3}
T72	Compounds	1	6.91×10^{1}	1.00×10^{-3}	4.00×10^{-3}
GST					
T0	Compounds	1	0	1	1
T24	Compounds	1	6.02×10^{1}	1.00×10^{-3}	4.00×10^{-3}
T48	Compounds	1	1.11×10^{1}	2.90×10^{-2}	1.16×10^{-1}
T72	Compounds	1	3.22×10^{0}	1.47×10^{-1}	5.88×10^{-1}
AChE					
T0	Compounds	1	0	1	1
T24	Compounds	1	5.17×10^{0}	8.50×10^{-2}	$3.4\ 0\times 10^{-1}$
T48	Compounds	1	5.53×10^{0}	7.80×10^{-2}	3.12×10^{-1}
T72	Compounds	1	2.52×10^{1}	7.00×10^{-3}	2.80×10^{-2}
α- esterase					
T0	Compounds	1	0	1	1
T24	Compounds	1	8.22×10^{-2}	4.16×10^{-1}	1
T48	Compounds	1	3.34×10^{-1}	5.94×10^{-1}	1
T72	Compounds	1	4.53×10^{1}	3.00×10^{-3}	1.20×10^{-2}
β- esterase					
T0	Compounds	1	0	1	1
T24	Compounds	1	8.99×10^{1}	6.90×10^{-4}	2.76×10^{-3}
T48	Compounds	1	2.82×10^{-1}	6.23×10^{-1}	1
T72	Compounds	1	9.01×10^{0}	4.00×10^{-2}	1.6×10^{-1}
Monooxygenase (Cytochrome p450)					
T0	Compounds	1	0	1	
T24	Compounds	1	4.87×10^{2}	2.49×10^{-5}	9.96×10^{-5}
T48	Compounds	1	1.59×10^{2}	2.28×10^{-4}	9.12×10^{-4}
T72	Compounds	1	9.58×10^{0}	3.60×10^{-2}	1.44×10^{-1}

Statistical significance p-value<0.05; Time Period: T0, T24, T48, T72; Compounds: SC4, CB1

Table 4.9 Post hoc Test of simple pairwise comparisons of compounds at each time Period

Time period	Group 1	Group2	n1	n2	P.adj
Total Protein					
T0	SC4	CB1	3	3	1
T24	SC4	CB1	3	3	7.21×10^{-2}
T48	SC4	CB1	3	3	1.75×10^{-3}
T72	SC4	CB1	3	3	1.14×10^{-3}
GST					
T0	SC4	CB1	3	3	1
T24	SC4	CB1	3	3	1.49×10^{-3}
T48	SC4	CB1	3	3	2.91×10^{-2}
T72	SC4	CB1	3	3	1.47×10^{-1}
AChE					
T0	SC4	CB1	3	3	1
T24	SC4	CB1	3	3	8.54×10^{-2}
T48	SC4	CB1	3	3	7.83×10^{-2}
T72	SC4	CB1	3	3	7.41×10^{-3}
α- esterase					
T0	SC4	CB1	3	3	1
T24	SC4	CB1	3	3	4.16×10^{-1}
T48	SC4	CB1	3	3	5.94×10^{-1}
T72	SC4	CB1	3	3	2.53×10^{-3}
β- esterase					
T0	SC4	CB1	3	3	1
T24	SC4	CB1	3	3	6.9×10^{-4}
T48	SC4	CB1	3	3	6.23×10^{-1}
T72	SC4	CB1	3	3	3.99×10^{-2}
Monooxygenase (Cytochrome p450)					
T0	SC4	CB1	3	3	1
T24	SC4	CB1	3	3	2.49×10^{-5}
T48	SC4	CB1	3	3	2.28×10^{-4}
T72	SC4	CB1	3	3	3.64×10^{-2}

Statistical significance p-value<0.05; n1, n2, number of test replicates of SC4 and CB1; p.adj=Bonferroni adjusted p-value; Time Period: T0, T24, T48, T72; Compounds: SC4, CB1

Table 4.10: Post hoc test of simple main effect of time periods with different compounds

Compound	Effect	DF	F value	p-value	p.adj
Total Protein					
SC4	Time period	3	940.0	1.58×10^{-10}	3.16×10^{-10}
CB1	Time period	3	1289.0	4.47×10^{-11}	8.94×10^{-11}
GST					
SC4	Time period	3	249.0	1.6×10^{-8}	3.20×10^{-8}
CB1	Time period	3	19.9	4.54×10^{-4}	9.08×10^{-4}
AChE					
SC4	Time period	3	13.5	2×10^{-3}	4.00×10^{-3}
CB1	Time period	3	4.48	4×10^{-2}	8×10^{-2}
α- esterase					
SC4	Time period	3	480.0	2.29×10^{-9}	4.58×10^{-9}
CB1	Time period	3	70.6	4.26×10^{-6}	8.52×10^{-6}
β-esterase					
SC4	Time period	3	80.2	2.60×10^{-6}	5.2×10^{-6}
CB1	Time period	3	13.5	2.00×10^{-3}	4×10^{-3}
Monooxygenase (Cytochrome p450)					
SC4	Time period	3	452.0	2.92×10^{-9}	5.84×10^{-9}
CB1	Time period	3	415.0	4.07×10^{-9}	8.14×10^{-9}

Statistical significance p-value<0.05; p.adj=Bonferroni adjusted p-value

Table 4.11: Post hoc test of simple pairwise comparisons of time periods for each compound

Compound	Group1	Group2	n1	n2	p-value	p.adj
Total Protein						
SC4	T0	T24	3	3	6.42×10^{-8}	3.85×10^{-7}
SC4	T0	T48	3	3	4.67×10^{-10}	2.80×10^{-9}
SC4	T24	T48	3	3	2.02×10^{-7}	1.21×10^{-6}
SC4	T0	T72	3	3	2.63×10^{-11}	1.58×10^{-10}
SC4	T24	T72	3	3	1.10×10^{-9}	6.57×10^{-9}
SC4	T48	T72	3	3	3.30×10^{-7}	1.98×10^{-6}
CB1	T0	T24	3	3	8.18×10^{-9}	4.91×10^{-8}
CB1	T0	T48	3	3	8.47×10^{-11}	5.08×10^{-10}
CB1	T24	T48	3	3	5.93×10^{-8}	3.56×10^{-7}
CB1	T0	T72	3	3	7.75×10^{-12}	4.65×10^{-11}
CB1	T24	T72	3	3	5.70×10^{-10}	3.42×10^{-9}
GST						
SC4	T0	T24	3	3	2.18×10^{-8}	1.31×10^{-7}
SC4	T0	T48	3	3	9.53×10^{-9}	5.72×10^{-8}
SC4	T24	T48	3	3	4.35×10^{-2}	2.61×10^{-1}
SC4	T0	T72	3	3	4.83×10^{-9}	2.90×10^{-8}
SC4	T24	T72	3	3	1.87×10^{-3}	1.12×10^{-2}
SC4	T48	T72	3	3	6.32×10^{-2}	3.79×10^{-1}
CB1	T0	T24	3	3	1.37×10^{-1}	8.21×10^{-1}
CB1	T0	T48	3	3	9.04×10^{-4}	5.42×10^{-3}
CB1	T24	T48	3	3	8.45×10^{-3}	5.07×10^{-2}
CB1	T0	T72	3	3	1.23×10^{-4}	7.4×10^{-4}
CB1	T24	T72	3	3	7.68×10^{-4}	4.61×10^{-3}
CB1	T48	T72	3	3	1.12×10^{-1}	6.7×10^{-1}
AChE						

