Biodiversity of Mosquitoes (Diptera) in Punjab, Pakistan and Screening of Bioactive Phytochemicals as a Larvicidal Potential against *Culex quinquefasciatus*

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Dedicated

To

My Parents and

Husband

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Attiya Iqbal

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LIST OF ABBREVIATIONS

AAE	Ascorbic acid equivalent
ANOVA	Analysis of variance
BHA	Butylated hydroxyanisole
BHT	butylated hydroxytoluene
BSA	Bovine serum albumin
CV	coefficient of variation
COI	Cytochrome c oxidase I
COII	Cytochrome c oxidase II
DPPH	2,2-diphenyl-1-picrylhydrazyl
DMSO	Dimethyl sulfoxide
df	Degree of freedom
DW	Dry weight
EI	Electron impact
FRAP	Ferric Reducing Antioxidant Power
FT-IR	Fourier transform infrared
GM	Geometric morphometric
GC-MS	Gas chromatography–mass spectrometry
HPLC	High performance liquid chromatography
ITS1	Internal transcribed spacers I
ITS2	Internal transcribed spacers 2
IC_{50}	Inhibitory concentration
KBr	Potassium bromide
LC-ESI-MS	Liquid Chromatography-Electrospray Ionization-Mass Spectrometry
LCL	Lower confident limit
MOE	Molecular operating environment
Mo (VI)	Molybdenum oxide (VI)

Mo (V)	Molybdenum oxide (V)
NCBI	National center for biotechnology information
NIST	National Institute of Standards and Technology
OR	Observed range
PCR	Polymerase chain reaction
PDB	Protein data base
PFTBA	Perfluorotributylamine
RFLP	Restriction fragment length polymorphism
RH	Relative humidity
rpm	Revolutions per minute
Rf	Retention factor
SD	Standard deviation
SE	Standard error
SDF	Structure data file
TLC	Thin layer chromatography
TBE	Tris-borate-EDTA
TAC	Total antioxidant capacity
TRP	Total reducing power
TBHQ	Tert-butylhydroquinone
TAC	Total antioxidant capacity
UV-VIS	Ultraviolet visible spectrometry
UCL	Upper confident limit
WHO	World health organization

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GENERAL ABSTRACT

Mosquitoes population are widely diverse in the whole Pakistan. Among all the Provinces of Pakistan, Punjab is the most populated area and constitutes highly favorable environment regarding temperature, rainfall and humidity for up breeding of insects like mosquitoes. The mosquitoes play a key role in causing number of diseases such as malaria, dengue fever, filariasis, yellow fever etc. being vector. Therefore, the current study mainly focused on biodiversity and control measures of mosquitoes using plants. For biodiversity assessment, mosquitoes were collected from eleven districts of Punjab from water containers, flowerpots, standing water, tires, sweep nets, aspirators etc. from April-November (2016-2019), and preserved in 70% ethanol for morphological study and in 95% ethanol for molecular analysis. A stereoscopic trinocular microscope was used to examine and measure all main body parts and identified with the help of key "The fauna of British India" (1934). The results revealed the presence of 27 mosquitoes spp. belonging to two subfamilies (Anophelinae and Culicinae) and five genera **(***Anopheles, Aedes, Culex, Armigeres, and Mansonia***).** The molecular identification was based upon the amplification of gene Cytochrome oxidase I (COI) which confirmed the presence of three species belonging to two genera *Anopheles* (*An. gambiae*) and *Aedes* (*Ae. aegypti* and *Ae. albopictus*).

For control measures, the ethanol and water dried leaves extract were evaluated from six plants (*Ocimum basilicum*, *Polygonum glabrum*, *Mentha piperita*, *Mallotus philippensis*, *Citrus aruntium*, *Fumaria officinalis*) for their larvicidal potential against *Culex quinquefasciatus larvae*. The cytotoxicity assay was applied on brine shrimps using five concentrations multiple of 80. The bioassay was applied in triplicates in 1000ml plastic containers with extracts, added 30 larvae of each four instars separately and fed with dog biscuits. A control excluding extract was setup parallel. Observations were made after every 12hrs till 72. By using probit analysis, the LC_{50} and LC_{90} values were determined. The DPPH radical scavenging activity, ferric reducing power, and total antioxidant capacity was estimated taken ascorbic acid as standard. The ethanolic extract of *M. piperita*, found to be the most effective (LC_{50}=208.98ppm) ones, it was characterized by preliminary qualitative and quantitative phytochemicals, UV-VIS spectroscopy, FT-IR, GC-MS, and molecular docking analysis. Biochemical analysis of dead mosquito larvae treated with ethanolic *M. piperita* extract showed the presence of significantly less value of proteins, carbohydrates, cholesterol, triglycerides and high-density lipid as compared with control.

Maximum total antioxidant capacity (125.46 ± 3.55, 115.63±1.76 (µg/mg), ferric reducing power (378.17± 1.04, 365.12±0.31 (µg/mg), and DPPH radical scavenging activity was demonstrated by *M. piperita* and *O. basilicum* leaf extracts (85.43±0.22, 68.64±0.41 (µg/ml). Except for *F. officinalis* (LC_{50}=569.31ppm), all of the experimental plants demonstrated cytotoxicity in a protective array >550ppm. The qualitative phytochemical study showed that *M. piperita* had the highest concentration of organic components (such as phenols, alkaloids, flavonoids, carbohydrates, and flavonoids etc.) compared to the other five plants used in the experiment. The quantitative phytochemical study showed that *M. piperita* had the highest levels of total phenolic (6.44±0.0018 µgGAE/mgDW) and flavonoid contents (8.47±0.0017 µgQA/mg DW). UV-VIS spectroscopy revealed two absorption peaks with absorption values of (2.34, 0.79) at 209.51 and 282.81nm, respectively. FT-IR results revealed the presences of alcohols, alkanes, aldehydes, aromatic rings, ether linkage, ester, and halo-compounds. The GC-MS analysis identified the compounds t-Butyl hydrogen phthalate (13.9%), 2H-Pyran-2,4(3H)-dione, 3-ethyl-5,5-dimethyl-6-phenyl (13.15%), and 1,2-Benzenedicarboxylic acid, mono-(2-ethylhexyl) ester (12.45%) with high % peak area 1,2-Benzenedicarboxylic acid and 3-ethyl-5,5-dimethyl-6-phenyl were the two compounds that best bound to the NS3 protease domain with PDB ID: 2FOM out of all the others.

To isolate bioactive secondary metabolites in the current study, the crude leaves extract of *M. piperita* was fractionated by column chromatography and further characterized by thin-layer chromatography using various solvents with multiple ratios, including n-hexane, ethyl acetate, chloroform, and methanol. The column chromatography showed that fractions F3, F4, F12, and F13 had the high potential of larvicides. The cytotoxic, antilarval and antioxidant properties (total antioxidant capacity, ferric reducing power, and DPPH) of 15 fractions were tested against *Cx. quinquefasciatus.* In the dose-response bioassay, various concentrations (80, 160, 240, 320, and 400ppm) were used. The highest antilarval activity was shown by fractions 3, 4, 12, and 13 against all four instars larvae, with LC_{50} values of (127.9, 121.7, 105.1, 112.5), (131.5, 128.1, 118.6, 95.1), (136.6, 112.5, 102.4, 52.6), and (104.1, 110.9, 130.7, 46.9) respectively. Maximum ferric reducing power (250±0.85, 255±0.65, 265±0.69, 275±0.94), total antioxidant capacity (70±0.54, 72±0.34, 77±0.21, 85±0.25), and DPPH radical scavenging (90±0.13, 96±0.11, 97±0.87, 98±0.34) activity were demonstrated by F3, F4, F12, and F13. The fraction (F13) had much higher antilarval activity, with an IC_{50} of 7.65±0.19ug/ml as compared with control 7.23±0.55ug/ml.

The active fractions were further put for chromatographic techniques preliminary phytochemical tests, UV-VIS, FT-IR, GC-MS analyses, and molecular docking. The phytochemical analysis executed the presences of 20 organic compounds. The UV-VIS profile exhibited peaks ranging from 200 to 800nm with a range of absorptions. The FT-IR spectrum of four active fractions demonstrated the presence of alcohol, primary amine, aliphatic primary amine, Alkyl aryl ether, secondary amine, alkane, carboxylic acid, alkene, anhydride, nitro-compound etc. The GC-MS analysis results showed the presence of various phytochemical, including ethanol, ammonium oxalate, carbazic acid, propanamide, isobornylthiocyanoacetate, 1, 2-Benzenedicarboxylic acid, N-[5-(5-phenyl-3,4-dihydro-2H-pyrrol-4-yl) nonan-5-yl] benzamide, eicosanoic acid.

Among all identified compounds from GC-MS, in silico molecular docking experiments, the 1, 2-benzenedicarboxylic acid of F3, eicosanoic acid of F4, phytol of F12, and N-[5-phenyl-3, 4-dihydro-2H-pyrrol-4-yl] nonan-5-yl] benzamide of F13 had the best binding affinity for the target NS3 protease. The results showed that all fractions F3, F4, F12, and F13 were effective against *Cx. quinquefasciatus*. It is suggested that the *M. piperita* could be a good source of larvicides.

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CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Mosquitoes are a diverse and extensive group of insects (Suhasini *et al*., 2014). The word mosquito means "small fly" in Spanish. Mosquitoes are creatures that belong to the family Culicidae, suborder Nematocera, and order Diptera. The term "two wings" refers to the defining feature that set flies apart from other creepy crawlies. Mosquitoes look like crane and chironomid flies from the outside (family Tipulidae) (Dong *et al*., 2008). Mosquitoes carry medically significant pathogens that transmit malaria, filariasis, japanese encephalitis, and other arboviral illnesses (Wilkerson *et al*., 2015).

1.1: GEOGRAPHICAL DISTRIBUTION OF MOSQUITOES

Biodiversity means the diversity of animals and their complex interactions with abiotic (nonliving) aspects of their environment. It can be divided into a structural, functional, and geographical component that fluctuate across time, location and refers to the wide range of genes, animals, and ecosystems (Gaston, 1996). To assess knowledge of these various aspects of mosquito biodiversity, it is essential to consider the current threat of new invasive species and disease transmission in a given area (Manguin and Bote, 2011).

Species richness and relative abundance based on morphological or genetic features are included in the structural biodiversity component. Their impact on ecosystem functioning and species interactions, are all part of the functional side as well as species ecology and habitat preferences (e.g: competition the influence of invasive species). On the other hand, spatial biodiversity refers to the number of species interactions in a certain area or habitat is important for anticipating species fluctuation in response to changing environmental and climatic circumstances (Schafer, 2004). In southern Europe, studies primarily focused on indigenous vector species like *Anopheles* spp. (Patsoula *et al*., 2007; Ponçon *et al*., 2007; Di Luca *et al.,* 2009) and invasive (potential vector) species such as *Aedes albopictus* (*Stegomyia albopicta* in the phylogenetic classification (Reinert *et al*., 2009) and *Ae. japonicus* (Medlock *et al*., 2012).

As a result of the loss of natural mosquito habitats (Florencio *et al*., 2014) and the worldwide spread of invasive mosquito species (Kumar and Nattuthurai, 2011)

are important in mosquito studies, distribution, relative abundance, and surveys. Global climate change has improved the possibility of vector-borne illness epidemics by increasing anthropogenic activity and allowing for faster communication (Usman *et al*., 2017). Mosquitoes are found in about 3,600 different species around the world. The Culicidae includes 42 genera, which are divided into three sub-families: Anophelinae, Culicinae, and Toxorhynchitinae, it includes 11 tribes and 113 genera (Knight and Stone, 1977; Harbach, 2007). Tropical and temperate climates can be found even above the Arctic Circle, but not in Antarctica. They can be found in caves and mines in hilly places at heights up to 6,000m and depths up to 1,250m (Lambrechts *et al*., 2010).

Mosquitoes biodiversity is a serious concern because the possibility of invading species as well as the occurrence and extent of vector-borne diseases. People are at risk from the spread of new strains of well-known or novel diseases because of the effectiveness, speed, and reach of modern transportation networks (Guimerà *et al*., 2005). With the globalization of economic activity, tourism, human migration, and mosquito biodiversity, disease vectors and viruses are also increasing more widely throughout the world (Tatem, 2009)

The expansion of primary disease areas suitable for the vectors is becoming a threat and some species are characterized as invasive in some countries. Ten percent of all mosquito species are thought to be effective vectors of infectious pathogens that have a significant influence on human welfare and assets. However, a small percentage of the mosquito population is responsible for most serious mosquito vector diseases belonging to three genera of Culicidae: *Anopheles*, *Aedes*, and *Culex*, among them which some species have been highly effective in large-scale invasions. An invasive species is the one that has a significant impact on its new environment and spreads over a significant distance (Daehler, 2001).

They can be found in a variety of forms all around the world. *Anopheles* mosquitoes are not present in Greenland, Iceland, the Seychelles, New Caledonia, or the islands of Central and Eastern Polynesia (Mouchet, 2000; Robert *et al*., 2011). According to several researches on mosquito biogeography, Southeast Asia and the Neotropics have the highest diversity of mosquitoes (Fang, 2010). The majority of mosquito species are found in many Asian nations, including Brazil, Indonesia,

Malaysia, and Thailand. There is a latitudinal biodiversity gradient, with species richness rising as one approach the equator. (Foley *et al*., 2007).

According to Harbach (2004), Manguin *et al*. (2010), and Krzywinski *et al*. (2011), *Anopheles* species are found in respective countries for example, Asia (50%), Americas (21%), Africa (16%), Australia-Pacific (14%), and Europe (13%), respectively. *Anopheles* species richness in collection sites is higher in Asia (4%), with more than 10 species per site and up to 20 in some localities (Coosemans *et al*., 2006), compared to less than 10 per site on the American and African continents based on a variety of different gathering techniques (Garros *et al*., 2008). The greatest diversity of mosquitoes (31%) is found in the Neotropics (Khan *et al*., 2015a).

1.2: BIODIVERSITY OF MOSQUITOES IN PAKISTAN

Pakistan lies in the northwest corner of South Asia. Although Pakistan's geographic location and ecology may make it a hotspot for mosquitoes-borne illnesses (Stark and Schoneberg, 2012). In Pakistan, the biodiversity of mosquitoes was discovered as early as 1971 (Khan, 1971). There are 134 species in Pakistan, including 4 subspecies, 89 species from East (now Bangladesh), and 91 from West Pakistan. **(**Tandina *et al*., 2018). The *Aedes* species have the most diverse species among the Culicidae and are particularly important in medical cases (Lemine *et al*., 2017).