SC4	T0	T24	3	3	1.47×10^{-3}	8.84×10^{-3}
SC4	T0	T48	3	3	1.03×10^{-3}	6.16×10^{-3}
SC4	T24	T48	3	3	7.83×10^{-1}	1
SC4	T0	T72	3	3	4.71×10^{-4}	2.82×10^{-3}
SC4	T24	T72	3	3	3.77×10^{-1}	1
SC4	T48	T72	3	3	5.34×10^{-1}	1
CB1	T0	T24	3	3	4.78×10^{-2}	2.87×10^{-1}
CB1	T0	T48	3	3	1.48×10^{-2}	8.9×10^{-2}
CB1	T24	T48	3	3	4.71×10^{-1}	1
CB1	T0	T72	3	3	1.20×10^{-2}	7.18×10^{-2}
CB1	T24	T72	3	3	3.95×10^{-1}	1
CB1	T48	T72	3	3	8.90×10^{-1}	1
α- esterase						
SC4	T0	T24	3	3	5.25×10^{-2}	3.15×10^{-1}
SC4	T0	T48	3	3	6.01×10^{-7}	3.61×10^{-6}
SC4	T24	T48	3	3	2.30×10^{-6}	1.38×10^{-5}
SC4	T0	T72	3	3	6.31×10^{-10}	3.79×10^{-10}
SC4	T24	T72	3	3	1.10×10^{-9}	6.58×10^{-9}
SC4	T48	T72	3	3	4.58×10^{-8}	2.75×10^{-7}
CB1	T0	T24	3	3	5.25×10^{-1}	1
CB1	T0	T48	3	3	2.81×10^{-4}	1.69×10^{-3}
CB1	T24	T48	3	3	6.00×10^{-4}	3.60×10^{-3}
CB1	T0	T72	3	3	1.31×10^{-6}	7.86×10^{-6}
CB1	T24	T72	3	3	1.97×10^{-6}	1.18×10^{-5}
CB1	T48	T72	3	3	1.57×10^{-4}	9.44×10^{-4}
β- esterase						
SC4	T0	T24	3	3	8.54×10^{-6}	5.12×10^{-5}
SC4	T0	T48	3	3	3.02×10^{-6}	1.81×10^{-5}
SC4	T24	T48	3	3	1.77×10^{-1}	1
SC4	T0	T72	3	3	4.56×10^{-7}	2.74×10^{-6}
SC4	T24	T72	3	3	1.57×10^{-3}	9.44×10^{-3}
SC4	T48	T72	3	3	1.25×10^{-2}	7.50×10^{-2}
CB1	T0	T24	3	3	1.39×10^{-2}	8.32×10^{-2}
CB1	T0	T48	3	3	1.44×10^{-3}	8.66×10^{-3}
CB1	T24	T48	3	3	1.45×10^{-1}	8.72×10^{-1}
CB1	T0	T72	3	3	3.22×10^{-4}	1.93×10^{-3}
CB1	T24	T72	3	3	2.09×10^{-2}	1.25×10^{-1}
CB1	T48	T72	3	3	2.45×10^{-1}	1
Monooxygenase (Cytochrome p450)						
SC4	T0	T24	3	3	3.85×10^{-7}	2.31×10^{-6}
SC4	T0	T48	3	3	6.68×10^{-9}	4.01×10^{-8}
SC4	T24	T48	3	3	7.58×10^{-6}	4.55×10^{-5}
SC4	T0	T72	3	3	4.64×10^{-10}	2.78×10^{-9}
SC4	T24	T72	3	3	3.76×10^{-8}	2.26×10^{-7}
SC4	T48	T72	3	3	8.17×10^{-6}	4.90×10^{-5}
CB1	T0	T24	3	3	7.52×10^{-2}	4.51×10^{-1}
CB1	T0	T48	3	3	5.53×10^{-6}	3.32×10^{-5}
CB1	T24	T48	3	3	2.71×10^{-5}	1.62×10^{-4}
CB1	T0	T72	3	3	1.11×10^{-10}	6.68×10^{-10}
CB1	T24	T72	3	3	1.89×10^{-10}	1.14×10^{-9}
CB1	T48	T72	3	3	2.83×10^{-9}	1.70×10^{-8}

Statistical significance p-value<0.05; n1, n2, number of test replicates of SC4 and CB1; p.adj=Bonferroni adjusted p-value

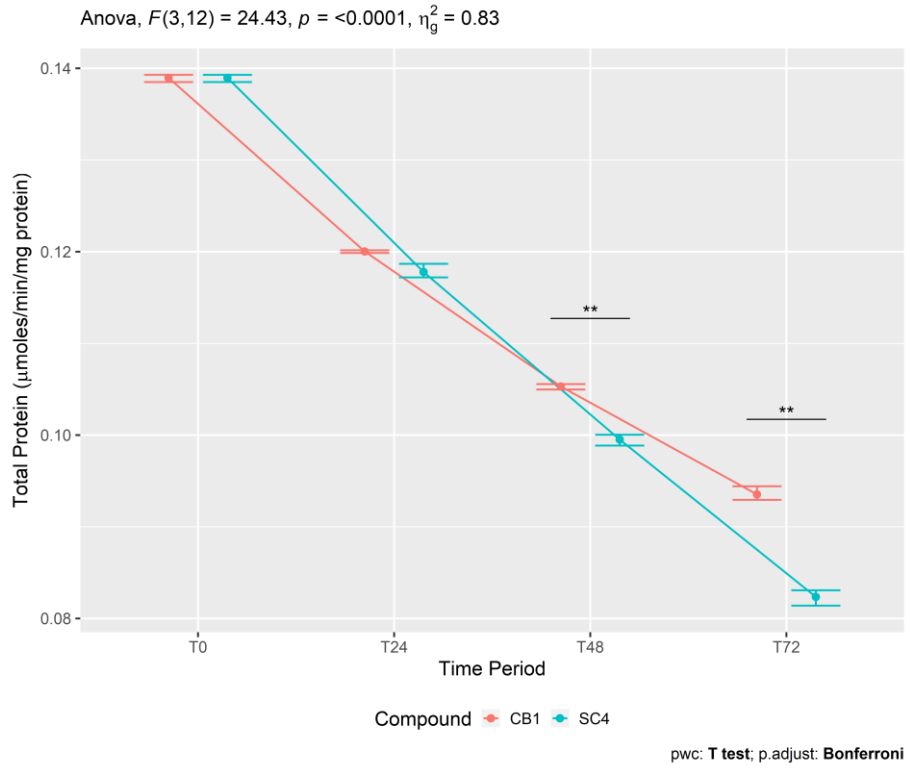

Figure 4.5 Comparison of the effect of CB1 and SC4 individually on the total protein concentration of susceptible (F0) *Ae. aegypti* strain at different time intervals T0, T24, T48 and T72 hours

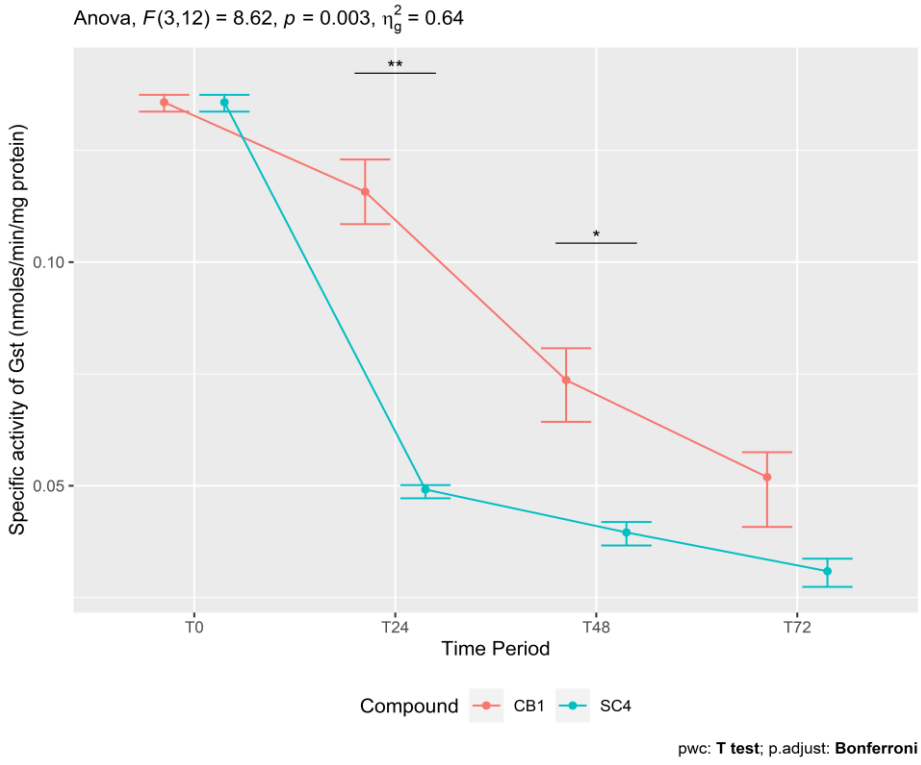

Figure 4.6 Comparison of the effect of CB1 and SC4 individually on specific enzymatic activity of GST of susceptible (F0) *Ae. aegypti* strain at different time intervals T0, T24, T48 and T72 hours

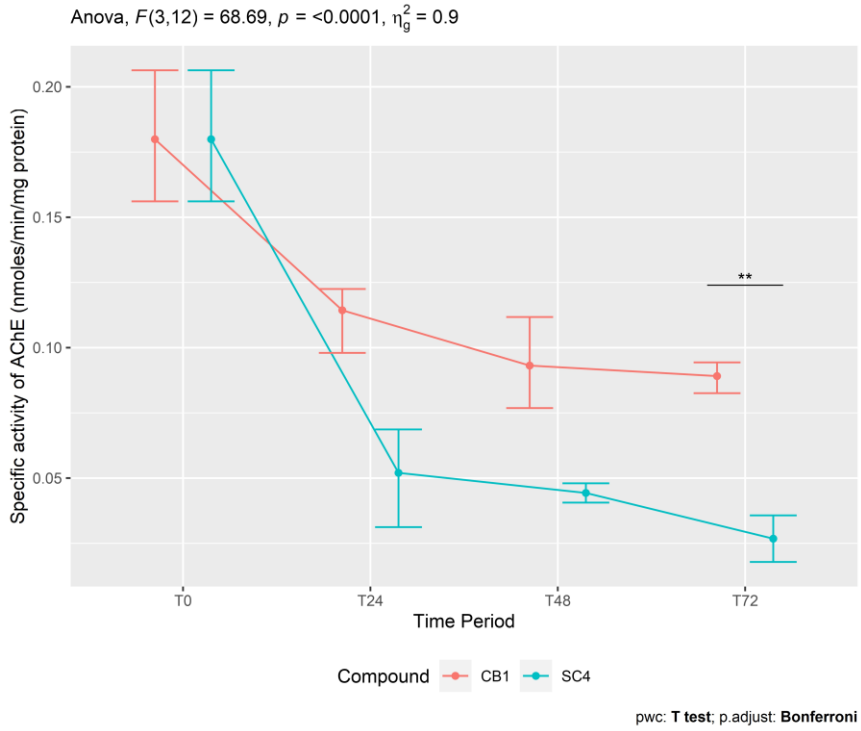

Figure 4.7 Comparison of the effect of CB1 and SC4 individually on specific enzymatic activity of AChE of susceptible (F0) *Ae. aegypti* strain at different time intervals T0, T24, T48 and T72 hours

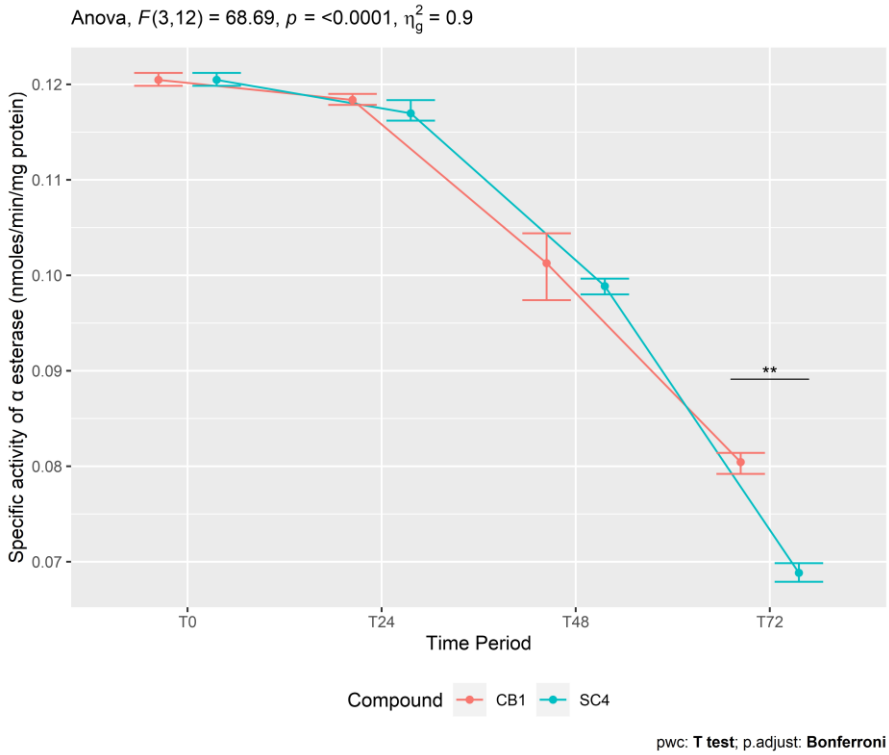

Figure 4.8 Comparison of the effect of CB1 and SC4 individually on specific enzymatic activity of α- esterase of susceptible (F0) *Ae. aegypti* strain at different time intervals T0, T24, T48 and T72 hours

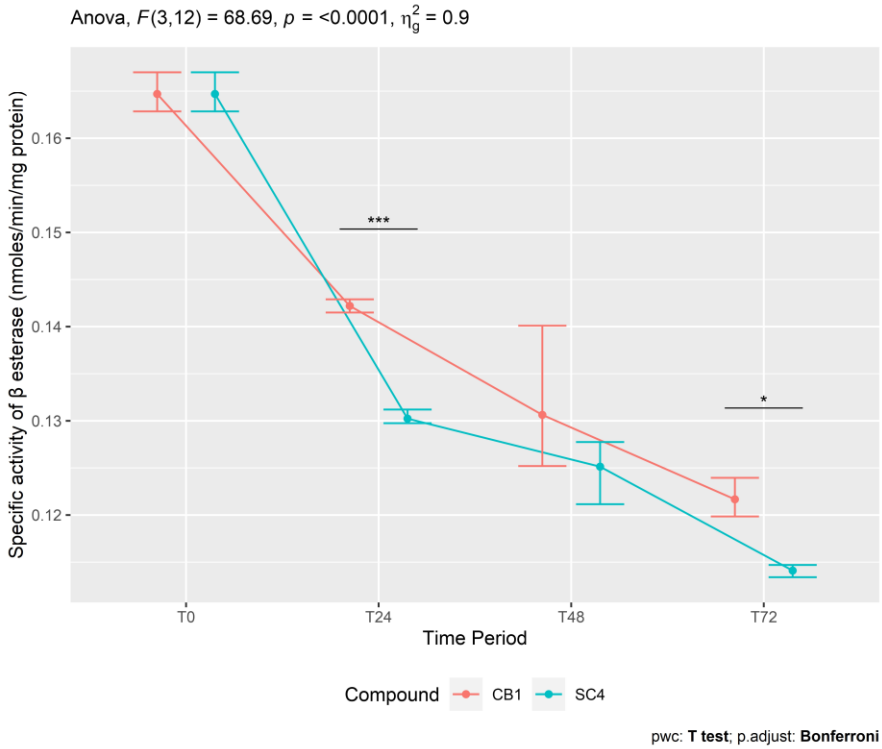

Figure 4.9 Comparison of the effect of CB1 and SC4 individually on specific enzymatic activity of β- esterase of susceptible (F0) *Ae. aegypti* strain at different time intervals T0, T24, T48 and T72 hours

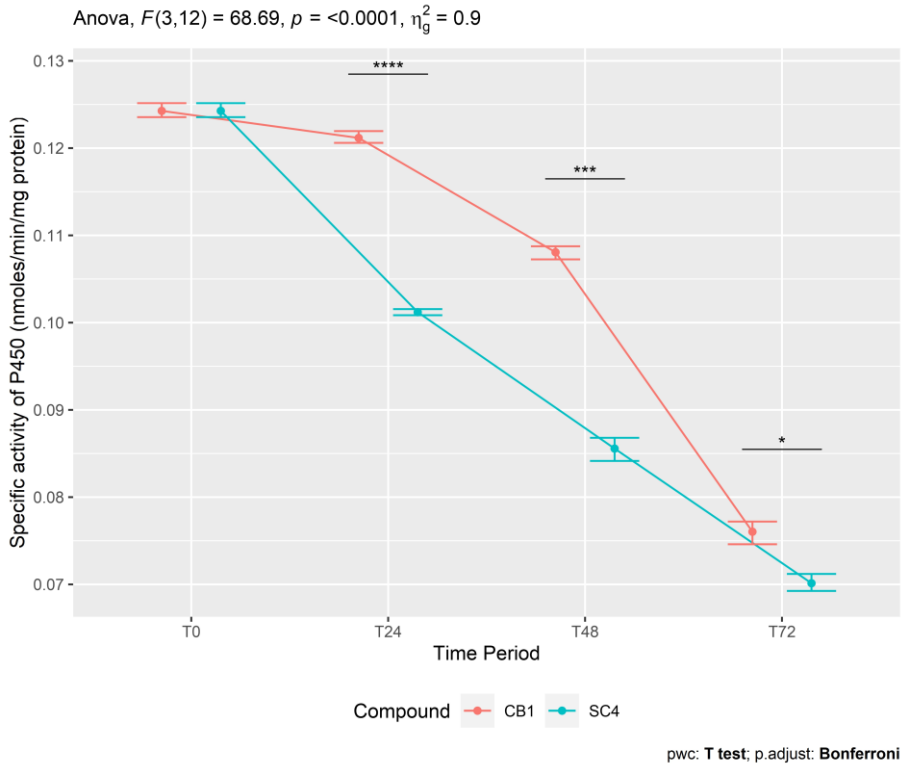

Figure 4.10 Comparison of the effect of CB1 and SC4 individually on specific enzymatic activity of monooxygenase of susceptible (F0) *Ae. aegypti* strain at different time intervals T0, T24, T48 and T72 hours

Table 4.12: Differential activity of detoxifying enzymes in F0 and SC4 selected F5 strains of *Ae. aegypti* larvae