Pakistan's mosquito biodiversity included one *Anopheles* and three *Culex* species between 1934 and 1971. In Pakistan, 16 indigenous mosquito species were discovered later (Aslam and Salman, 1969). Until now, almost 3523 species from 111 genera have been reported from all over the world (Harbach, 2012). The *Culex* genus contains 763 species classified into 26 subgenera. In the genus *Aedes*, there are 927 species split into 70 subgenera. The majority members of the Culicidae are vital to public health (Wilkerson *et al*., 2015; Freitas *et al*., 2015).

Dengue fever outbreaks in Pakistan have sparked renewed interest in mosquitoes research (Mukhtar *et al*., 2011; Shakoor *et al*., 2012; Ilahi and Suleman, 2013; Rasheed *et al*., 2013). Changa Manga National Forest researchers studied mosquito bionomics and described 29 mosquito species, many of which were unique, rare, or unknown in Pakistan at the time of 1969. Culicidae is divided into three

subfamilies: Culicinae (Culicines), Anophelinae (Anophelines), and Toxorhynchites (Service, 2008).

1.2.1: Subfamily Culicinae

Culicidae is the largest subfamily of Nematocera Dipterans. There are 3,046 different species of Culicinae mosquitos. *Culex*, *Aedes*, *Mansonia*, *Sabethes*, *Haemagogus*, and *Psorophora* are among the 30 mosquito genera in the Culicinae, being the most medically significant. Small flies have forewings for flying and hindwings reduced to halters for balancing. With 1,257 species divided into 10 genera, Aedini is the largest tribe in the subfamily. *Aedes* class contains 931 species divided into 78 subgenera. The Stegomyia large subgenus contains 128 species and pathologically important. Mosquito ability to transmit the diseases to humans results in millions of deaths each year. Around 17% of all contagious infections are caused by the major vector-borne pathogens (Jabeen *et al*., 2019).

1.2.2: Subfamily Anophelinae

Anopheles is a genus comprising 460 morphologically recognizable species and seven subgenera have been presented within the Anophelinae, out of them 70% have been concerned in malaria parasite transmission. Few individuals of the *Anopheles* class are well-known enough to form complexes of more than 500 sibling/cryptic species with ambiguous ordered features, bringing the total number of species (Chhilar, 2014). Mosquito vector can transmit a variety of pathogens, including filarial, arboviruses, and protozoans which can produce persistent infections and serious health problems in humans. They may however spread bacterial illnesses to a lower amount. Mosquitoes of therapeutic importance belong to the Culicidae family and are found all over the world. Mosquitoes belonging to the *Anopheles* genus carry Plasmodium species, which is the malaria-causing agent (Sinka *et al*. 2012).

The female *Anopheles* obtains a blood meal from a sick vertebrate host and changes into the gametocytic form found in the blood, which starts the biological vector's life cycle (Pimenta *et al*., 2015). They are nasty bloodsuckers who feed on mammals, birds, reptiles, land and water animals. Because of their biting and bloodsucking ability to transfer diseases that cause severe illnesses, mosquito biodiversity is an important aspect of medical science that is expected to grow into a modern, substantial, and vital

aspect of human life. Around the world, mosquito species diversity varies according to different geographical regions (Suhasini and Sammaiah, 2014).

1.2.3: Subfamily Toxorhynchitinae

The tribe Toxorhynchitini belongs to the Toxorhynchitini (Ribeiro, 2004). Toxorhynchites is the only genus in the Toxorhynchitini tribe includes 90 species belonging to four subgenera: Toxorhynchites (51), Lynchiella (16), Afrorhynchus (19), and Ankylorhynchus (4) (Focks, 2007). While Toxorhynchites (old world), Afrorhynchus and Lynchiella (New world) and Ankylorhynchus (Africa) are found. Members of the genus are physically similar to one another despite having a wide geographic distribution, which makes species identification difficult (Steffan, 1975). Although Toxorhynchites was first thought to be a separate subfamily of the Culicidae, subsequent investigations put it within the Culicinae (Mitchell *et al*., 2002).

1.3: ECOLOGY OF MOSQUITOES

Mosquitoes are dangerous vectors because they are found all over the world and have adapted to a wide range of natural environments. They are restricted to aquatic and land domains as immature and adults, respectively. Stalled pools, tree gaps, slow-moving streams, freshwater or brackish water from lakes, water pooling in artificial containers, fissures, footprints, and geothermal pools are all possible habitats. Depending on nutritional levels, competition for available resources, and temperature, the hatchlings may occur in a matter of days or weeks (Norris, 2004).

The larval and adult life-history phases of mosquitoes are not separated despite such disparities in their habitats. The expansions of larval forms, physiological and behavioral properties of adult mosquitoes are influenced by biological elements, both biotic and abiotic. For example, as larvae mosquitos secure and store resources, and the number of nutrients will influence how adult females seek sustenance and blood meals (Chandrasegaran *et al*., 2020).

Changes in disease vector survival and pathogen development, as well as newly emergent sanitary and environmental concerns are all closely linked to a variety of socioeconomic consequences as a result of global warming (Vora, 2008). Numerous environmental and socioeconomic changes, as well as increasing populations of people and animals (both domestic and peridomestic) may all contribute to the spread

of vector-borne diseases, according to recent studies (Gubler, 2011; White, 2016).

Predation, parasitism, interspecific competition, and ecosystem engineering are a few examples of the biotic interactions between invasive species, and ecosystems (Williamson, 1996). Potential biotic difficulties of some invasive species include harm to human and animal health. Native or non-native invasive vector diseases may directly affect human health, and may change the cycles of pathogen transmission (McMichael and Bouma, 2000).

Mosquito invasions that have affects the most prevalent category of disease-carrying insects are the result of ecological interactions. Invading mosquitoes have detrimental effects on **i)** resident species or ecosystems, and **ii)** human or vertebrate animal health, both of which are usually negative. The first illustrates a set of impacts that may be experienced by any invading species. The second category is a subset of impacts that are primarily relevant to vectors. The two types of mosquito impacts are thought to be caused by different life cycle stages. While the terrestrial adult phase provides a health danger to people, the aquatic larval phase is more likely to interact with and influence other species (Lounibos, 2002).

Multiple hypothesis concerning the biological processes occurring during mosquito invasions have been explored in numerous sites for one well-studied invasive species (*Aedes albopictus* Skuse, Asian tiger mosquito). We categorize ecological processes into two groups**: i)** Species interactions that may influence invasions by altering resident species or acting as barriers to invaders, and **(ii)** climate factors that may promote invasion success. Inter-specific competition, predation, and apparent competition via shared diseases are the most studied species interactions. The term "vector control" refers to a sort of applied population dynamics that falls within the applied ecology umbrella (Sakai *et al*., 2001).

1.4: ECONOMIC IMPACT OF MOSQUITOES

Mosquito larvae are aquatic insects that are crucial to the aquatic food web. It eats by collecting small natural particles from the water, such as unicellular green algae, and storing them in their body tissues, which are then eaten by fish. The larvae of mosquito are essentially nutrient-rich appetizers for fish and other marine animals. Furthermore, mosquito hatchlings consume excrement which supplies nutrients like

nitrogen to the plant ecology, but some mosquito species feed on insect carcasses found in water (Endy and Nisalak, 2002).

As a result, the extinction of some mosquito species may have an impact on how plants grow in particular areas. For birds, bats, spiders, and mosquito larvae are just as nutritious as adult mosquitoes are. It appears that mosquitoes are a significant source of food for animals at the base of the food chain. Only a few mosquito species, females need blood to generate the proteins they need to lay eggs. Adult mosquitoes, both male and female, rely on nectar to stay alive for the most part. Mosquitoes pollinate plants while gathering nectar, ensuring the survival of a diverse spectrum of plant life. Mosquitoes pollinate plants which helps them survive especially those near the seaside, where they spend a lot of time. Other living creatures and species seek refuge and protection amid these plants (Oki *et al*., 2011).

Dengue fever, an endemic and epidemic mosquitoes-borne illness that is a fast-expanding public health burden in tropical and sub-tropical nations has multi-country economic repercussions. Mosquitoes spread the diseases which affects people of all ages and socioeconomic backgrounds (Knerer *et al*., 2015).

Dengue affects over 2.5 billion people worldwide or two thirds of the world's population, and 120 million people travel to dengue-affected areas every year. The World Health Organization estimates that each year between 50 and 100 million people become infected with the number rising as a result of the expansion of the human population and the spread of vector mosquitoes. Unlike malaria, dengue fever is more common in cities than in rural areas. Dengue fever causes a severe headache, muscular and joint discomfort, diarrhea, vomiting, and a high fever that lasts about a week. It can proceed to dengue hemorrhagic fever, which is fatal in a tiny percentage of individuals (Bouzid *et al*., 2016).

1.5: MOSQUITOES GENERAL MORPHOLOGY

Mosquitoes are symmetrical on both sides, as are other arthropods. An adult mosquito body is made up of the head, thorax, and abdomen. An exoskeleton covers each of these areas to provide protection. Large compound eyes and an oval-shaped cranium are both present. Its five appendages are the proboscis, two maxillary palps, and two antennae. The three body sections that make up the thorax,

or the region between the head and the abdomen, are the prothorax, mesothorax, and metathorax. Each segment has two operable mesothorax wings, two metathorax halters, and two jointed legs (Rattanarithikul *et al*., 2005).

Three of the 10 regions that make up the abdomen are used for reproduction and excretion. The aquatic immature stages of mosquitoes are comparable to those of the Chironomidae, Dixidae, Chaoboridae, and other Nematocera families, which have mosquitoes-like adults. The veins and wing margins of mosquitoes have scales, which set them apart from other dipterous insects. They also have a large proboscis that projects forward and is designed for piercing and sucking (Darsie and Ward, 2005). In contrast to an adult, the larva body is mostly made up of firm sclerotized plates in the head and soft membrane tissues in the thorax and abdomen. This facilitates body doubling and swimming motions when using palatal brushes or cleaning the mouth (Gadahi *et al*., 2012).

Mosquito larvae and pupae are freshwater organisms that are main components of the food chain and serve as indicators of the quality of the water (Qasim *et al*., 2014). Numerous mosquito species, including those of the genus *Aedes* are adapted to their particular habitats, such as cooler climates where their eggs are more prevalent than in warmer areas (Kumar and Nattuthurai, 2011). Species differences in mosquito behavior are evident. Some species are active both during the day and at night, whereas many species only emerge at night. The temperature has an impact on mosquitoes eating patterns as well as where they reproduce (Steib *et al*., 2001).

Almost any animal that can feed them with a blood meal will cause a mosquito to bite. In disease transmission, mosquitoes host selectivity for blood-feeding is critical. Mosquitoes can be classified using morphology-based identification keys, male and female species can be recognized by their wings and palps (Shepard *et al*., 2006).

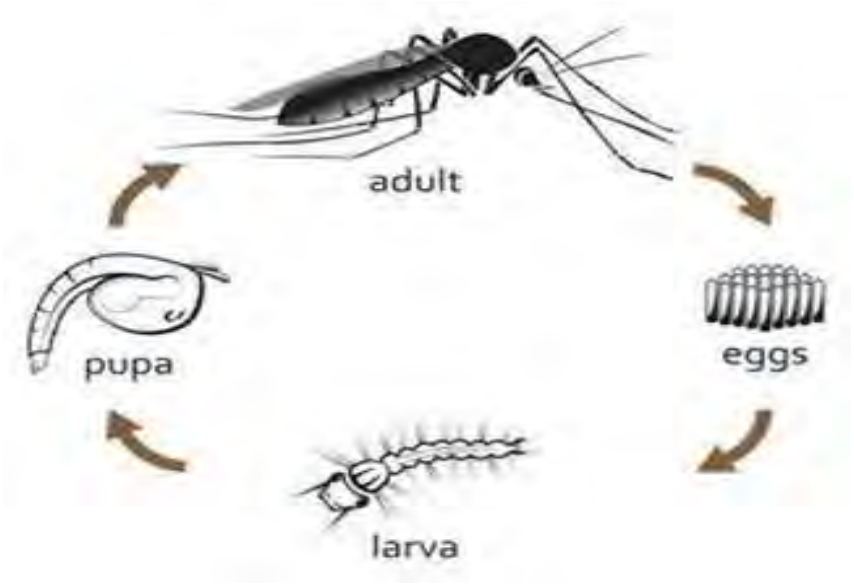


Figure 1.1: Life cycle of Mosquito (Abd, 2020)

1.6: BIOLOGY OF MOSQUITOES

Mosquitoes have four unique life stages: egg, larva, pupa, and adult. Their development is holometabolous. Mosquito larvae and pupae need a place with standing or moving water to develop properly. The female adult lays up to several hundred eggs at a time, either in single eggs (like those laid by *Aedes* or *Anopheles*) or in clusters, on the water's surface, on the upper surface of floating vegetation, along the margins of quiet water pools, on the walls of artificial containers, or in moist habitat subject to flooding (e.g., *Culex*, *Culiseta*). Before becoming pupa, the larvae, often known as wrigglers, shed (or moult) their skin four times (Harris *et al*., 1969).

Most species' larvae filter out organic detritus and other bacteria and feed on them in the water for 1–3 weeks or more depending on the water temperature. The larvae of the mosquito predator species Toxorhynchites and Lutzia eat the larvae of other mosquitoes. The earliest instars of several predatory species are filter feeders with predaceous feeding mechanisms only developing in the second instar. After the fourth larval moult, the pupae (sometimes known as tumblers) or resting stage appears (Rueda *et al*., 2001).

Pupae do not eat and exist for one to three days before becoming adults, in contrast to larvae. Humans and animals are only bitten by adult female mosquitoes.

Male mosquitoes eat largely flower nectars, whereas female require a blood meal to lay healthy eggs. Some species (Anthropophilous) like to eat wild humans, while others (Zoophilous) prefer to eat other wild animals like bees and birds. Blood is not consumed by female Toxorhynchites (Harwood and James, 1979).

Certain autogenous females can generate viable eggs without a blood meal. Females feed every 3–5 days and during each feeding, a female usually consumes more blood than she weighs. Some mosquito species (such as *Anopheles*) prefer to feed during dusk, twilight, or overnight. Those species that exchange hosts on a seasonal basis allowing disease transmission from animals to humans (called zoonotic disease transmission). Diapause occurs in a variety of life stages, including eggs in *Psorophora* and most *Aedes* larvae in *Coquillettidia*, *Culiseta*, *Mansonia*, *Orthopodomyia*, *Toxorhynchites*, *Wyeomyia*, and adults often fertilized females in *Uranotaenia* (Stojanovitch and Scott, 1997).

Mosquitoes like other biting arthropods search for a suitable host using a combination of visual, thermal, and olfactory signals. Although olfactory signals tend to be the most important as a mosquito approaches a host, visual stimuli appear to be crucial for in-flight orientation, particularly over long distances. For daylight biters, the host's activity may cause them to orient toward that person or animal. Out of the more than 300 compounds produced by the human body as by-products of metabolism, more than 100 volatile substances have been discovered in human breath. Mosquitoes can sense carbon dioxide which is released mostly by the skin and breathing. Mosquito attractants like carbon dioxide and octenol are frequently used in mosquito monitoring and surveillance in their natural habitats. Mosquito antennae feature chemoreceptors that are activated by lactic acid but can be inhibited by repellents. Repellents (such as deet or N, N-diethyl-3-methylbenzamide) can help to reduce or prevent the transmission of vector-borne diseases by providing effective personal protection against biting insects (Rueda *et al*., 1998).

1.7: HABITAT OF MOSQUITOES

Mosquitoes may live in a variety of environments due to their wide range of habitats. Mosquitoes larvae can be found in ponds, streams, ditches, swamps, marshes, temporary and permanent pools, rock holes, tree holes, crab holes, lake

margins, plant containers (leaves, fruits, husks, tree holes, bamboo nodes), and artificial containers (tyres, tin, cans, flower vases, bird feeders, and other habitats) (Laird, 1988).

The value of diverse habitats in regulating mosquito populations has long been recognized by aquatic ecologists and public health professionals. Furthermore, knowing where larvae live is crucial for vector management and disease prevention. It is essential for the creation of successful vector control programs. Eliminating as many of their habitats as you can, especially sources of standing water like old, abandoned tyres, clogged gutters, and stumped tree holes, among other things, is the most practical method for controlling a local mosquito population (Rueda *et al*., 2005).

Changes can be made to larval environments that cannot be eliminated (e.g., cleaning clogged ditches, open water management in salt marshes). Such techniques, such as biological control agents (predatory fish, microbiological), selective insecticides for permanent habitats (ponds, lakes), or other environmental modification tactics can be helpful for control of mosquito development (Rueda *et al*., 2006).

1.8: MORPHOMETRIC IDENTIFICATION

Infectious diseases like dengue fever, malaria, west Nile virus infection, and filariasis are spread by mosquitoes of the genera *Culex*, *Anopheles*, and *Aedes*. Collectively, these illnesses result in millions of passing annually and put at risk roughly 3 billion individuals throughout the world residing in endemic regions. The reemergence of illnesses follows the expansion of mosquito geographic distribution, making it critical to reliably distinguish epidemiologically important mosquito species (Lorenz *et al*., 2017).

1.8.1: Importance of Identification

Mosquito species identification is critical for determining epidemiological patterns of illness transmission, which are linked to the quantity, infectivity, vector capacity, and competence of the vector mosquitoes. The biological sciences have used body form research to better comprehend scientific classification, evolution, and systematics (Ballard *et al*., 2009).

1.8.2: Geometric Morphometric Approach

The geometric morphometric is a valuable method for distinguishing species based on morphological characters. Since the current accessibility of inexpensive computational control, a specific programme, and digitizing applications affordable indeed for less experienced clients, it is a convincing and typically simple method that has gained popularity. The GM approach may be an effective instrument for assessing relationships between shape and natural or hereditary factors. For mosquitoes and other insects, this is especially true. GM considers the frame of natural structures in two or three spatial dimensions, provides a few quantifiable assessments, and allows for graphical representation of shape and estimate. The shapes of life forms may show either similitudes or contrasts, depending on sex, topographical area, phylogenetic relationship, environmental connections, and sorts of medications endured. It is now possible to differentiate morphological variants and examine their causes both within and between populations thanks to genetic modification (Lorenz *et al*., 2015).

Morphometric has also been used to differentiate complicated species that are difficult to distinguish using traditional morphology. GM may not be as accurate as hereditary sequencing in all instances of species recognition proof, but the results have demonstrated that it can still be quite instructive. Because it is a quick and inexpensive technique, even untrained operators can use it in the field to solve biological and ordered problems. The analyzed mosquitoes may speak to diverse genotypes and likely come from one separate quality pool, thus the geometric morphometric analyses the form contrasts of a morphological highlight using competent and thorough factual procedures (Mondal *et al*., 2015).

1.9: VECTOR CONTROL STRATEGIES

Effective vector control techniques continue to be the dominant strategy for mosquitoes-borne disease control and prevention, despite significant advancements in the creation of medicines and vaccines for illnesses brought on by mosquitoes over the past few decades (Amer and Mehlhorn, 2006).

Vector-based therapies are the most effective approaches for lowering the public health burden of most mosquitoes-borne diseases. Before the development

of pesticides, these interventions depend on environmental management with an emphasis on eliminating mosquito breeding areas and improving housing with screens to prevent mosquitoes from entering through doors and windows. DDT, dieldrin, and other pesticides were developed after WWII for indoor and outdoor use, and insecticides were later included in bed nets (Wilson *et al*., 2020).

Insecticides of various kinds have typically been used in mosquito management to eradicate mosquitoes. In places where endemic mosquito-borne diseases exist, environmental management (via the decrease or elimination of mosquitoes breeding sites) is frequently utilized alongside chemical or microbiological ovicides, larvicides, and pupicides (Semmler *et al*., 2009; Benelli, 2015; Liu, 2015). Because insecticide resistance is widespread (Hemingway and Ranson, 2000; Strode *et al*., 2014; Naqqash *et al*., 2016; Ranson and Lissenden, 2016), and there is concern about environmental damage and effects on non-target organisms, the use of synthetic insecticides must be regulated. Despite these advancements and ongoing efforts to control mosquitoes, epidemics and the spread of mosquitoes-borne diseases continue to endanger the health of billions of people around the world and obstruct economic progress (Sarma *et al*., 2019).

Vector control products have been used to reduce the spread of disease agents transmitted by *Aedes* mosquitoes, just as they have been used to limit the transmission of malaria, and there is worry regarding the continued use of existing methods against these mosquitoes. High prices, poor community adoption, and slow operational implementation make larviciding or space spraying of pyrethroids and organophosphates difficult, and pesticide resistance in *Aedes* is already common (Moyes, 2017; Weetman *et al*., 2018).

Numerous mosquito species have developed insecticide resistance, prompting the creation of creative, affordable, and efficient mosquitoes control methods (Benelli, 2015). The price burden of insecticide-based vector control programs is prohibitive to widespread adoption in many areas where mosquitoes-related diseases are endemic. To assist lessen the selection pressure for pesticide resistance; environmentally friendly alternatives have been investigated. These varied biocontrol techniques are designed to target distinct stages of the mosquito lifecycle while being environmentally friendly and long-lasting. Natural creatures

that kill mosquitoes, force mosquito behavior to improve mosquito mortality, and releasing mosquitoes that are either sterile or unable to spread illness are among the several biocontrol tactics (Jeffries and Walker, 2015; Yakob and Walker, 2016).

Mosquitoes control can be divided into three categories: prevention, source reduction, larviciding, and adulticiding. The most desirable method is to prevent mosquitos from breeding. This fundamental distinction has significant implications for vector management techniques, such as global control programs that may be applied at a continental size in the former situation, and regional strategies for mosquitoes in the latter one. **1)** Physical control, such as the destruction of larval homes by environmental alterations, **(2)** chemical control, such as the use of insecticides, and **(3)** biological control, such as the use of biolarvicides, are the three main ways used to control vectors (Robert *et al*., 2011).

1.9.1: Physical control

Physical control can be achieved by removing larval habitats (containers, water deposits), which is particularly effective against *Aedes* and *Culex* species. Drainage operations, intermittent irrigation, changes in river discharge or layout, and the removal of water plants or algae can all modify larval environments, making them unsuitable. There have been a few promising results against *Anopheles* mosquitoes, but they require significant technical efforts (Mouchet, 2000).

1.9.2: Chemical control

Chemical control is based on material impregnation with insecticides, as well as regular spraying of insecticides/larvicides by terrestrial or airways according to the nature of the habitats and their size, which is inevitably challenged by insecticide resistance and off-target effects on other arthropod species. Insecticide-treated nets (ITNs), long-lasting insecticidal nets (LLINs), and indoor residual spraying (IRS) are the most common vector control strategies against *Anopheles* mosquitoes, and they are still effective when used appropriately. Larviciding can also be used in unusual circumstances (well- known breeding sites mainly man-made). The use of different pesticides in a rotation or mosaic pattern can also be recommended to avoid (or at least postpone) resistance issues (Lenormand and Raymond, 1998).

Insecticides used to control mosquitoes, such as organophosphates, carbamates, and pyrethroids, can have harmful consequences on human health. Vector control against *Aedes* mosquitoes based on insecticidal spraying (organophosphates, pyrethroids) utilizing hand-held gear; road vehicle or aircraft has some efficacy in conjunction with larval habitat clearance (Hemingway *et al*., 1997).

Indoor spraying is more ideal for *Ae. aegypti*, which prefers to rest within houses. Because of its capacity to breed away from humans, vector management for *Ae. albopictus* appears to be more challenging, yet its outside resting behavior makes outdoor spraying an effective control technique. The efficacy of two pyrethroids insecticides, alphacypermethrin, and lambda-cyhalothrin, when applied uniformly to the inside surfaces of either dry tyres partially filled with water 24 hours after spraying or wet tyres partially filled with water prior to spraying, in preventing mosquito larval colonization in abandoned car tyres was demonstrated. Both insecticides, when used in either application mode, have a high potential for preventing invasion by *Ae. aegypti* and *Ae. albopictus* (Pettit *et al*., 2010).

1.9.3: Biological control

Predatory or parasitic organisms that target disease vectors are used in biological control. Furthermore, removing habitats from the environment can aid in disease vector population decrease. These diverse vector control tactics necessitate an understanding of the biology of the targeted mosquito species, the pharmacological actions of the insecticides used, or the behavior of the introduced predatory or competitive species to quickly eradicate mosquito larvae or adults. Indoor residual spray, for example, is more likely to help control indoor species like *Ae. aegypti*, but has little influence on mosquito populations of diverse *Culex* species that rest live outdoors (Blanford *et al*., 2005; Scholte *et al*., 2009).

Larvivorous fish (Gambusia, Poecilia), predatory insect larvae (dragonfly, toxorhynchites mosquitoes), copepods, nematode worms, fungi, and bacterial larvicides have all been found to be effective against mosquito larvae. *Bacillus sphaericus* is commonly utilized against *Culex* species (Skovmand *et al*., 2009), but mega doses of *Bacillus thuringiensis* subspecies israelensis (Bti) dry formulations demonstrated effective for residual control of *Aedes aegypti* in small containers

(Ritchie *et al.,* 2010). Recent research has also indicated that using entomopathogenic fungus could be a useful technique with the added benefit of being evolution-proof (Read *et al*., 2009).

1.9.3.1: Plant-based control of Mosquitoes

1.9.3.1.1: Plant-Borne Mosquitocides, Repellents, and Oviposition Deterrents

Exploring floral biodiversity and entering the field of employing safer insecticides of botanical origin as a simple and sustainable means of mosquito control is one of the most effective alternative approaches under biological control programs. In addition, unlike traditional insecticides, which are made up of a single active component, plant-derived insecticides are made up of botanical blends of chemical compounds that work together to affect both behavioral and physiological processes. As a result, pests are unlikely to acquire resistance to such compounds. Identification of efficient bio-insecticides that are also suited and adaptable to ecological settings is critical for sustained effective vector control management. Botanicals offer a wide range of insecticidal qualities and will undoubtedly become a new weapon in the arsenal of synthetic pesticides, as well as a viable alternative solution to combat mosquito-borne diseases in the future (Singh *et al*., 2006).

Roark (1947) classified and discussed roughly 1200 plant species with insecticidal potential, while Sukumar *et al.* (1991) listed and discussed 344 plant species with only mosquitocidal activity. The current state of knowledge on larvicidal plant species, extraction processes, growth and reproduction inhibiting phytochemicals, botanical ovicides, synergistic, additive, and antagonistic joint action effects of mixtures, residual capacity, non-target organism effects, resistance, and screening methodologies, and discussed some promising advances in phytochemical research. Plant-borne compounds, in particular, are generally effective against *Aedes, Anopheles*, and *Culex* immature larval instars at a few parts per million (ppm). Currently, over 80 plant species have been used to successfully synthesize nanomosquitocides, with a focus on larvicidal applications (Shaalan *et al*., 2005).

Botanicals can also be used as reducing and capping agents in the quick synthesis of mosquitocidal nanoformulations, as well as to make low-cost repellents with low human toxicity (Russell *et al*., 2009). Much remains to be discovered about

this fast-growing research area, particularly with regard to the following topics: **i)** chemical characterization and standardization of plant-borne botanicals used for Nano-biosynthesis **(ii)** the potential of plant-synthesized nanoparticles as mosquitoes ovicides and ovideterrent (Sakthivadivel and Daniel, 2008) and **(iii)** the potential of plant-synthesis (Karmegan *et al*., 1997) **(iv)** the biofabrication of nanomosquitocides using industrial by-products of plant origin (e.g., neem cake) (Bhatt and Khanal, 2009), **(v)** Field study of green nanoparticles' mosquitocidal activities against Culicidae (Rahuman and Venkatesan, 2008; Maurya *et al*., 2009), **(vi)** the non-target impacts and environmental fate of mosquito vector-killing plant-derived nanoparticles (Rahuman *et al*., 2007). Because natural plant products include a plentiful supply of bioactive compounds that might be utilized in integrated management programmes, they have been considered as prototypes for new pesticide agents (Iqbal *et al*., 2022).

Botanicals that are naturally occurring insecticides acquired from floral resources are known as phytochemicals. Botanicals are secondary metabolites that help plants endure constant selection pressure from herbivore predators and other environmental variables (Akpaso *et al*., 2011). Phytochemicals were taken from the full body of small herbs or various sections of bigger plants or trees, such as fruits, leaves, stems, barks, roots, and so on. The most dangerous chemicals were concentrated on, located, and extracted for mosquito control in all cases. Plants produce a wide range of compounds, many of which are therapeutic or pesticide. There are around 2000 plant species that produce chemical components and metabolites that are useful in pest management programs. Solanaceae, Asteraceae, Cladophoraceae, Labiatae, Miliaceae, Oocystaceae, and Rutaceae have distinct forms of larval, adulticidal, and repellent actions against different mosquito species (Anbuselvi *et al*., 2012).

Phytochemicals such as alkaloids, steroids, terpenoids, essential oils, and phenolics from various plants have previously been found to have insecticidal properties. To extract mosquito poisons, a variety of plants including herbs, shrubs, and huge trees were used. The insecticidal capabilities of plant extracts vary based on the plant species, mosquito species, regional differences, and components used, as well as the extraction methods and polarity of the solvents used during extraction (Hamzah *et al*., 2013).