Enzyme	Susceptible F0 strain (Mean±SD)	SC4 Selected F5strain (Mean±SD)	t- value	df	p-value
Glutathione-s transferase (µMoles/min/mg protein)	1.36×10^{-1} $\pm3.80\times10^{-3}$	4.28×10^{-2} $\pm8.7\times10^{-3}$	16.90	4	p <0.01
Acetylcholine esterase (nMoles/min/mg protein)	1.80×10^{-2} $\pm8.1\times10^{-3}$	3.70×10^{-3} $\pm2.44\times10^{-2}$	4.40	4	p <0.01
α- esterase (nMoles/min/mg protein)	1.21×10^{-1} $\pm1.30\times10^{-3}$	8.97×10^{-2} $\pm1.30\times10^{-3}$	28.98	4	p <0.01
β- esterase (nMoles/min/mg protein)	1.65×10^{-1} $\pm 4.2\times10^{-3}$	1.18×10^{-1} $\pm4.2\times10^{-3}$	13.59	4	p <0.01
Monooxygenase (Cytochrome p450) (nMoles/min/mg protein)	1.24×10^{-1} $\pm1.60\times10^{-3}$	7.87×10^{-2} $\pm2.7\times10^{-3}$	25.33	4	p <0.01
Total protein (mg/ml)	1.39×10^{-1} $\pm8.00\times10^{-4}$	9.19×10^{-2} $\pm1.50\times10^{-3}$	47.84	4	p <0.01

Statistical significance p-value<0.05

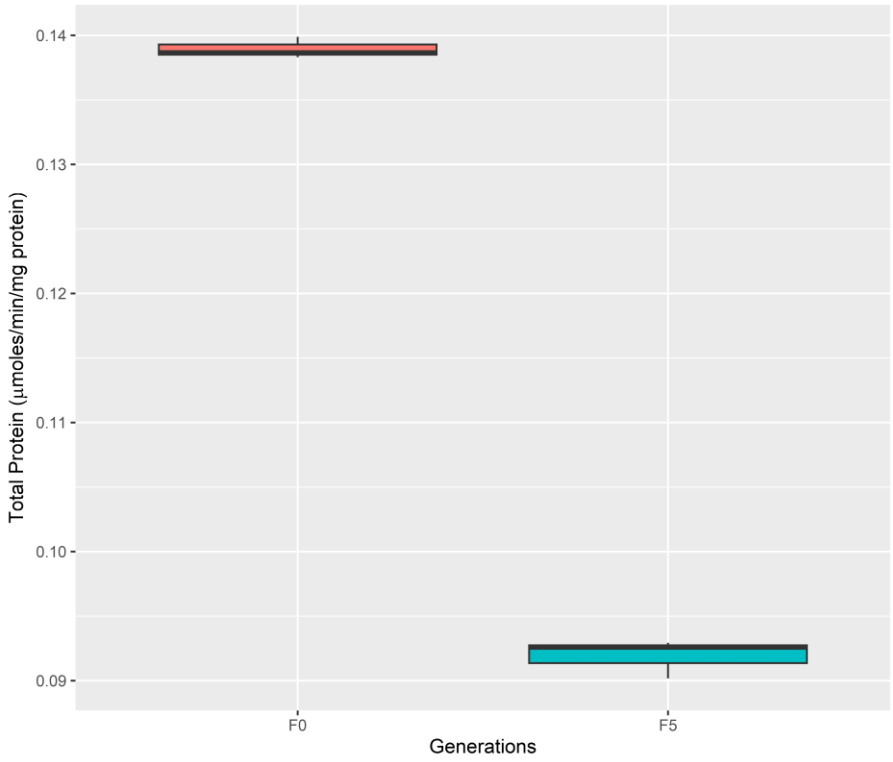

Figure 4.11 Comparison of total protein concentration in susceptible F0 and SC4 selected F5 *Ae. aegypti* strain

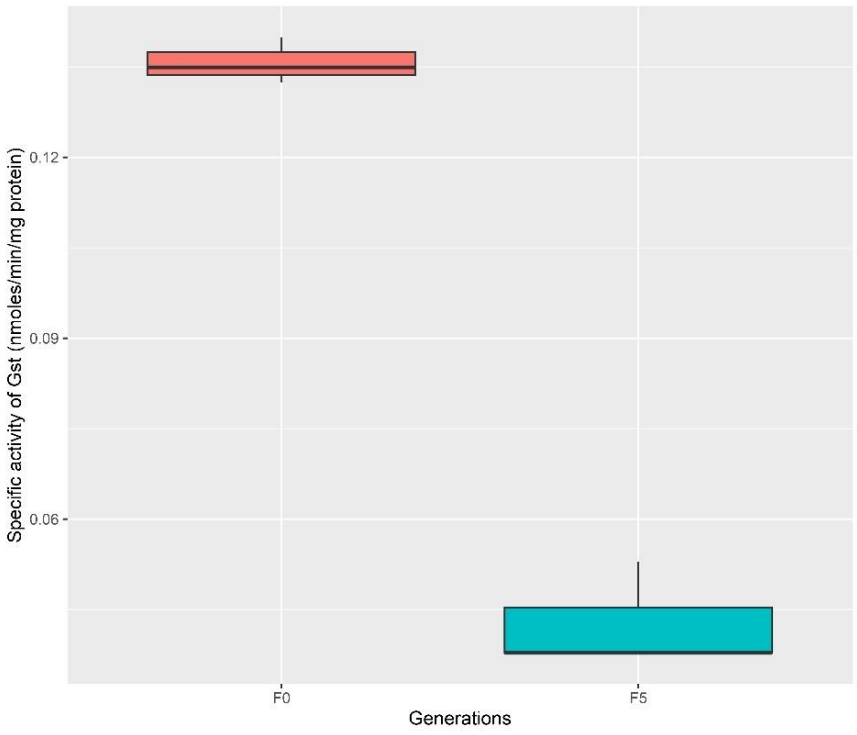

Figure 4.12 Comparison of Specific activity of GST in susceptible F0 and SC4 selected F5 *Ae. aegypti* strain

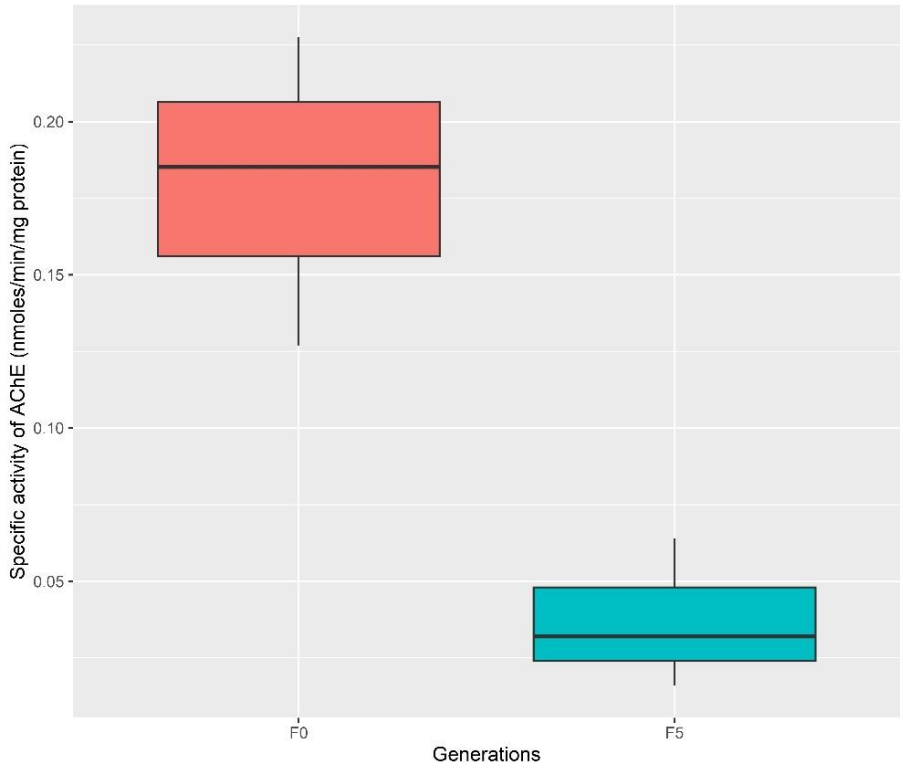

Figure 4.13 Comparison of Specific activity of AChE in susceptible F0 and SC4 selected F5 *Ae. aegypti* strain