1.9.3.1.2: Mosquitoes Predators

Natural enemies in aquatic habitats that feed on mosquito larvae and pupae can help to reduce Culicidae numbers (Kumar and Hwang, 2006; Louca *et al*., 2009). Indeed, mosquitoes young instars are preyed upon by a variety of aquatic organisms, including fish (Kamareddine, 2012; Subramaniam *et al*., 2016), amphibians (Brodman and Dorton, 2006; Bowatte *et al*., 2013), copepods (Vu *et al*., 1998; Schaper, 1999), odonate young instars (Singh *et al*., 2003), water bugs (Venkatesan and Jeyachandra, 1985; Bailey, 1989; Cloarec, 1990; Singh *et al*., 2003), and even larvae of other mosquitoes species (Steffan and Evenhuis, 1981). The importance of larvivorous fish in mosquito biological control has largely been overlooked. Larvivorous fish eat mosquito larvae in the water (Griffin and Knight, 2012).

Fish predation of mosquito larvae has been documented in a variety of habitats, ranging from small plastic containers (Connor, 1922) to complex natural ecosystems, such as coastal wetlands (Harrington and Harrington, 1982). Larvivorous fish are particularly successful at reducing mosquito larvae populations in a range of ecosystems around the world (Van and Walton, 2007). More than 60 countries have introduced larvivorous fish from the genus Gambusia and Poecilia (Poeciliidae) for mosquito control (Das and Prasad, 1991; Walton, 2007; Ohba *et al*., 2010; Kweka *et al*., 2011; Chobu *et al*., 2015). Larvivorous fish, on the other hand, are frequently regarded as a threat to native aquatic fauna, especially frogs (Rupp, 1996; Kats and Ferrer, 2003), underlining the importance of carefully considering the ecological cost of adding predatory species to help with mosquito control (Chitra *et al*., 2013).

A variety of omnivorous copepods (small aquatic cyclopoid crustaceans) can eat mosquito larvae in their early stages (Marten *et al*., 1989). Several copepod species have been identified as active predators of mosquitoes juvenile instars, including *Cyclops vernalis*, *Megacyclops formosanus*, *Mesocyclops (M.) aspericornis, M. edax, M. guangxiensis, M. longisetus, and M. thermocyclopoides* (Rawlins *et al*., 1997; Manrique-Saide *et al*., 1998; Mahesh *et al*., 2012; Murugan *et al*., 2015; Anbu *et al*., 2015; Chandramohan *et al*., 2016). The largest and most successful use of copepods for mosquito control was in Vietnam, where the main vector of dengue virus (DENV), *Ae. aegypti* was targeted (Nam *et al*., 2005).

Some Culicidae species have larvae that prey on other mosquito species that are important vectors for public health. Toxorhynchites (T.) is a big, worldwide mosquito genus that does not ingest blood. It is also known as the "elephant mosquito" or "mosquito eater" (Rawlins *et al*., 1991; Aditya *et al*., 2006). The larvae prey on the larvae of other mosquitoes as well as other nektonic (free-swimming) creatures, while the adults feed on sugar-rich items like honeydew, fruit, and nectar (Soumare and Cilek, 2011). Toxorhynchites are mostly found in woods; however, one jungle species, *T. splendens,* feeds on mosquito larvae in tree cracks (particularly those belonging to the genus *Aedes*). Adult Toxorhynchites are larger than *Aedes* and are considered harmless to people since they do not feed on blood (Kumar *et al*., 2016). The potential for anurans (especially frogs and toads) to reduce mosquitoes has gotten very little attention (Marian *et al*., 1983; Raghavendra *et al*., 2008). Tadpoles with varied life histories, for example, actively prey on the eggs of *Ae. aegypti*. This mosquito species prefers to lay eggs in tadpole water, and tadpoles of *Polypedates cruciger*, as well as those of the *Bufo*, *Ramanella*, *Euphlyctis*, and *Hoplobatrachus* genera, have been shown to feed on the eggs (Kay *et al*., 2010).

1.9.3.1.3: *Bacillus thuringiensis israelensi*s (Bti) and Entomopathogenic Fungi

Biocontrol measures can also include naturally occurring organisms that are pathogenic to mosquitoes. *Bacillus thuringiensis var. israelensis* (Bti) is the most widely used mosquito larvicides in Europe right now. Bti is a gram-positive, spore-forming bacteria that produce insecticidal toxins and virulence factors that are specific for insect larvae (Becker, 1997; Lacey, 2007). Bti has been used to reduce the number of *Ae. aegypti* (Novak *et al*., 1985; Armengol *et al*., 2006; Ritchie, 2010) and *Ae. albopictus* larvae (Lam *et al*., 2010), but longer-term use is subject to the development of Bti toxins resistance (Georghiou and Wirth, 1997), and the use of Bti in large mosquitoes breeding sites in urban environments is logistically demanding (Gómez-Dantés and Willoquet, 2009).

Infectious spores (conidia) produced by entomopathogenic fungus attach to and penetrate the cuticle of mosquitoes, releasing poisons that cause mosquitoes death (Scholte *et al*., 2004). Several researches on malaria mosquito vectors (Blanford *et al*., 2005; Knols *et al*., 2010) and *Ae. aegypti* have shown the harmful effect (Paula *et al*., 2011; Darbro *et al*., 2012). Because adult mosquitoes are the primary target of

entomopathogenic fungi, and various distinct toxins released during fungal infection are fatal to mosquitoes. When compared to insecticides that kill quickly, the selection pressure for resistance is likely to be lower (Scholte *et al*., 2007).

Table 1.1: Plants used against various mosquitoes spp. reported from previous studies.

Plant species	**Family**	**Plant parts used**	**Target mosquito species**	**References**
Acacia nilotica	Fabaceae	Leaf	*An. stephensi*	Saktivadivel and Daniel, 2008
Aloe barbadensi	Liliaceae	Leaf		
Argemone Mexicana	Papaveraceae	Leaf, seed		
Eucalyptus globulus	Myrtaceae	Seed, Leaf	*Cx. pipiens*	Sheeren *et al*., 2006
Citrus aurantium	Rutaceae	Fruit peel	*Cx. quinquefasciatus*	Kassir, 1989
Argemone Mexicana	Papaveraceae	Leaf	*Cx. quinquefasciatus*	Karmegan *et al*., 1997
Piper nigrum	Piperaceae	Seed	*Cx. pipiens*	Shaalan *et al*., 2005
Euphorbia hirta	Euphorbiaceae	Stem Bark	*Cx. quinquefasciatus*	Rahuman *et al*., 2007
Ocimum basilicum	Lamiaceae	Leaf	*An. stephensi* and *Cx. quinquefasciatus*	Maurya *et al*., 2009
Momordica charantia	Cucurbitaceae	Fruit	*An. stephensi, Cx. quinquefasciatus, Ae. aegypti*	Singh *et al*., 2006
Curcuma	Zingiberaceae	Rhizome	*Ae. aegypti*	Choochate

aromatic				*et al*., 2005
Solanum nigrum	Solanaceae	Dried fruit	*An. culicifacies, An. stephensi, Cx. quinquefasciatus, Ae. aegypti*	Raghavendra *et al*., 2009
Tridax procumbens	Compositae	Leaf	*An. subpictus*	Kamaraj *et al.,* 2011
Plumbago zeylanica, P. dawei and *P. Stenophylla*	Plumbaginaceae	Root	*An. gambiae*	Maniafu *et al.,* 2009
Euphorbia tirucalli	Euphorbiaceae	Latex and stem bark	*Cx. pipiens Pallens*	Yadav *et al.,*2002
Nyctanthes Arbortristis	Nyctantheceae	Flower	*Cx. quinquefasciatus*	Khatune *et al.,* 2001
Citrus sinensis	Rutaceae	Fruit peel	*An. subpictus*	Bagavan *et al.,*2009
Cassia obtusifolia	Leguminosae	Seed	*Ae. aegypti*, *Ae. togoi*, and *Cx. pipiens pallens*	Yang *et al.,* 2003
Melia azedarach	Meliaceae	Leaf and seeds	*An. stephensi*	Nathan *et al.,* 2006
Moringa oleifera	Moringaceae	Bark	*Cx. gelidus*	Kamaraj and Rahuman, 2010
Solenostemma argel	Apocynaceae	Aerial parts	*Cx. pipens*	Al-Doghairi *et al.,* 2004
Chrysanthemum Indicum	Asteraceae	Leaf	*Cx. tritaeniorhynchus*	Kamaraj *et al.,* 2010
Coccinia indica, Cucumis sativus,	Cucurbitaceae	Leaf	*Ae. albopictus*	Rahuman and Venkatesan, 2008

Momordica charantia				
Azadirachta indica	Meliaceae	Leaf	*Cx. fatigans*	Azmi *et al.,* 1998
Piper retrofractum	Piperaceae	Unripe and ripe fruit	*Cx. quinquefasciatus*	Chansang *et al.,* 2005
Rhizophora Mucronata	Rhizophoraceae	Bark, pith, stem Wood	*Ae. aegypti*	Kabaru and Gichia, 2001
Piper Longum	Piperaceae	Fruit exocarp	*Ae. aegypti*	Chaithong *et al*., 2006
Annona crassiflora	Annonaceae	Root wood, root bark, stem	*Ae. aegypti*	De Omena *et al.,* 2007
Tagetes minuta	Asteraceae	Aerial parts	*Ae. fluviatilis*	Macedo *et al.,* 1997
Citrullus vulgaris	Cucurbitaceae	Leaf	*Ae. stephensi*	Mullai *et al.,* 2008
Dysoxylum beddomei *Aloe turkanensis*	Meliaceae	Leaf	*An. gambiae*	Matasyoh *et al.,* 2008
Carica papaya	Caricaceae	Seed	*Cx. quinquefasciatus*	Rawani *et al.,* 2009
Paullinia Clavigera	Sapindaceae	Leaf	*An. benarrochi*	Iannacone and Pérez, 2004

1.10: TECHNIQUES

Purification and isolation of bioactive chemicals from plants is a technology that has seen a lot of progress in the last few years (Mulinacci *et al*., 2004). On the one hand, this new technique allows researchers to keep up with the development and availability of many complex bioassays, while on the other, it provides precise

isolation, separation, and purification techniques. When looking for bioactive compounds, the goal is to design a method that can screen the source material for bioactivities, such as cytotoxicity, antioxidant, antibacterial, while also being simple, specific, and fast (Altemimi *et al.,* 2015).

Because animal tests are costly, time-consuming, and willing to ethical difficulties, in vitro procedures are frequently preferred over in vivo trials. Some obstacles make finding final methodologies or protocols to isolate and identify certain bioactive compounds impossible (Sarajlija *et al*., 2012). Isolation and purification of bioactive substances can be accomplished using column chromatographic methods. The purified compounds can be identified using a variety of spectroscopic techniques such as Phytochemical characterization, Ultraviolet-Visible spectroscopy (UV-Vis), Fourier transform infrared (FT-IR), Gas Chromatographic Mass Spectrometer (GC-MS), and molecular docking (Popova *et al*., 2009).

1.10.1: Purification and Characterization of the Bioactive Molecule

To assess the chemical fractions, silica gel, thin-layer, and column chromatography technologies have historically been used. Many bioactive substances have been discovered and purified. Because of their ease, economy, availability in diverse stationary phases, column chromatography, and thin-layer chromatography (TLC) are still widely utilized. The most useful materials for separating phytochemicals are silica, alumina, cellulose, and polyamide. Plant materials include a high concentration of complex phytochemicals, making separation difficult. As a result, using multiple mobile phases to increase polarity is beneficial for highly valued separations (Zhang *et al*., 2005).

1.10.2: Clarification of the Bioactive Molecules' Structure

Data from a variety of spectroscopic techniques, such as Ultraviolet visible and infrared spectroscopy is used to determine the structure of particular compounds (FT-IR). The fundamental premise of spectroscopy is that electromagnetic energy is passed through an organic molecule, which absorbs some but not all of it. A spectrum can be created by measuring the amount of electromagnetic energy that is absorbed. The spectra are unique to each of a molecule’s bonds. The structure of the organic molecule can be determined using these spectra. For structural clarity, scientists

primarily utilize spectra from three or four regions ultraviolet (UV) visible and infrared (IR) (Kemp, 1991).

1.10.3: Phytochemical characterization

Photochemistry is a discipline of chemistry that deals with phytochemicals, or compounds that come from plants. Secondary plant metabolites are molecules with biological features such as antioxidant activity, antibacterial activity, detoxification enzyme modulation, immune system stimulation, platelet aggregation reduction, hormone metabolism regulation, and anticancer effects (Selvi *et al*., 2016). Alkaloids, tannins, flavonoids, and phenolic chemicals are the most important bioactive elements of plants (Hill, 1952).

1.10.4: Ultraviolet–visible spectroscopy

A spectrophotometer is a device that measures the transmittance or absorbance of a sample as a function of concentration and is used to examine both quantitative and qualitative chemicals (Theng and Korpenwar, 2015). Quantitative determination is based on the absorbance value created from the spectrum, while qualitative determination based on the peaks produced on the spectrum of a certain element at a specific wavelength. The absorption spectrum in the ultraviolet and visible light ranges is made up of one or more absorption bands. This transition occurs in the spectrum between 200 and 800nm (Perkampus, 2013).

1.10.5: Fourier-transform infrared spectroscopy

The FT-IR has proven to be a useful method for identifying and characterizing chemicals or functional groups (chemical bonds) in an unknown combination of plant extracts (Mariswamy *et al*., 2012; Maobe and Nyarango, 2013). It is a quick, non-destructive method that requires very little sample preparation. It allows for the qualitative identification of organic compounds by observing the appearance of bands in the infrared spectrum at a given frequency, which is modified further by the functional groups in the vicinity (Pramila *et al*., 2012; Murugan and Mohan, 2014).

1.10.6: Gas chromatography–mass spectrometry

Gas chromatography-mass spectrometry (GC-MS) is an instrumental technique that combines the features of gas chromatograph (GC) and a mass

spectrometer (MS) to separate, identify, and quantify complicated mixtures of compounds. It is suitable for analyzing the hundreds of low-molecular-weight chemicals prevalent in environmental materials because of this. A chemical must be sufficiently volatile and thermally stable to be examined by GC-MS (Dhar *et al*., 2013).

1.10.7: Molecular docking

A computer-assisted pharmaceutical technique in which two molecules are positioned in three dimensions is known as molecular docking. The molecular docking method may be used to describe the atomic level interaction between a small molecule and a protein, allowing us to characterize small molecule behavior in target protein binding sites and interpret critical biochemical processes. It is the most important tool in structural biology and antilarval chemical discovery. Molecular docking methods are commonly utilized in the contemporary drug design process to understand protein-ligand interactions. Predicting the ligand structure as well as its position and orientation within these sites (known as pose) and estimating the binding affinity are the two primary processes in the docking procedure (Arinaminpathy *et al*., 2009). The three-dimensional structure of the protein-ligand complex should help researchers better understand how proteins interact to perform biological functions. Because of its crucial function in the viral life cycle, the NS3 protein is an appealing molecular target for antiviral drugs. Because the active protease and helicase of NS3 are structurally similar, a drug that could change the dynamics of the multifunctional enzyme would be intriguing (Edris, 2007).

Bioactive phytochemicals from medicinal plants can be examined as antilarval medicines because they are freely accessible and affordable (Elango *et al*., 2012). There is a growing need to identify novel control measures that will stay effective even if insecticidal resistance develops against them (Achs and Malaney, 2002).

The aim of this study was to find out larvicidal product within the available natural resources, which is economical and safe in use and control of mosquitoes through natural plants.

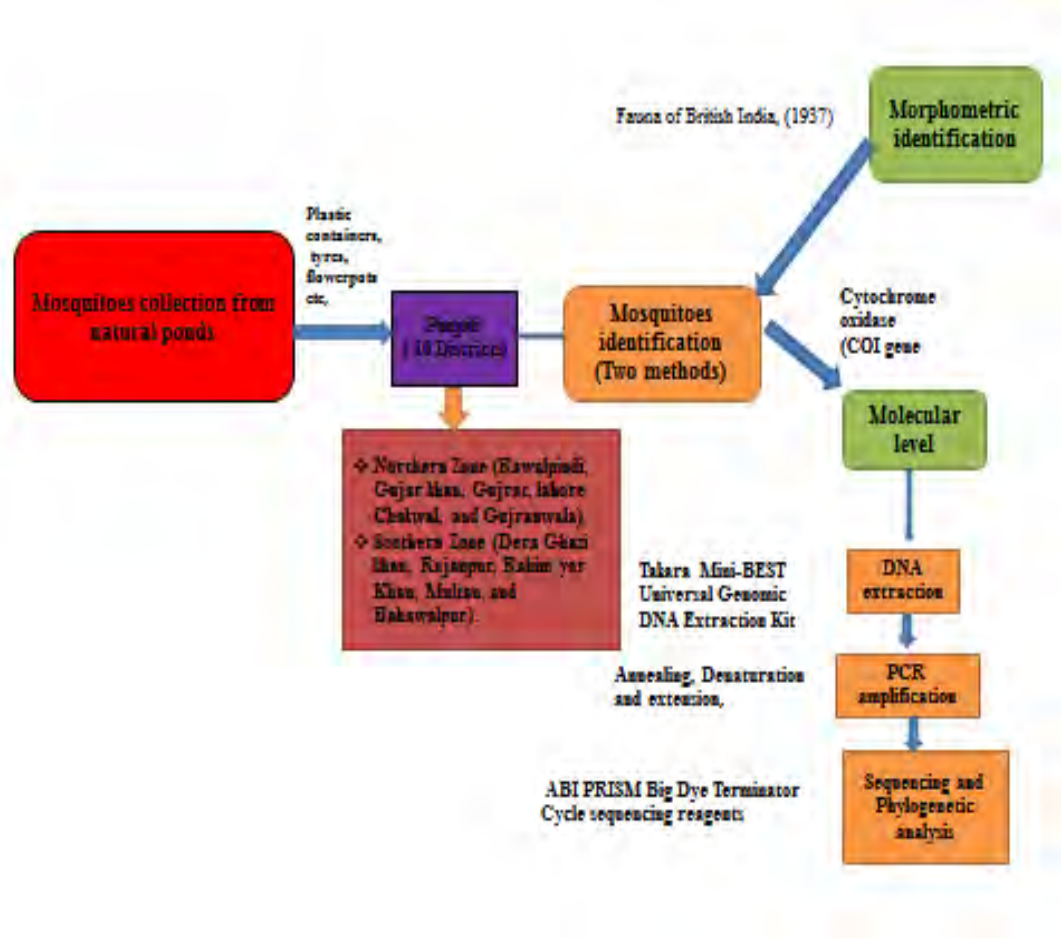
Objectives

- Molecular and morphological identification of Mosquitoes from Punjab, (Pakistan).
- Basic Screening of different plants extracts by conducting cytotoxicity, antilarval, and antioxidant bioassays.
- Column and thin Chromatography of most effective plant for isolation of bioactive fractions.
- Cytotoxicity, antilarval, antioxidant bioassays of isolated fractions to sort out bioactive compounds.
- Phytochemical characterization of bioactive fractions by using a preliminary phytochemical test, UV-VIS spectroscopy, FT-IR, GC-MS analysis, and Molecular docking.

CHAPTER 2

MORPHOMETRIC AND MOLECULAR CHARACTERIZATION OF MOSQUITOES FROM PUNJAB, PAKISTAN

GRAPHICAL ABSTRACT



ABSTRACT

Biodiversity of mosquitoes varied in all provinces of Pakistan. However Punjab is the largest subtropical region exhibiting a great variety of mosquitoes. The current study is based upon the morphological and molecular identification of mosquitoes from Punjab, Pakistan. The sampling includes dwellings, parks, junkyards, tyres, animal sheds, standing water, and woodland areas, etc. The study was done between 2016 and 2019 in summer (April-November). Adult mosquitoes were collected with sweep nets and aspirators preserved in 70% ethanol for morphological studies and in 95% ethanol for molecular identification. A stereoscopic trinocular microscope was used to examine and measure all main body parts and identified with the help of key "The fauna of British India" (1934). The results revealed morphological identification of 27 mosquito spp. belonging to two subfamilies (Anophelinae and culicinae) and five genera of *Anopheles* (*An. culicifacies, An. annularis, An. subpictus, An. stephensi, An. maculatus, An. sinensis , An. fluviatilis, An. splendidus koidzum, An. tesselatus, An. aconitus)*, *Culex* (*Cx. quinquefasciatus, Cx. tritaeniorhynchus, Cx. vagans, Cx.vishnui, Cx. fatigans , Cx. raptor, Cx. pipiens, Cx. gelidus, Cx. bitaeniorhynchus, Cx. fuscocephala)*, *Aedes* (*Ae. agypti , Ae. albopictus), Mansonia* (*Ma. uniformis)* and *Armigeres (Ar. subalbatus, Ar. obturbans, Ar. flavus,* and *Ar. kuchingiensi*). The molecular identification was based upon the amplification of COI genes which confirmed three species belonging to two genera *Anopheles* (*An*. *gambiae*) and *Aedes* (*Ae. aegypti* and *Ae. albopictus*). Taxonomic classification and molecular study are helpful for classifying mosquito species.

INTRODUCTION

Mosquitoes are monophyletic taxon of order Diptera, suborder Nematocera , and family Culicidae (Ilahi and Suleman, 2013) are responsible to spread a variety of diseases such as west Nile virus, yellow fever, filariasis, dengue fever, and malaria (Charrel *et al*., 2007; Stark and Schöneberg, 2012). Out of 3500 mosquito species recognized worldwide (www.mosquito-taxonomicinventory.info), 104 have been renowned in Pakistan and Bangladesh (AslamKhan, 1971), and have been classified into 41 genera (Aina *et al*., 2009).

` In Punjab and Pakistan, three mosquito genera dominate: *Culex*, *Anopheles,* and *Aedes* (Jahan and Hussain, 2011). The genus *Anopheles* (Weeraratne *et al*., 2017), in the oriental region discovered 45 species and eight genera and 440 species of Culicinae may also be detected (Knight and Stone, 1978).

Pakistan has 64 species of the culicinae subfamily and 23 species of *Anopheles*. On e species each belongs to the genera Tripteroides, Ficalbia, Coquilletidia, Uranotaenia, and Armigere, while two species each belong to *Mansonia*, three (*Culiseta)*, 24 (*Culex)*, and 3 0 (*Aedes)* (Aslamkhan, 1971).

Different mosquitoes species have been reported from different regions of the world like 113 species from Bangladesh (Ameen and Moizuddin, 1973; Ahmed, 1987); 22 species of *Culex, Aedes,* and *Anopheles* from India (Sathe, 2011); southern Israel (25) (Margalit *et al*., 1987); Egypt (39) (Glick, 1992); Lombok Island (41) (Arifin and Dachlan, 1994); Turkey (21) (Sengil *et al*., 2011); Thailand (384), Malaysian (249) (Tsukamoto *et al.,* 1987); the Island of Japan (20) (Toma *et al.,* 2011); Guatemala (124) (Clark-Gil and Darsie, 1983); Philippine (309) (Tsukamoto *et al.,* 1985) and Vietnam (191) (Phuong and Darsie, 2008). Bangladesh has discovered 34 and three *Anopheles* species in Iran (Banafshi *et al*., 2013).

Barraud (1934) and Christopher (1933) described 252 *Culex* and 43 *Anopheles* species of mosquitoes in the Indian subcontinent including 66 *Culex* and 34 *Anopheles* species in Pakistan. Three *Culex* and one *Anopheles* species were added to the mosquito

fauna of Pakistan from 1934 to 1971 (Khan, 1971). Khan (1972) reported 16 valid species of mosquito from Pakistan. Twenty-four Anopheline (seventeen species of subgenus *Cellia* and seven of subgenus *Anopheles*) were recorded from Pakistan (Amerasinghe *et al.,* 2002).

Suleman *et al*. (1977) reported 30 species of mosquitoes from Changa Manga Lahore, and described *Cx. tritaeniorhynchus, Cx. quinquefasciatus* and *Ae. lineatopennis* responsible in the transmission of west Nile virus. Twenty- nine species of mosquitoes were described for the first time in Lahore, Pakistan (AslamKhan and Salman, 1969). Suleman *et al*. (1993) reported various species of mosquitoes from Peshawar Valley and adjoining areas along with their relative abundance.

In Murree, four Anophlinae subfamily species have recently been discovered (Qasim *et al*., 2014), *Armigerus subalbatus* has been found in Peshawar (Naz *et al*., 2014) and Swat has discovered four different types of *Anopheles* mosquitoes (Illahi and Suleman, 2013a). In Malakand District, six *Anopheles* species and one *Armigerus* were identified (Ali *et al*., 2013). Narowal has discovered five *opheles* and one *Armigeres* species (Rasool *et al*., 2015).

In Pakistan, the disease has been documented in both rural and urban areas, with outbreaks in Lahore, Peshawar, and the country's northern regions (WHO, 2012). Filariasis and Japanese encephalitis are transmitted by mosquitos of the *Culex* genus (Hemingway and Ranso, 2000). In urban environments, mosquitoes of the genus *Armigeres* have been found to transmit lymphatic and animal filariasis (Paily *et al*., 2009), as well as dog heartworm (Cheong *et al*., 1981). Pakistan is one of the most important mosquitoes-borne virus host (Braack *et al*., 2018), which are important medically and contributing to the current global public health issue (Ilahi and Suleman, 2013).

Two of the 23 Anopheline species *(An. culicifacies* and *An. stephensi*) reported from Pakistan are regarded as main malaria vectors. In rural (*An. culicifacies)* (Covell, 1931; Hick and Majid, 1937; Reisen and Boreham, 1982; Mahmood *et al*., 1984; Pervez

and Shah, 1989), and urban areas (*An. stephensi)* is the most common vector (Rehman and Mutalib, 1967). The two more species, *An. pulcherrimus* and *An. fluviaitlis* are thought to be vectors, notably in the mountains and foothills of NWFP and Punjab's northern regions (Suleman *et al*., 1993).

Pakistan's severity differed significantly around the country. Seasonal and climatic fluctuations influence the global distribution of mosquito species and the arboviruses they transmit (Anyamba *et al*., 2001; Hasnan *et al*., 2016). Furthermore, many closely related mosquito species with diverse ecology and host preferences are virtually indistinguishable morphologically (Walton *et al*., 2008). For establishing vector-borne illness management approaches, accurate vector identification is crucial (Otranto *et al*., 2009). Since considerable taxonomic investigations have concentrated on mosquitoes that carry human diseases (Munstermann and Conn, 1997), other species have become less attention (Zavortink, 1990; Krzywinski and Besansky, 2003).

Mosquitoes identification of a species or even a genus is frequently challenging (Reinert, 2000; Reinert *et al*., 2009). As a result, DNA-based mosquito identification (Kang and Sim, 2013), genetic diversity (Low *et al*., 2013), and molecular phylogeny (Shepard *et al*., 2006) are becoming more widely used. Prior research has used both morphological (Coetzee *et al*., 2000) and genetic techniques to track mosquito populations. Several biological kinds of researches require species identification (Bass *et al*., 2010). To study the variation in insect morphology, entomologists use morphometric analysis. The technique of measuring, calculating, and scoring morphological traits is known as morphometry (Rueda, 2008). In statistical analysis, quantification is employed to confirm the link between morphological traits. Morphometry is the precise study of anatomical characters by measurement (Klinberg, 2002; Mokosuli, 2013).

It has long been assumed that differences in environmental processes, genetics, and numerous demographic factors caused genetic variety in insects at both the intraspecies and interspecies levels. Insect species, on the other hand, have genetic uniformity due to free movement and a lack of obstacles (Batool, 2012; Ashraf, 2013; De

Lourdes *et al*. 2013), migration as well as transportation through various means (De Souza *et al*., 2001; Franco *et al*., 2002; Ayres *et al*., 2003). Identification of mosquitoes is important because the speed of genetic modification of mosquitoes is very high (Beebe *et al*., 2005; Andrew and Bar, 2013).

For efficiently analyzing mosquito population dynamics, molecular approaches are a valuable tool. It enables a more thorough understanding of the connections between a mosquito's vectorial capacity, genetic makeup, and geographic origin, as well as more precise taxonomy and evolutionary studies (Kaura *et al*., 2010; Kamali *et al*., 2012). The use of comparative DNA analysis to estimate genetic (variation, relatedness, and exchange) within species is a powerful tool. Based on a polymerase chain reaction, molecular genetic researches are extremely important. For this study, a variety of gene markers were utilized. Internal Transcribed Spacer 1 and 2 (ITS1 & 2), mitochondrial cytochrome c oxidase subunit I and II (COI & COII), and D3 (28S rDNA) gene sequences are valuable in phylogenetic analysis and species identification (Takano *et al*., 2010; Sarma *et al*., 2012).