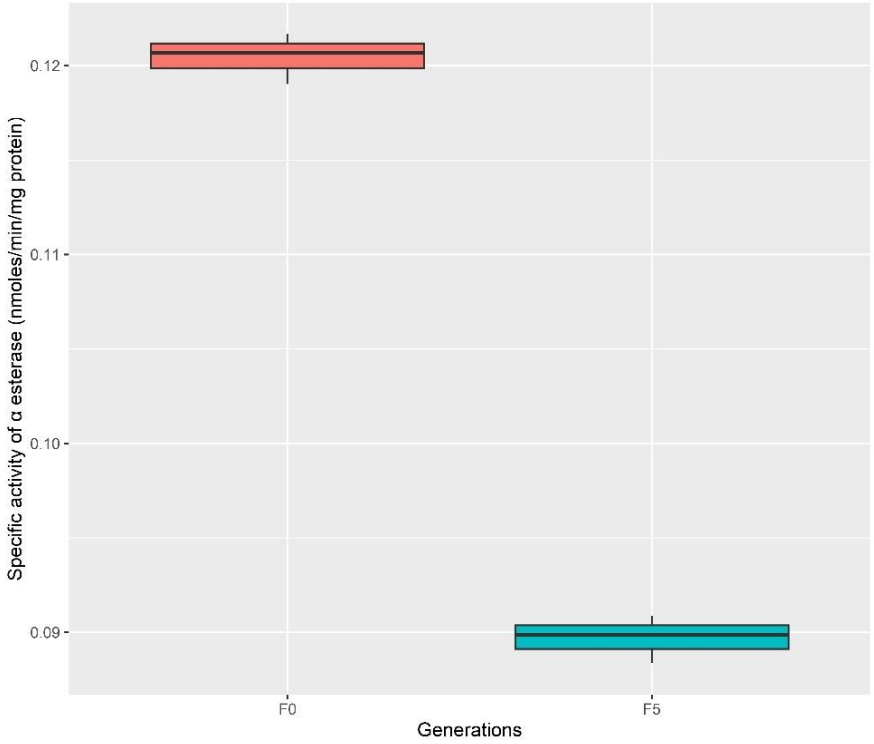

Figure 4.14 Comparison of Specific activity of α- esterase in susceptible F0 and SC4 selected F5 *Ae. aegypti* strain

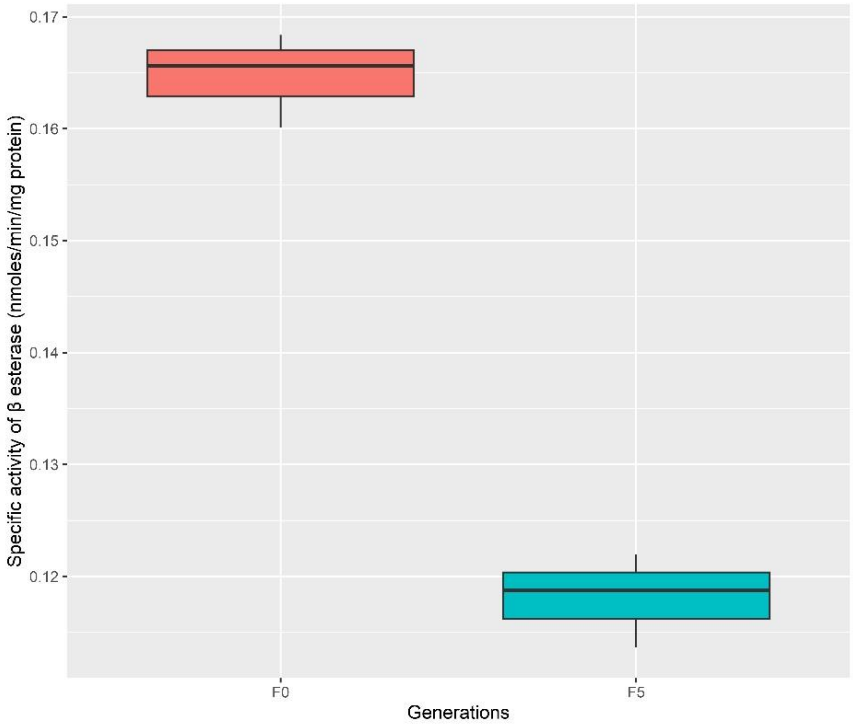

Figure 4.15 Comparison of Specific activity of β- esterase in susceptible F0 and SC4 selected F5 *Ae. aegypti* strain

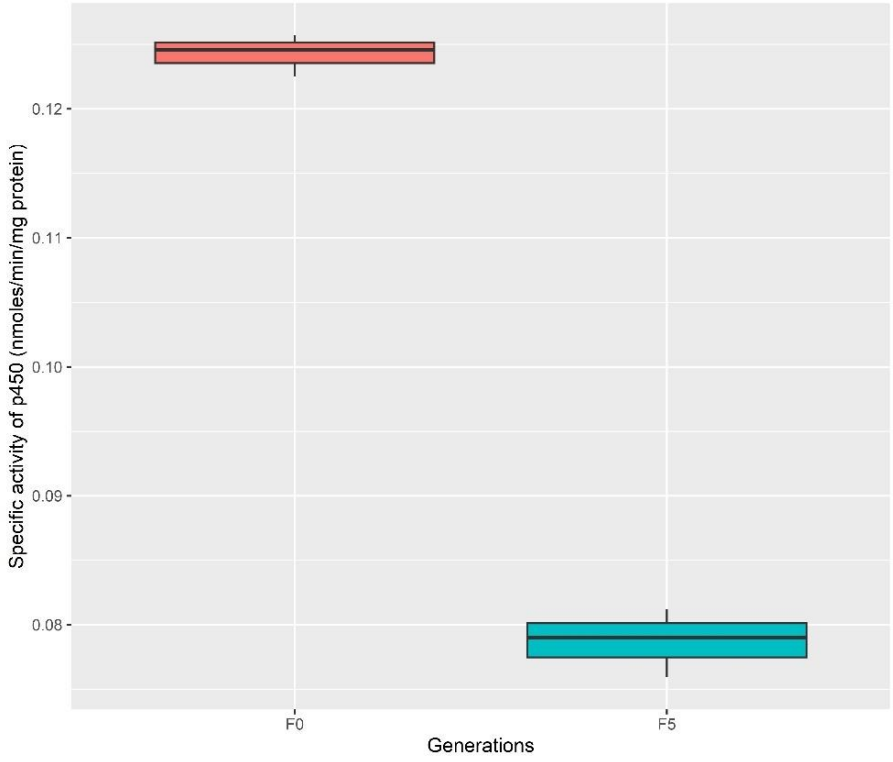

Figure 4.16 Comparison of Specific activity of monooxygenase in susceptible F0 and SC4 selected F5 *Ae. aegypti* strain

4.4 Discussion

The findings of this investigation unveiled the synergistic potential of a bioactive compound extracted from the plant *C. bonplandianum,* CB1, when combined with four different conventional insecticides against *Ae. aegypti*. All four insecticides demonstrated increased effectiveness when synergized with the Phyto-compound, exhibiting a positive cytotoxicity coefficient. The study included two distinct classes of insecticides, namely organophosphates and pyrethroids. The outcomes showed effectiveness in all tested combinations, with lambda-cyhalothrin (SC4) showing the highest efficacy, followed by cypermethrin (SC3), temephos (SC2), and malathion (SC1), with synergistic factors of 8.33, 3.11, 1.9, and 1.75, respectively.

Various studies have documented comparable synergistic effects resulting from combining different plant extracts or integrating plant extracts with insecticides to control pest populations. Considering the exceptional adaptability of mosquitoes, it becomes evident that more than depending solely on a single control strategy is required. While synthetic insecticides are known for their rapid action, high potency, and cost-effectiveness, their prolonged usage has contributed to ongoing environmental degradation. Mosquitoes, particularly species like *Ae. aegypti*, possess a remarkable ability to develop resistance against synthetic insecticides due to their short life cycle and high reproductive rates. This development has demanded alternate strategies, such as utilising renewable resources like plant extracts, which could offer more durable and less hazardous mosquito control solutions. The combined effects of synthetic pesticides and plant extracts demonstrate synergism, improving the efficacy of pest management techniques (Tyagi, 2016). Synergists are substances that, when combined with insecticides in sublethal concentrations, increase the toxicity and general effectiveness of the insecticides against pest species. According to studies by Brindley and Selim (1984), insecticide synergists like piperonyl butoxide (PBO) can drastically increase the toxicity of insecticides when used in sublethal amounts. Synergists were first used to stabilise pyrethrins to produce a competitive insecticide that could be used to fight mosquitoes, houseflies, and other household pests. The scarcity and high price of natural pyrethrum imported from Kenya led to the development of PBO, the first effective and commercially viable synergist (Wachs, 1947). Synergistic effects were demonstrated through bioassays employing a blend of botanical extracts and various synthetic

insecticides, which had been a recurring observation in several earlier research efforts (Kalyanasundaram and Babu, 1982; Kalyanasundaram and Das, 1985; Thangam and Kathiresan, 1990).