The species is identified by amplification of the ribosomal DNA region, followed by restriction fragment length polymorphism (RFLP) analysis and, if necessary, sequencing of the amplification products. Gene flow among mosquito populations is demonstrated by monomorphism in the amplification profiles of the same species from various regions (Ayres *et al*., 2002); the polymorphic pattern of the DNA profile, on the other hand, reveals genetic variety from various places (Zahoor *et al*., 2013). As a result, it became important to use molecular techniques to deduce the genetic composition of mosquito populations in order to design and implement a successful mosquito control programmes in specific areas. As a result, it is inclined to a wide range of mutations, making it an excellent choice for researching phylogenetic of closely related mosquito species, as well as biodiversity and regional races of a particular mosquito species (Marrelli *et al*., 2006).

DNA barcoding is a molecular method of identifying species that uses a short DNA sequence with substantially less variation within species than it does across species.

Elongation factor-1 alpha (EF-1a), acetylcholinesterase 2 (ace-2), alpha amylase, and internal transcribed spacer subunit 2 have all been utilized as nuclear markers in mosquito barcoding investigations (ITS2) (Foley *et al*., 2007; Hasan *et al*., 2009; Hemmerter *et al*., 2009; Puslednik *et al*., 2012). The 'Universal' or 'Folmer' region of the mitochondrial gene Cytochrome Oxidase I (COI) is currently the most widely utilized barcode region for animals. The Barcode of Life Database (BOLD), an online platform for aggregating and curating DNA barcoding information from around the world, has designated this region as the standard marker (Ratnasingham and Hebert, 2007). While COI has been utilized in a variety of ways in mosquito barcoding investigations. Multiple markers are frequently used, with mitochondrial and nuclear genes both proving beneficial in identifying species (Lin and Danforth, 2004).

It can be difficult to distinguish members of species complexes and subgroups, which are closely related species that may not be genetically distinct, when only one barcoding region is used (Foster *et al.,* 2013; Jiang *et al.,* 2014). The maternal inheritance of mitochondrial genes is also advantageous for barcoding (Tamura *et al.,* 2007). Non-coding DNA makes up the ITS2 rDNA sequence. Among these gene sequences, the ITS2 of rDNA has been discovered to be beneficial for taxonomic classification (Park *et al*., 2008; Coleman, 2003).

Genetic approaches are thought to be largely devoid of the subjectivity of morphological features identification, and they can show the presence of cryptic species complexes that are frequently neglected (e.g., Hemmerter *et al*., 2007). As a result, barcoding as a way of identifying mosquitos is critical to a monitoring program's accuracy. To identify and describe mosquito species in Punjab, Pakistan, we used molecular genetic techniques such as PCR and sequencing of the cytochrome oxidase subunit 1 (CO1) gene.

MATERIALS AND METHODS

2.1: Ethics statement

This research does not require any special approvals. Mosquitoes from private dwellings were only collected with the permission of the owners. There were no endangered or protected species in the research.

2.2: Sampling strategies and study sites

The research region consists of eleven districts of Punjab from two opposing zones: Northern (Rawalpindi, Gujar Khan, Gujrat, Chakwal, Gujranwala and Lahore) and Southern (Dera Ghazi khan, Rajanpur, Rahim yar Khan, Multan, and Bahawalpur). Between March 2016 and August 2019, the collection of mosquitoes (700) was made.

Mosquitoes were captured utilizing baits and trap nets from both indoor and outdoor places such as water bodies and terrestrial habitats such as flowerpots, plastic containers, tyres, and mud pots. Private dwellings, building sites, junkyards, water catchments, marshes, ponds, and woodlands were among the sampling locations. Mosquitoes that were biting both peoples and animals were captured using an aspirator. Adult mosquitoes were removed from the chosen sampling locations using an aerial net and aspirator.

After emerging, the adult mosquitoes were gently sucked through an aspirator and removed from various plastic jars. Using a cotton swab dipped in chloroform and placed in the jar's entrance, adult mosquitoes were killed in two to four minutes (Ali *et al*., 2013). In test tubes, mosquitoes were preserved and had a little amount of silica gel as a preservative, which was capped with cork to keep it dry. When the hue changed, silica gel was replaced. The adults who were killed were later recognized morphologically using the taxonomic keys from Christophers and Barraud's "The fauna of British India, including Ceylon and Burma. Using (http://www.simplemappr.net), GPS coordinates were obtained (Table) and collection sites were mapped (Figure 2.1)

For molecular identification, specimens were kept in 70% ethyl alcohol and

examined under a stereoscopic trinocular microscope. A total of 100 samples were picked at random for DNA testing. The adult's mosquitoes were recognized using common taxonomic keys after being given a specimen number and being photographed.

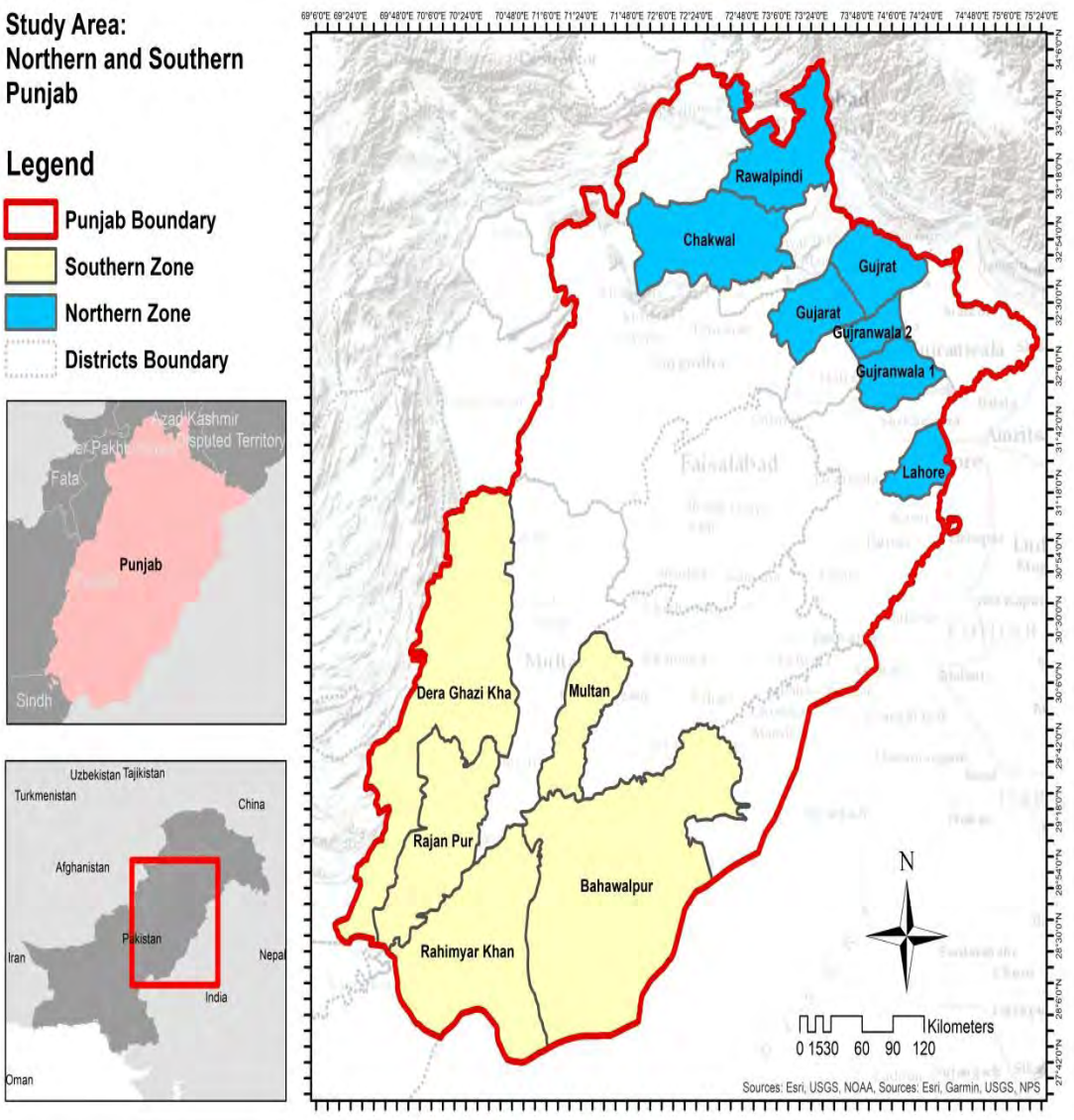


Figure 2.1: Map of the mosquitoes sampling areas (Punjab)

2.3: Climatic data of study areas

2.3.1: Punjab

Weather of Punjab is humid, semi-arid, hot and subtropical monsoon type with cold winter and hot summer. The temperature in the Punjab region varies from 2° to 45°C, however it can get as high as 50°C (122°F) in the summer and as low as 10°C in the winter. Punjab has four distinct seasons: hot and humid (April through June), with highs of 123 °F (51 °C), and wet (July to September).

2.4: MOSQUITOES IDENTIFICATION

2.4.1: MORPHOLOGICAL IDENTIFICATION

2.4.1.1: External morphology of the mosquitoes

Using a stereoscopic trinocular microscope, mosquitoes collected from these sample locations were morphologically identified, measurements were taken using ocular scales and Image J software. Species were identified using several Taxonomic keys including The Fauna of British India (Barraud, 1934), Mosquito (Diptera: Culicidae) of Murree Hills, Punjab, Pakistan (Qasim *et al*., 2014) and a Study of Mosquitoes Fauna of District Upper Dir, Khyber Pakhtunkhwa Pakistan (Khan *et al*., 2015). A detailed discussion of the taxonomic terms and measurements utilized in this work was conducted (Van der Beek, 2013; Chhilar, 2014).

The length (µm) of following characters was measured: (full body, proboscis, palpi, antennae and wing), fore, mid and hind leg (femur, tibia, and tarsus). Range, mean, standard deviation, standard error, and coefficient of variance of these characters were also examined in the data.

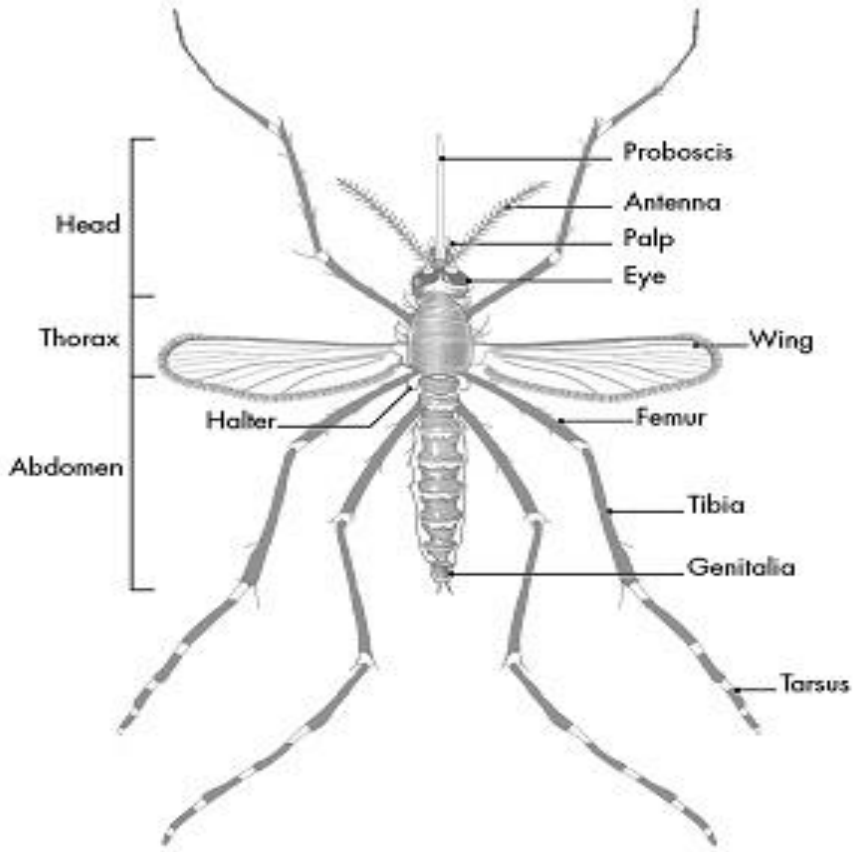


Figure 2.2: Image depicts different body parts of mosquito

2.5: Data Analysis

Captured adult mosquitoes were used for analysis. Using the suggested formula, variations of selected mosquito genera in terms of distribution and relative abundance were observed (Ali and Rasheed, 2009).

Relative abundance $= n/L \times 100$

Where n = number of each species specimens

N = total number of specimens.

Different mosquito species were classified in different relative abundance classes given in Table 2.1.

Table 2.1: Classification of species/genus based on their relative abundance.

Relative abundance (%)	Classification
Less than 1	Satellite species
Less than 5	sub-dominant species
More than 5	Dominant species

The % distribution of all mosquito species/genus in diverse habitats was determined using the formula below (Ali *et al.,* 2013).

Distribution (C) =Relative abundance $= n/N \times 100$ Where

n = number of those habitats where species were found,

N = total number of habitats

After %age distribution different classes of mosquito's species/genus were also analyzed being illustrated in Table 2.2.

Table 2.2: Shows different classes of species/genus based on their distribution.

Classes	Distribution	Classification
C_1	0-20%	Sporadic
C_2	20.1%-40%	Infrequent
C_3	40.1%-60%	Moderate
C_4	60.1%-80%	Frequent
C5	80.1%-100%	Constant

2.6: Molecular identification and analysis

2.6.1: Sample selection for Molecular analysis

In total 100 specimens from subfamilies Anophelinae and Culicinae were initially selected for the molecular analysis.

2.6.2: Selection of marker genes for molecular identifications

CO1 genes were selected as marker genes for the amplifications of the sequences from the selected specimens' genes for their molecular identification.

2.7: DNA extraction

Before DNA extraction, distilled water was used to rehydrate the alcohol-preserved and dry-pinned specimens for at least 30min. Prior to DNA extraction, the head, thorax, wings, legs, and abdomen of about 100 specimens were dissected. Using the Takara Mini-BEST Universal Genomic DNA Extraction Kit and standard DNA extraction techniques, genomic DNA was extracted from one whole mosquito sample (Takara, Dalian, China). All extracted DNA samples were stored at -20°C until analysis.

2.8: Gel electrophoresis:

In order to verify and check the DNA extracted after following the above-mentioned protocol, the gel electrophoresis technique was used. 1% gel was prepared by mixing 0.5g agarose gel, 5ml 10X TBE buffer and 45ml distilled water. The mixture was heated for 1min in microwave oven. 5µl of ethidium bromide was added to the mixture immediately after heating. 16 wells gel electrophoresis tray was used. Both combs were placed in the tray prior to pouring the heated mixture, after pouring the mixture it was left until solidified. The tray was then placed in the chamber. 2µL of loading dye and 4µL of samples DNA was mixed and laden in the wells. The electrophoresis runs at 120V for 35min. The agarose gel was observed in gel doc apparatus and the image was recorded.

2.9: PCR Amplifications

For the amplification, the mitochondrial genes COI were chosen.

Table 2.3: List of primers used in this study.

Primer name	**Primer sequence**	**Size (bp)**
Anopheles species identification		
Forward, COI	5'TTGATTTTTTGGTCATCCAGAAGT3'	980bp
Reverse, CUL	5'TAGAGCTTAAATTCATTGCACTAATC3'	
Aedes species identification		
Forward, C1-J-1718	5'GGAGGATTTGGAAATTGATTAGTTC3'	418-440bp
Reverse, C1-N-2191-5	5'CCCGGTAAAATTAAAATATAAACTTC3'	

2.10: PCR procedure:

A master mix was prepared containing all reagents for PCR amplification; 25µl volume for each reaction was used. To avoid contamination only one tube was opened at one time. All the reagents were vortexes before use. The DNA template was added at the end. Standard PCR amplification involved 8µL (40ng/µL Template) DNA, **2.5µL** PCR 10X buffer, **1.5µL (**25mM) $MgCl_2$, **1µL (**2mM) dNTPs,**1µL of each of** Forward and Reverse Primers, **0.3µL (**5U/µL) Taq DNA polymerase and **9.7µL** of dH2O.

2.11: Amplification reaction:

The following were the PCR conditions: 94°C for 5min; 35 cycles at 94°C for 45s, with varied annealing temperatures for 45s and 72°C for 45s. The last extension took 10min at 72 °C.

2.12: Amplification of COI gene:

The 980bps fragment of the COI gene from all specimens was amplified using the published primers composed of forward primer named as described above. The PCR reaction and other conditions used are described above. All PCR amplification products were verified by electrophoresis of 7µl on a 1.5% agarose gel.

2.13: Purification of Amplified Product

i) After the successful amplifications of all gene fragments, the PCR products were manually purified by using the following protocol. To the PCR products 35µL of 10M ammonium acetate was added ($1/10^{th}$ of the PCR product volume). It was followed by the addition of the 40µl of chilled absolute ethanol. The samples were placed at -20°C for 20min. The entire product samples were then spun at 14000rpm for 10min. 100µl of chilled 70% ethanol was added to each sample and all samples were then vortexes for 10s after which they were rotated at 14000rpm for 10min. The supernatant was discarded from each sample and the pellets were air dried followed by suspension of the samples in 15µl autoclaved deionized water.

ii) The amplified products of the CO1 gene were purified using a gene jet PCR purification kit (Cat # K0701, Thermo Fisher Scientific, EU Lithuania) and were sent to Korea (macrogen Inc.) for sequencing.

2.12: PCR Products quantification by using Nano drop

In order to measure the quantity and quality of the purified PCR products, each sample was subjected to the Nano drop quantification by dropping 1µl of PCR product into the Nano drop equipment measuring unit.

2.14: Sequencing

Sequencing of the purified PCR products was carried out on an Applied Biosystems (310 Genetic Analyser) according to manufacturer's protocols. Both forward and reverse samples were sequenced for CO1 gene amplified products. Column cycle sequencing was performed on both forward and reverse strands for CO1 with the same primers used for PCR by using ABI PRISM Big Dye Terminator Cycle Sequencing reagents.

2.15: Comparison of sequences

Sequences were initially analysed using Bio Edit Sequence Alignment Editor Software to check for any kind of discrepancies or failure in the sequencing. To identify species, the COI sequences were compared with related mosquito species sequences on the NCBI web site via the BLAST function.

2.16: Sequences analysis and phylogenetic tree construction

The sequences were deposited to the gene bank under the accession numbers i.e. MN919391, MN919392, MN919393, MN919394, MN909288, MN909289, MN909290, MN909291, MN906911, MN906912, MN906913.The CO1 sequences were then tested using MEGA6 (Tamura *et al*., 2013) software to check that whether the amplified sequences are of mitochondrial and nuclear DNA origins respectively or not. The resultant sequences were aligned using ClustalW. Other sequences were retrieved for comparison from the NCBI database. The evolutionary history was inferred based on the

1270bp of the CO1 and 500bp of the ITS2 fragments by the Neighbour-Joining analysis using nucleotide substitution model and p-distance method. The bootstrap support for the sequences was calculated from 1000 replications to represent the evolutionary history of the identified specimens. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated from the dataset by using pairwise deletion option.

2.17: Statistical analysis:

Minitab version 16 software was used to analyse the results of morphometric variations among the specimens. The values like mean, standard deviations, and Coefficient of variability were found out by applying "1 *sample t test"* to the data obtained from the morphometric measurements.

RESULTS

2.1: Distribution of Mosquitoes in different districts of Punjab

Twenty-seven different species were identified from eleven districts of Punjab belonging to five genera i.e., *Anopheles, Culex, Aedes, Armigeres, and Manosoni.*

From Rawalpindi six species of *Culex viz Cx. quinquefasciatus, Cx. vishnui, Cx. fatigans, Cx. pipiens, Cx. gelidus,* and *Cx. fuscocephala,* six species of *Anopheles viz An. culicifacies, An. subpictus, An. annularis, An. tesselatus, An. splendidus koidzum, and, An. sinensis,* two species of *Aedes viz Ae. aegypti, and Ae. albopictus,* one species of *Armigeres* viz *Ar. subalbatus,* and one species of *Mansonia viz Ma. uniformis* were identified.

Four species of *Culex viz Cx. raptor, Cx. bitaeniorhynchus*, *Cx. pipiens,* and *Cx. fuscocephala,* five species of *Anopheles viz An. stephensi, An. subpictus, An. maculatus, An. fluviatilis, and An. aconitus,* one species of *Aedes viz Ae. albopictus,* two species of *Armigeres* viz *Ar. kuchingiensis,* and *Ar. flavus,* were recorded from Gujar khan.

From Gujrat five species of *Culex viz Cx. quinquefasciatus, Cx. fatigans, Cx. pipiens, Cx. gelidus,* and *Cx. fuscocephala,* six species of *Anopheles viz An. culicifacies, An. stephensi, An. subpictus, An. maculatus, An. fluviatilis,* and *An. splendidus koidzum,* two species of *Aedes viz Ae. albopictus,* two species of *Armigeres* viz *Ar. subalbatus, and Ar. kuchingiensis,* and one species of *Mansonia viz Ma. uniformis* were identified.

Four species of *Culex viz Cx. vagans, Cx. vishnui, Cx. fatigans, and Cx. bitaeniorhynchu,* four species of *Anopheles viz An. subpictus, An. sinensis, An. annularis, and An. aconitus,* one species of *Aedes viz Ae. aegypti,* and one species of *Armigeres* viz *Ar. obturbans* were documented rom Chakwal

From Gujranwala five species of *Culex viz Cx. quinquefasciatus, Cx. vagans, Cx. vishnui, Cx. pipiens,* and *Cx. gelidus,* four species of *Anopheles viz, An. annularis, An. sinensis An. splendidus koidzum,* and *An. tesselatus,* one species of *Aedes viz Ae.*

albopictus two species of *Armigeres* viz *Ar. kuchingiensis,* and *Ar. flavus,* were found.

Six species of *Culex*, including *Cx. quinquefasciatus, Cx. tritaeniorhynchus*, *Cx. pipens*, *Cx. fatigans*, Cx. *raptor* and *Cx. vishnui* as well as four *Anopheles* species, including *An. subpictus*, *An. stephensi*, *An. annularis*, and *An. maculatus*, and two species of *Aedes* viz *Ae. aegypti* and *Ae. albopictus,* two species of *Armigeres* viz *Ar. flavus* and *Ar. obturbans,* one species of *Mansonia viz Ma. uniformis* were reported from Lahore.

Five species of *Culex viz Cx. tritaeniorhynchus, Cx. raptor, Cx. pipiens, Cx. gelidus,* and *Cx. bitaeniorhynchus*, five species of *Anopheles viz An. culicifacies, An. annularis, An. sinensis, An. fluviatilis, and An. aconitus,* one species of *Ae. albopictus,* one species of *Armigeres* viz *Ar. subalbatus* were documented from Dera Ghazi Khan.

Five species of *Culex viz Cx. quinquefasciatus, Cx. raptor, Cx.pipiens, Cx. gelidus,* and *Cx. fuscocephala,* six species of *Anopheles viz An. culicifacies, An. stephensi, An. subpictus, An. maculatus, An. splendidus koidzum,* and *An. aconitus,* one species of *Aedes viz Ae. aegypti,* and one species of *Armigeres* viz *Ar. flavus* were identified from Rajanpur.

From Rahim yar khan five species of *Culex viz Cx. tritaeniorhynchus, Cx. fatigans, Cx. raptor, Cx. pipiens,* and *Cx. bitaeniorhynchus*, six species of *Anopheles viz An. culicifacies, An. annularis, An. sinensis, An. fluviatilis, An. tesselatus, and An. stephensi,* one species of *Aedes viz Ae. albopictus,* two species of *Armigeres* viz *Ar. subalbatus,* and *Ar. kuchingiensis,* were reported.

Six species of *Culex viz Cx. quinquefasciatus, Cx. tritaeniorhynchus, Cx.raptor, Cx. fatigans, Cx. gelidus,*and *Cx. fuscocephala,* five species of *Anopheles viz An. culicifacies, An. annularis, An. subpictus, An. fluviatilis,* and *An. splendidus koidzum,* two species of *Aedes viz Ae. aegypti, and Ae. albopictus,* two species of *Armigeres* viz *Ar. obturbans,* and *Ar. flavus,* and one species of *Mansonia viz Ma. uniformis* were documented from multan.

Five species of *Culex viz Cx. bitaeniorhynchus, Cx.vishnui, Cx. vagans, Cx.*

gelidus, and *Cx. quinquefasciatus,* six species of *Anopheles viz An. culicifacies, An. aconitus, An. sinensis, An. maculatus, An. stephensi,* and *An. splendidus koidzum,* two species of *Aedes viz Ae. aegypti, and Ae. albopictus,* two species of *Armigeres* viz *Ar. subalbatus, and Ar. kuchingiensis* from Bahawalpur were identified.

Table 2.4: Distribution of various species of mosquitoes in different districts of Punjab.

Species	Districts of Punjab										
	Rawal pindi	**Gujar Khan**	**Gujrat**	**Chak wal**	**Gujran Wala**	**Lahore**	**Dera Ghazi Khan**	**Rajan pur**	**Rahim yar Khan**	**Multan**	**Bha wal pur**
Cx. quinquefasciatus	+	-	+	-	+	+	-	+	-	+	+
Cx. tritaeniorhynchus	-	-	-	-	-	+	+	-	+	+	-
Cx. vagans	-	-	-	+	+	-	-	-	-	-	+
Cx. vishnui	+	-	-	+	+	+	-	-	-	-	+
Cx. fatigans	+	-	+	+	-	+	-	-	+	+	-
Cx. raptor	-	+	-	-	-	+	+	+	+	+	-
Cx. pipiens	+	+	+	-	+	+	+	+	+	-	-
Cx. gelidus	+	-	+	-	+	-	+	+	-	+	+
Cx. bitaeniorhynchus	-	+	-	+	-	-	+	-	+	-	+
Cx. fuscocephala	+	+	+	-	-	-	-	+	-	+	-
An. culicifacies	+	-	+	-	-	-	-	+	+	+	+
An. annularis	+	-	-	+	+	+	+	-	+	+	-
An. stephensi	-	+	+	-	-	+	-	+	+	-	+
An. subpictus	+	+	+	+	-	+	-	+	-	+	-
An. maculatus	-	+	+	-	-	+	-	+	-	+	+
An. sinensis	+	-	-	+	+	-	+	-	+	-	+
An. fluviatilis	-	+	+	-	-	-	+	-	+	+	-

An. splendidus koidzum	+	-	+	-	+	-	-	+	-	+	+
An. tesselatus	+	-	-	-	+	-	-	-	+	-	-
An. aconitus	-	+	-	+	-	-	+	+	-	-	+
Ae. aegypti	+	-	-	+	+	+	-	+	-	+	+
Ae. albopictus	+	+	+	-	-	+	+	-	+	+	+
Ar. subalbatus	+	-	+	-	-	-	+	-	+	-	+
Ar. kuchingiensis	-	+	+	-	-	-	-	-	+	-	+
Ar. flavus	-	+	-	-	+	+	-	+	-	+	-
Ar. obturbans	-	-	-	+	+	+	-	-	-	+	-
Ma. uniformis	+	-	+	-	-	+	-	-	-	+	+

+ Indicates species were found - Indicates species were not found

Table 2.5: Coordinates and elevation of the localities in Punjab.

Localities	Coordinates	Elevation(m)
Rawalpindi	33.5651° N, 73.0169° E	508m
Gujar Khan	33.2616° N, 73.3058° E	461m
Lahore	31.5204° N, 74.3587° E	217m
Gujrat	32.5731° N, 74.1005° E	232m
Chakwal	32.9328° N, 72.8630° E	498m
Gujranwala	32.1877° N, 74.1945° E	231m
Dera Ghazi khan	30.0489° N, 70.6455° E	390m
Rajanpur	29.1044° N, 70.3301° E	99m
Rahim yar Khan	28.4212° N, 70.2989° E	83m
Multan	30.1575° N, 71.5249° E	122m
Bahawalpur	29.3544° N, 71.6911° E	214m

2.3: Classification of Mosquitoes

Phylum:	Arthropoda
Class:	Insecta
Order:	Diptera (i.e., two-winged insects)
Suborder:	Nematocera
Family:	Culicidae
Subfamily:	Anophelinae / Culicinae
Genus:	*Anopheles* / *Culex* / *Aedes* / *Armigeres*/*Mansonia*

2.3.1: Genus identification

Genus: A*nopheles*

- *Anopheles* genus belongs to subfamily Anophelinae and is worldwide in distribution.
- Usually blackish to dark brown in appearance, with an eight-segmented dark abdomen; when feeding, the female holds herself at a 45-degree angle to the host's skin.
- *Anopheles* is distinguished by its spotted wings, palpi that are equal to proboscis in both sexes but have inflated apical segments in males, and lengthy segments with claws and spurs on the forelegs of males.