In a comparable investigation by Shaalan et al., 2005a, the synergistic effectiveness of botanical combinations was explored both with and without synthetic insecticides against *Cx. annulirostris and Ae. aegypti* mosquitoes. The outcomes of the study suggested that these combinations are more potent than using either insecticides or phytochemicals individually. Fakoorziba et al. (2009) reported the synergistic effectiveness of piperonyl butoxide in combination with deltamethrin, a pyrethroid insecticide, against *Cx. tritaeniorhynchus* and other mosquito species. Aivazi and Vijayan (2010) illustrated the synergistic impact of combining *Ruta graveolens* with cypermethrin to effectively combat *An. stephensi* larvae in a study conducted at Mysore. In a study conducted by Raghavendra et al. (2013), the synergistic potential of two plant extracts, *Solidago canadensis* and *E. jambolana* was investigated against *Ae. aegypti* mosquito larvae. The research focused on deltamethrin, and the results demonstrated that combining phytochemicals and insecticides exhibited greater efficacy than using insecticides or phytochemicals individually. Bhan et al. (2015) conducted a study to assess the larvicidal efficacy of temephos and extracts of *Cuscuta reflexa*, individually and combined, against Anopheline and Culicine larvae, revealing synergistic activity within the combinations. In an investigation done by Dhinakaran et al. (2019), four monoterpenes, γ-terpinene (T), R-(+)-limonene (L), carvacrol (C), and trans-anethole (A), were assessed for their impact on late third instar *Ae. aegypti* larvae, individually and in combination, revealed promising synergistic mixtures within the two tested binary combinations.

From the outcomes of the current study, it was clear that both SF and CTC demonstrated significant synergistic effects ($P<0.01$) at LC_{50} and LC_{90} levels for all the tested combinations. Additionally, the extent of synergism varied according to the concentration of insecticides used in combination with the plant isolate. The study results revealed that the various insecticides showed varying effectiveness, and SC4 (lambda-cyhalothrin combination) exhibited the most efficient synergistic pairing. Hence, the synergistic combination of SC4 was the focus of the resistance studies against *Ae. aegypti*. This study conducted a sequential selection experiment with five successive generations of *Ae. aegypti* (F0-F5), during which they were

exposed to different concentrations of SC4. The results showed that the LC_{50} values increased slightly in each generation. However, the RR values were calculated, and the outcomes showed that the increase in the lethal concentration of SC4 remained less than two even after five generations of exposure. This result indicated that the species remained susceptible to the selected synergistic combination despite the selective pressure exerted over the experimental generations.

Recognising the mechanism of action of plant extracts and insecticides is critical because it allows the evaluation of possible risks to human health and the probability of resistance evolution. Moreover, this knowledge enables researchers to formulate novel compounds with diverse modes of action. Additionally, it provides knowledge about the emergence of resistance among pests, particularly in situations involving target insensitivity (Karunaratne et al., 2018). In this present study, four detoxifying enzymes, namely Esterases (α and β), AChE, Monooxygenase (Cytochrome P450), and GST, were analysed to determine any potential elevation in the selected strain compared to the susceptible F0 strain, as part of the investigation into resistance development. The same enzymes were also tested with the susceptible F0 strain of *Ae. aegypti* larvae using a 10-fold dilution of its original LC_{50} to assess the impact of the synergistic combination SC4 on the detoxifying enzyme mechanism. The findings revealed no significant elevations in quantifying these enzymes between the selected F5 strain and the susceptible F0 strain, suggesting the absence of resistance development.

The insecticide sequestration or biodegradation catalysed by insect enzymes is a significant reason behind the emergence of metabolic resistance (Hemingway et al., 1985; Chen et al., 2003; Das and Dutta, 2014). Many studies suggested that insecticide metabolism primarily relies on the activities of three prominent enzyme families: cytochrome P450 monooxygenases, GST, and carboxy/cholinesterases. These enzymes play a principal role in coordinating a wide array of detoxification reactions, serving as the primary line of enzymatic defence against xenobiotic substances and supporting the elimination of diverse metabolic byproducts (Feyereisen, 2005; Oakeshott et al., 2010). Among the various mechanisms of resistance found in mosquitoes, metabolic resistance is a prevailing and most challenging one. Esterases, monooxygenases, and GST constitute the primary enzyme groups responsible for the metabolism of mosquitoes. There have been

documented instances of heightened esterase levels, increased cytochrome P450 activity, and their correlation with insecticide resistance across diverse mosquito species (Wan-Norafikah et al., 2013; Muthusamy and Shivakumar, 2015b).

In mosquitoes, esterases take an essential role as the primary mechanism responsible for conferring resistance to insecticides like organophosphates, carbamates, and pyrethroids (Polson et al., 2011; Marcombe et al., 2012). Multiple studies have presented compelling evidence that GST enzymes also play a significant role in conferring resistance to these categories of insecticides (Enayati et al., 2005). In addition, AChE, a serine esterase found at nerve synapses, constitutes the primary target for insecticides (Osta et al., 2012). In the endless struggle against diseases spread by mosquitoes, researchers have been exploring the complex mechanisms that constitute insecticide resistance in these insects. These studies aimed to understand how mosquitoes become resistant to insecticides and adapt to them, as is an essential element limiting vector control strategies. The detoxifying enzymes break down insecticides into less toxic or non-toxic compounds. Increased detoxification is a typical resistance mechanism that causes the inhibition of the target sites of the insecticides. This increase in detoxifying enzymes may be triggered by changes in the catalytic characteristics of the enzymes, an increase in the production of enzymes, or a combination of these two. Sometimes, amplification of a gene results in increased production of an enzyme (Oppenoorth, 1985). In this study, compared to the susceptible strain, which was not exposed to insecticides, the activity of each tested detoxifying enzyme in the mosquitoes decreased in both the generations treated with CB1 and those treated with the SC4. As the scientific literature in this area suggests that mosquito strains that have developed pesticide resistance have higher concentrations of these enzymes, this observation may indicate that the compounds under test effectively reduce the rate at which insecticide resistance develops.

The results of this study align with other research findings conducted in this area. In a research investigation carried out by Parthiban et al. in 2020, where they evaluated the larvicidal effectiveness of *A. muricata*, it was observed that the levels of AChE, GST, and esterase enzymes steadily declined in the tested mosquito strains, in contrast to the control strain that remained untreated with the extract. This decline indicated the susceptibility of the mosquito species to the plant extract under

examination. Another research focused on the larvicidal activity of *Artemisia absinthium* extracts, particularly regarding the inhibition of detoxifying enzymes in *Ae. aegypti* larvae, the ethanolic extract demonstrated significant inhibitory effects on detoxifying enzymes, including AChE and α and β-carboxylesterases. Additionally, exposure to the ethanolic extract for 24 hours led to a notable reduction in protein levels in fourth-instar larvae. The authors proposed that these results strongly imply that the active ethanolic extract derived from this plant holds promise as a potent solution for pest and vector control (Sofi et al., 2022).

4.5 Conclusion

While synthetic insecticides offer rapid and potent effects, their uncontrolled use often causes resistance, allowing vector populations to resurge along with disease. Conversely, botanical insecticides are increasingly favoured for their eco-friendly attributes, yet their application can be time-consuming, quantity-intensive, and harmful to non-target organisms. An integrated approach is essential for efficient, eco-friendly, and cost-effective management to optimise the efficacy of botanical insecticides and minimise resource demands. Synergistic interactions between synthetic insecticides and botanical agents can offer a potent and low-risk approach, offering effectiveness and less environmental impact. This chapter focused on examining the synergistic activity of the bioactive compound CB1 when combined with chemical insecticides, including malathion, temephos, cypermethrin, and lambda-cyhalothrin. The aim was to analyse the synergistic impact of these combinations on *Ae. aegypti* larvae. Significantly, all the examined combinations exhibited synergistic effects, established to be relevant in managing the vector population. Furthermore, the chapter explored the impacts of SC4 on the detoxifying enzymes of the *Ae. aegypti* mosquito. The potential for resistance development within the mosquito population against this synergistic compound was also investigated by assessing the detoxifying enzyme activity over five consecutive generations exposed to the treatment. The consistent decline in the activity of these tested enzymes across all treated generations suggested a possible hindrance to the rapid development of resistance against this compound. In summary, the identified synergistic interactions between CB1 and chemical insecticides offered a prevailing approach to enhance vector control efficacy while slowing down the development of resistance in mosquito populations.

SUMMARY

Mosquitoes, tiny but potent disease vectors, have played a significant role in influencing public health outcomes worldwide. These blood-feeding insects can transmit a variety of deadly pathogens to humans and animals in addition to being an annoyance. The list of diseases that mosquitoes are able to transmit includes a number of severe public health problems, such as dengue fever, malaria, Zika virus, West Nile virus, chikungunya, and many more. Each of these diseases can have devastating effects on individuals and communities, leading to illness, disability, and even death. Mosquito-borne diseases are particularly prevalent in tropical and subtropical regions, where the environmental conditions favour both the mosquito vectors and the pathogens they carry. In the continuous worldwide effort to protect public health, it is essential to understand the intricate relationships between mosquitoes and the diseases they transmit.

In the first chapter of this study, molecular identification and diversity analyses were conducted on vector mosquitoes, yielding valuable information on the genetic composition and diversity of disease-transmitting vectors. The collection data revealed a diverse range of mosquito vector species, spanning *Anopheles*, *Aedes*, *Culex*, and *Armigeres* genera, emphasizing the complexity of vector-borne diseases. These species were accurately identified and categorized using molecular techniques, providing information about their vectorial capacities. The diversity indices of the collected species with respect to their collection sites were also discussed in this chapter.

Chapter two shifted focus towards screening diverse plant species to evaluate their effectiveness in controlling the dengue fever vector, *Ae. aegypti*. Notably, *C. bonplandianus*, when subjected to hexane extraction, demonstrated remarkable larvicidal efficacy, emphasizing its potential as a natural solution. Further investigation identified Eicosane as the bioactive compound responsible for this efficacy in controlling *Ae. aegypti* populations. These findings determined the significance of botanical insecticides as eco-friendly resources in the battle against mosquito vectors, offering practical and sustainable alternatives for vector control.

In the third chapter, a thorough assessment was conducted to determine the larvicidal effectiveness of four conventional insecticides when applied against the *Ae. aegypti* mosquito species. Among these insecticides, Lambda-cyhalothrin demonstrated the highest potency, effectively controlling *Ae. aegypti* larvae. It was followed by Cypermethrin, Temephos, and Malathion in decreasing order of effectiveness. These findings highlighted the importance of using a balanced strategy in mosquito control efforts, combining chemical insecticides with natural substitutes like eicosane. This multilayered approach is vital for the effective management of mosquito-borne diseases while simultaneously minimizing potential ecological repercussions. This equilibrium could minimize environmental harm and optimize the effectiveness of vector control methods.

The final chapter examined the synergistic interactions between chemical insecticides and the bioactive compound extracted from *C. bonplandianus*. These combinations showed remarkable outcomes, as they displayed strong synergistic effects against *Ae. aegypti*. This result provided a promising path for more effective management of mosquitoes by suggesting a relatively safe but highly effective vector control strategy. The chapter also covered the effectiveness of the most effective synergistic compound and plant isolate on the detoxifying enzymes of *Ae. aegypti*. This aspect of the study suggested that these synergistic compounds might hinder the rapid development of resistance within the selected *Ae. aegypti* populations. This is a significant finding because resistance development is a pressing concern in the field of mosquito control. The objective outlined in this chapter demonstrated important alternatives for potential mosquito management strategies that would provide efficiency and prioritize environmental sustainability and cost-effectiveness. Combining the strengths of natural compounds like the one found in *C. bonplandianus* with chemical insecticides can potentially revolutionize vector control strategies. This approach maximizes the impact of mosquito vector management while minimizing the ecological footprint, making it a valuable contribution to the ongoing battle against mosquito-borne diseases.

RECOMMENDATIONS

Effective disease control requires understanding and managing mosquito vectors, which can provide information regarding ecosystems, early detection of emerging diseases, and targeted interventions. Combining molecular techniques with classical taxonomy enables high accuracy in rapid and species-specific identification.

- **Targeted disease control**: Mosquito vector identification and diversity studies enable the implementation of precise and targeted control measures, reducing the spread of mosquito-borne diseases. It also reduces excessive use of insecticides hence minimizing environmental hazards.
- **Early detection of emerging diseases**: These studies provide early warning signs of emerging mosquito-borne diseases by monitoring their vectors, allowing for proactive public health responses and the development of preventive strategies.
- **High Precision**: Molecular techniques allow for precise identification of mosquito species, including species that may be difficult to distinguish based on morphology alone, enhancing the accuracy of vector surveillance.
- **Rapid detection of vector**: Molecular methods enable quick and reliable identification of mosquito vectors, accelerating disease surveillance and response efforts in areas prone to vector-borne diseases.
- **Species-level vector monitoring**: Barcoding and molecular studies provide species-level information, helping researchers and public health officials understand the specific vector species involved in disease transmission, which is crucial for targeted control measures. By detecting the presence of potential disease vectors before outbreaks occur, these studies contribute to the establishment of early warning systems, improving vigilance and reducing the impact of disease epidemics.
- **Research opportunities**: These techniques provide opportunities for progressive research, such as the discovery of novel mosquito-borne vectors, genetic adaptations, and vector behavior, advancing the understanding of vector-borne diseases.

- **Global health impact**: Accurate molecular identification and monitoring of mosquito vectors have a direct impact on global health by facilitating more effective disease control and prevention strategies.

  Exploring the diverse aspects of mosquito control, from enhanced efficacy through the synergistic approach to reduce resistance development and its cost-efficiency, highlights the potential for environmentally friendly and sustainable strategies.

- **Enhanced efficacy:** When plant extracts are combined with conventional insecticides, their efficacy is enhanced, leading to more effective mosquito control and a reduction in disease transmission.
- **Delaying resistance development:** Synergistic combinations have the potential to slow down the development of insecticide resistance among mosquito populations, thereby extending the period of control methods.
- **Environmentally friendly approach:** Plant extracts are often biodegradable and pose fewer risks to non-target organisms and the environment than synthetic chemicals and thus helps to attain environmentally sustainable mosquito control.
- **Reduced health hazards:** Decreasing reliance exclusively on synthetic insecticides can lower the potential health risks associated with exposure to these chemicals for both humans and animals.
- **Cost-efficiency:** The Synergistic approach can be cost-effective as it requires lower concentrations of plant products. This makes them a practical and affordable option for mosquito control, especially in resource-limited regions.
- **Community involvement:** Including resident people in the production and application of plant extracts would promote community involvement in vector control programs.
- **Conservation of biodiversity:** Minimizing the use of synthetic chemicals contributes to the protection of local ecosystems and the preservation of biodiversity.
- **Long-term sustainability:** The development of synergistic combinations represents a sustainable and enduring approach to the prevention of mosquito-borne diseases.

- **Prospects for research:** There are many opportunities for research on synergistic mosquito control methods, providing ways to investigate the efficiency, environmental effects, and long-term approaches of combining different chemical and botanical insecticide combinations.

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PUBLICATIONS & PARTICIPATIONS

Publications

- Synergistic effect of *Croton bonplandianum* Baill. With Cypermethrin and Lambda-cyhalothrin against *Aedes aegypti* Linn, a Dengue fever vector.

  Authors: **Kalarikkal Venugopalan Lakshmi**, Ambalaparambil, Vasu Sudhikumar, Embalil Mathachan Aneesh.

  South African Journal of Botany, 140, 103-109.

- Larvicidal activity of phytoextracts against dengue fever vector, *Aedes aegypti*- A Review

  Authors: **Kalarikkal Venugopalan Lakshmi**, Ambalaparambil Vasu Sudhikumar, Embalil Mathachan Aneesh.

  Plant Science Today, 5(4), 167-174.

- Mosquitoes as Pesticide Pollution Indicators- A Comparative Susceptibility Analysis of Field and Laboratory Strains of Mosquitoes Against Different Conventional Insecticides.

  Authors: Ambadath Velayudhan Asha, **Kalarikkal Venugopalan Lakshmi**, Ambalaparambil Vasu Sudhikumar, Embalil Mathachan Aneesh.

  Journal of Applied Biology and Biotechnology, 9(6), 115-121.

Presentations and Participations

- Presented a paper on "Molecular Identification through the amplification and sequencing of the gene Cytochrome oxidase subunit I of different mosquito vectors from Thrissur District, Kerala, India." At 12th National Conference on Vector-Borne and Zoonotic Diseases Identification to Management held on 26th and 27th November 2019 at Zoological Survey of India, New Alipore, Kolkata.
- National workshop on "Intellectual Property Rights" on 15th March 2018 at St. Joseph's College Irinjalakuda.
- Training on 'Molecular Biology and Bioinformatic tools for Advanced Life Science Research' from February 20th to March 5th, 2018, at College of Veterinary and Animal Sciences, Mannuthy, Thrissur.

PLATES

Plate 1: Mosquito species collected from selected study sites of Thrissur District

Figure 1.4a

Figure 1.5a

Figure 1.6a

Figure 1.7a

Figure 1.8a

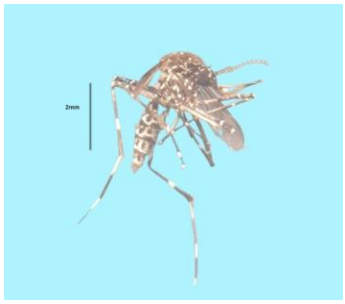

Figure 1.9a

Figure 1.10a

Figure 1.11a

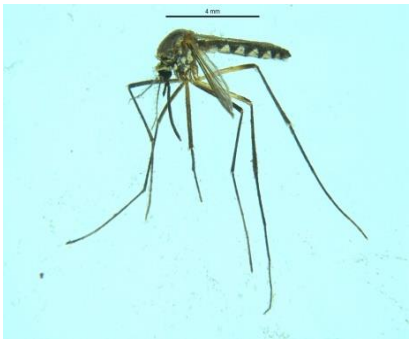

Figure 1.12a

Figure 1.13a

Figure 1.14a

Figure 1.4a *Anopheles stephensi*
Figure 1.5a *Anopheles subpictus*
Figure 1.6a *Anopheles vagus*
Figure 1.7a *Aedes aegypti*
Figure 1.8a *Aedes albopictus*
Figure 1.9a *Aedes vittatus*
Figure 1.10a *Armigeres subalbatus*
Figure 1.11a *Culex gelidus*
Figure 1.12a *Culex tritaeniorhynchus*
Figure 1.13a *Culex pipiens*
Figure 1.14a *Culex quinquefasciatus*

Plate 2: Plants collected for larvicidal activity screening against *Ae. aegypti* larvae

Figure 2.1.1
10.556N,76.178E

Figure 2.1.2
10.473N,76.212E

Figure 2.1.3
10.464N,76.242E

Figure 2.1.4
10.554N,76.173E

Figure 2.1.5
10.543N.76.144E

Figure 2.1.6
10.459N.76.252E

Figure 2.1.7
10.473N,76.212E

Figure 2.1.8
10.467N,76.224E

Figure 2.1.9
10.467N.76.224E

Figure 2.1.10
10.543N,76.145E

Figure 2.1.11
10.464N.76.212E

Figure 2.1.12
10.552N,76.182E

Figure 2.1.13
10.505N,76.246E

Figure 2.1.14
10.556N,76.178E

Figure 2.1.15
10.532N.76.139E

Plate 2: Plants collected for larvicidal activity screening against *Ae. aegypti* larvae

Figure 2.1.16
10.557N,76.177E

Figure 2.1.17
10.467N,76.224E

Figure 2.1.18
10.532N,76.139E

Figure 2.1.19
10.463N,76.227E

Figure 2.1.20
10.464N,76.242E

Figure 2.1.21
10.467N.76.224E

Figure 2.1.22
10.556N,76.178E

Figure 2.1.23
10.477N,76.214E

Figure 2.1.24
10.552N.76.182E

Figure 2.1.25
10.467N.76.224E

Figure 2.1.26
10.473N,76.212E

Figure 2.1.27
10.544N,76.184E

Figure 2.1.28
10.464N.76.242E

Figure 2.1.29
10.479N,76.215E

Figure 2.1.30
10.552N.76.182E

Plate 3: Instruments Used for Phyto-extraction

Plate 4: Separation techniques used for CB1 compound isolation (Figure 2.3 and figure 2.4)

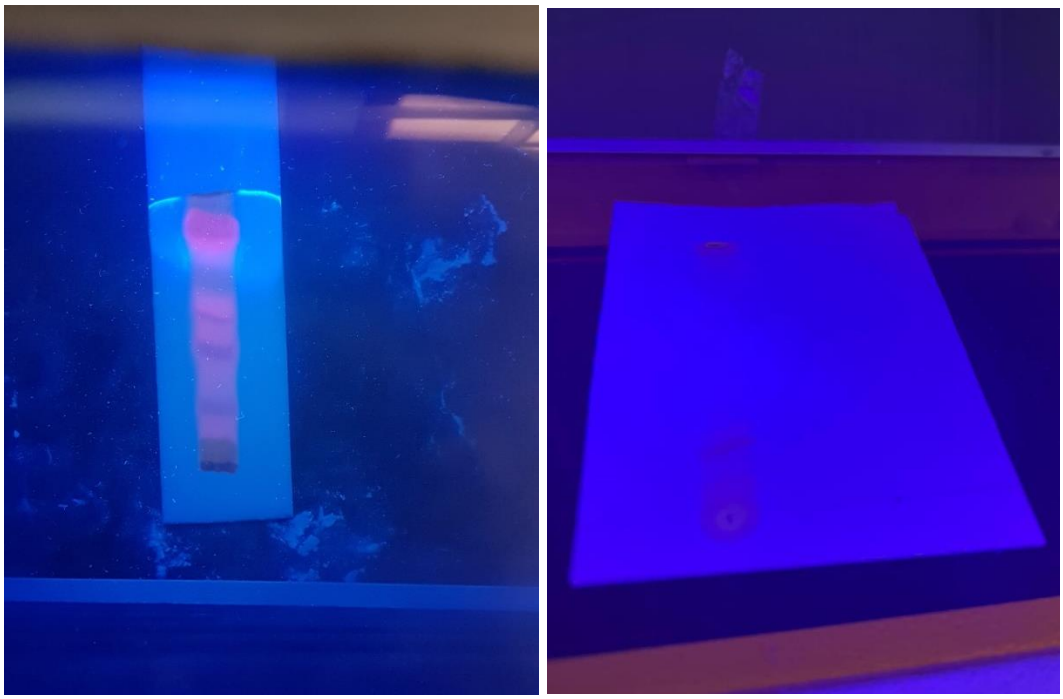

Figure 2.3

Figure 2.4

Plate 5: Mosquito Colony Maintenance and Susceptibility Assays

Plate 6: Antimicrobial Activity of CB1

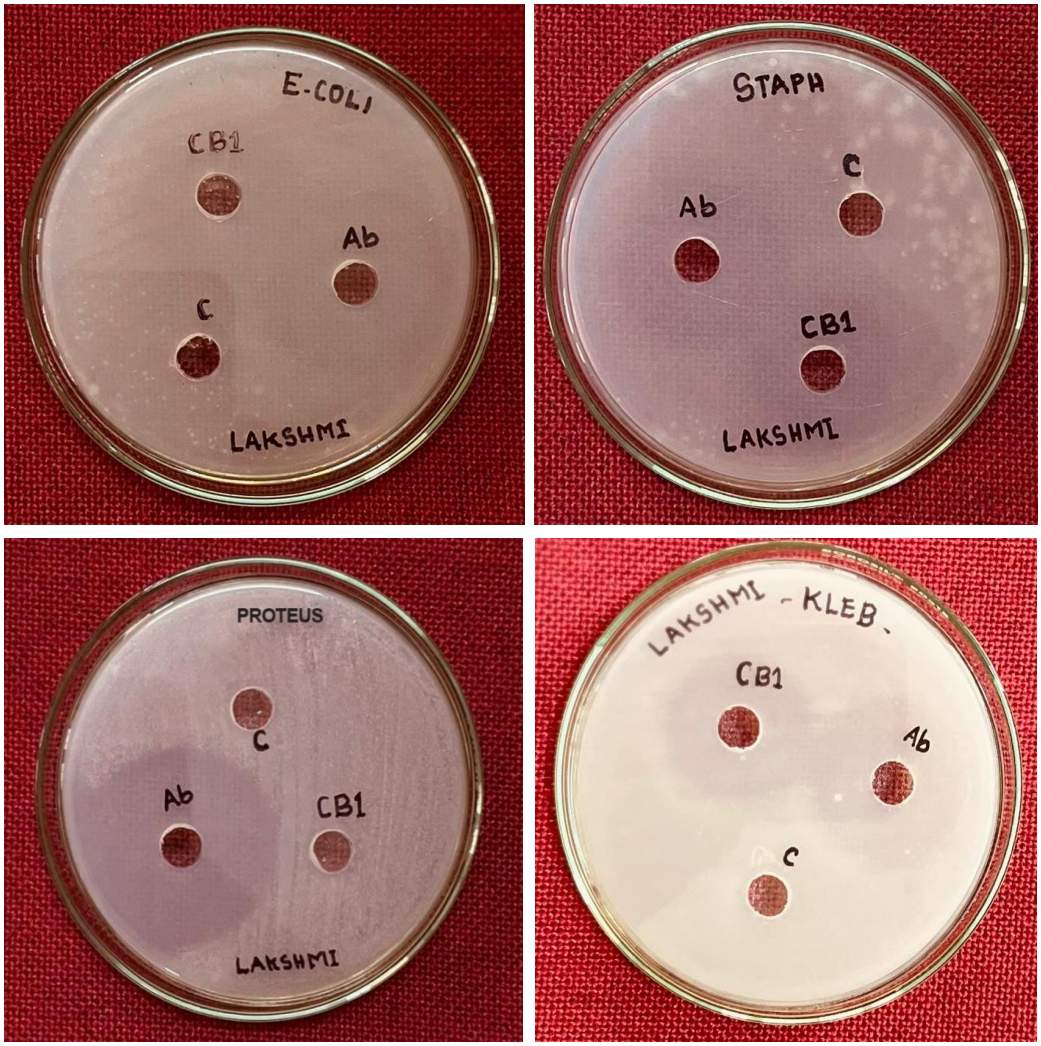

Plate 7: Specimen Collection of *Croton bonplandianus* Baill.

Plate 8: Quantitative Assay of Detoxifying Enzymes of *Aedes aegypti*