Genus: *Culex*

- *Culex* genus belongs to subfamily Culicinae.
- They have relatively blunt abdomen and while feeding female keeps her bodyerect (parallel).
- The main identifying feature of *Culex* is the length of palps and their wing structure.
- In males, palps are somewhat equal to proboscis but are thin and erect while in femalepalps are relatively shorter than proboscis.
- Wings are quite clear without any spots.
-

Genus: *Armigeres*

- *Armigeres* genus belongs to subfamily Culicinae
- Proboscis is stout and tip laterally bent downward
- Palpi in female are one-third the length of proboscis

Genus: *Aedes*

- *Aedes* genus belongs to subfamily Culicinae
- The key feature is black and white markings on their bodies and legs
- They have pointed abdomen with unique patterns of light and dark scales
- Wings are uniformly colored and have short scales

Genus: *Mansonia*

- Big in size, black or brown in color.
- Characterized by the presence of broad, asymmetrical scales on the wing veins and legs

2.4: Species identification

2.4.1: Subfamily *Anophelinae*

1. Tarsomeres 3rd, 4th, and 5th white in colour, white scales present, front femur swollen and cerci contain dark scales (Figure 2.3) ……*Anopheles annularis*

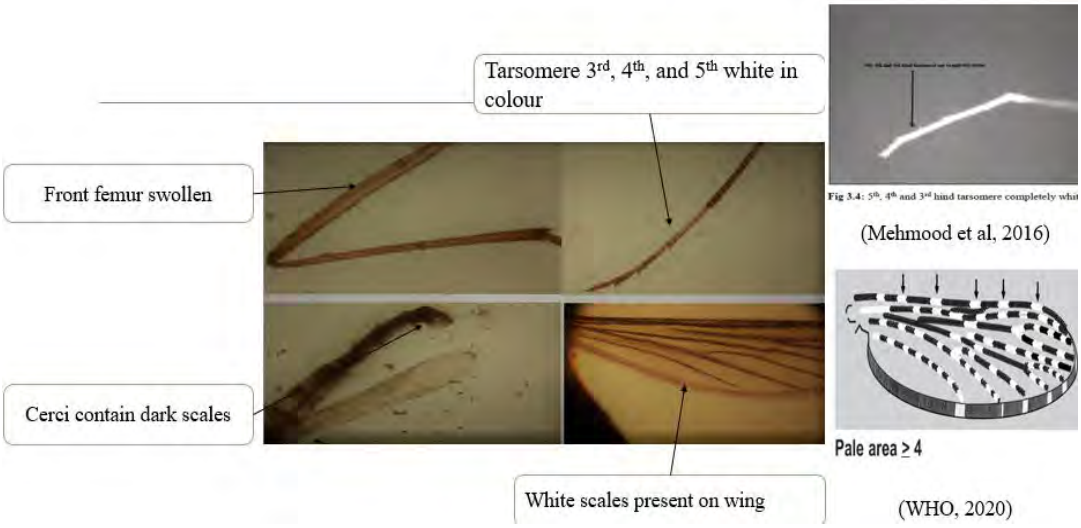


Figure 2.3: Identifying features of *An. annularis*

2. Scales on antennae are absent or rudimentary, femur not swollen, tibia dark, tarsomeres not banded, abdominal scales absent and have no scales on cerci, dark black legs without banding patterns (Figure 2.4) …*Anopheles culicifacies*

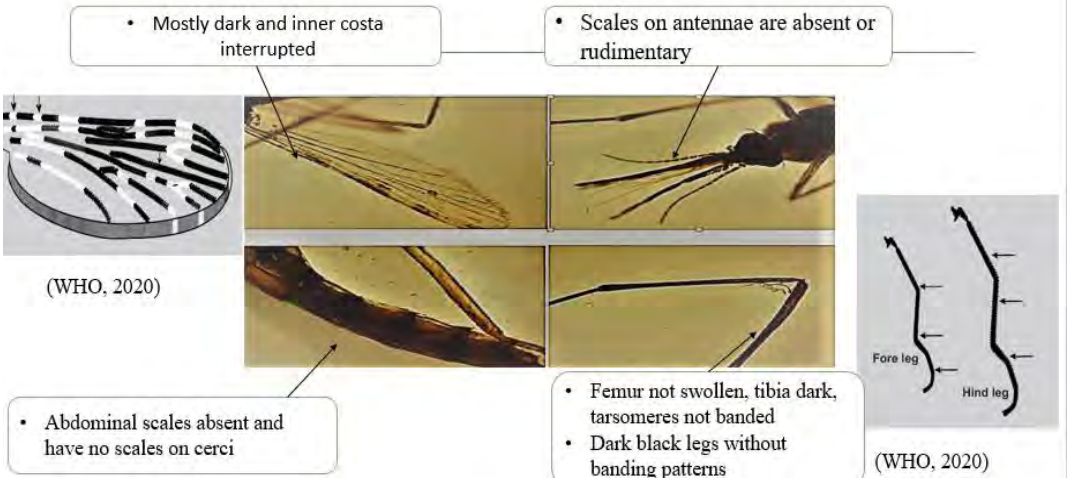


Figure 2.4: Identifying features of *An. culicifacies.*

3. Male palpi are swollen and equal in length to that of proboscis, fore femur swollen, hind femur and tibia dark colored, narrow scales are present on 2nd, 3rd, and 8th tergites, covered with dark spots, cerci covered with dark scales (Figure 2.5).… *Anopheles stephensi*

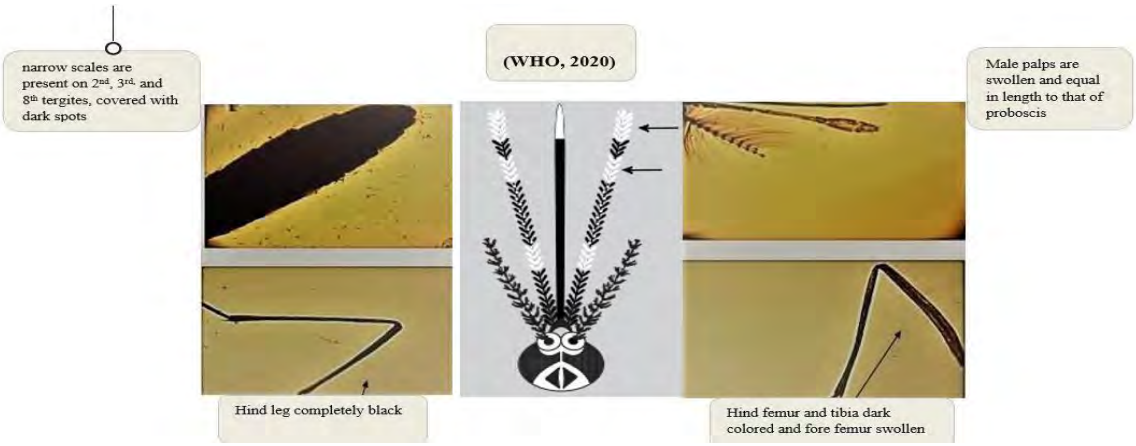


Figure 2.5: Identifying features of *An. stephensi.*

4. Tip of proboscis banded, specking in legs, tarsal segment has dark bands, narrow to broadscales on abdominal segments VI to VIII, wings have two dark spots (Figure 2.6)…….. *Anopheles maculatus*

Figure 2.6: Identifying features of *An. maculatus.*

5. Antennae have no scales, straight, cylindrical and thin palpi, abdominal scales absent and have no scales on cerci, no bands on legs (Figure 2.7) ……... *Anopheles fluviatilis*

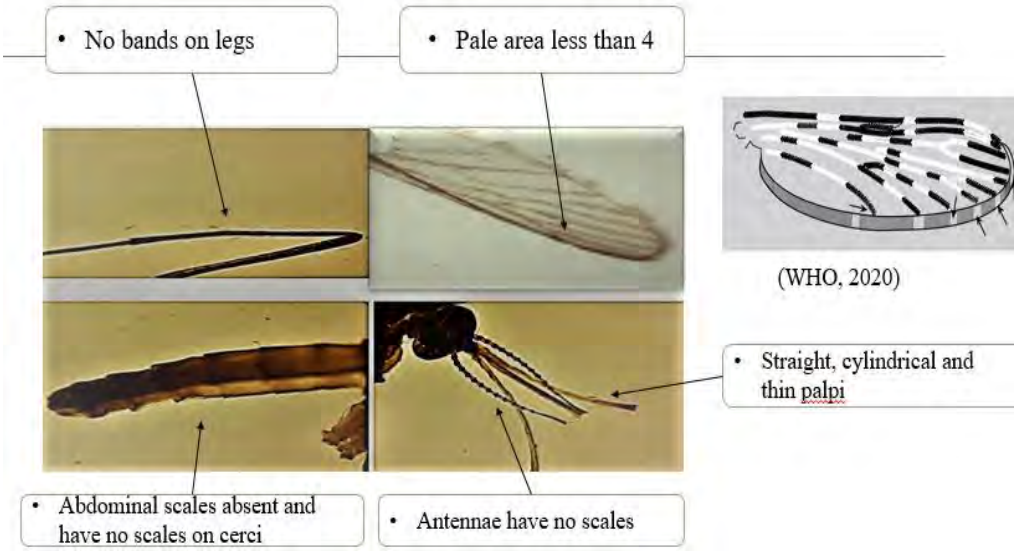


Figure 2.7: Identifying features of *An. fluviatilis*

6. Broad pale bands on foreleg tarsomeres, speckling in legs is absent, apical pale band nearly equal to pre-apical dark band (Figure 2.8)……. *Anopheles subpictus*

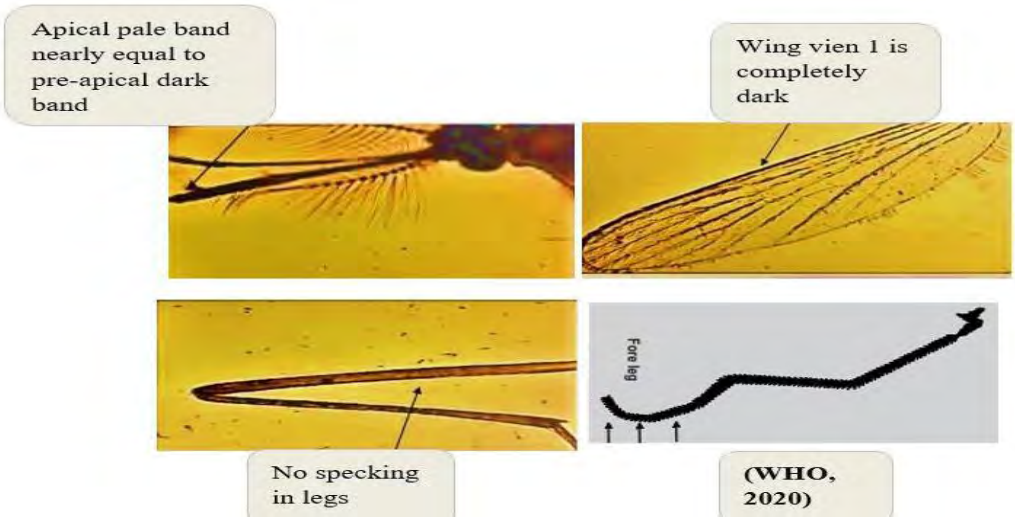


Figure 2.8: Identifying features of *An. subpictus*

7. Apical segment of palpi long, spots present on femora and tibia, hind tarsomere 5th not white, abdomen dark colored having hairs and cerci have no scales (Figure 2.9) …… *Anopheles tessellatus*

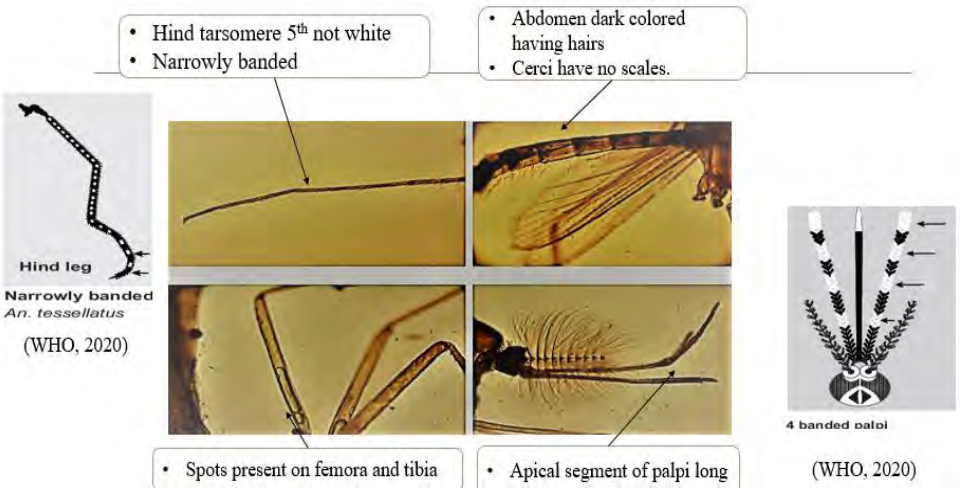


Figure 2.9: Identifying features of *An. tesselatus*

8. Tip of the palpi pale, wings spotted, vein 2 is scaled, femur and tibia having spherical and round white spots, hind tarsal segments white, abdomen dark colored and cerci contain black scales (Figure 2.10) …….....*Anopheles sinensis*

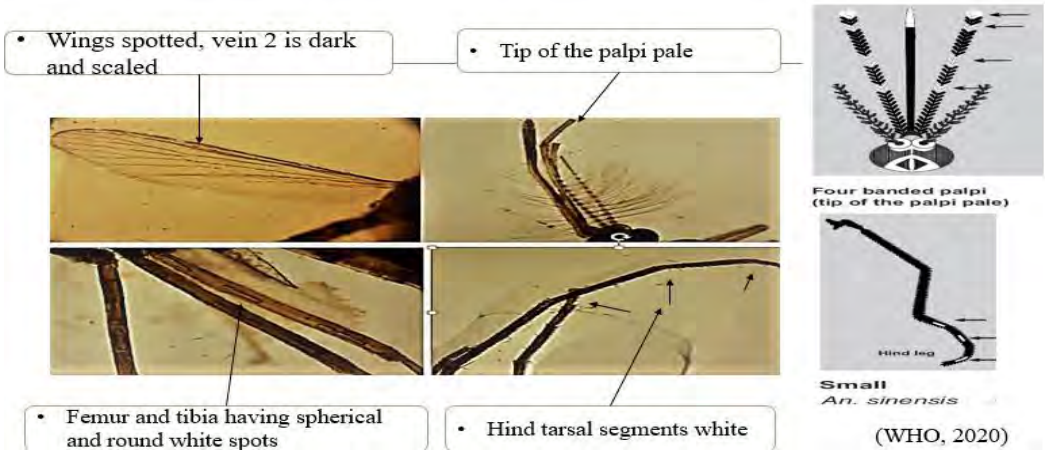


Figure 2.10: Identifying features of *An. sinensis*

9. Intervening dark and pale bands on palpi, complete dark legs, apical and subapical pale bands on palpi are equal in length (Figure 2.11) ……*Anopheles aconitus*

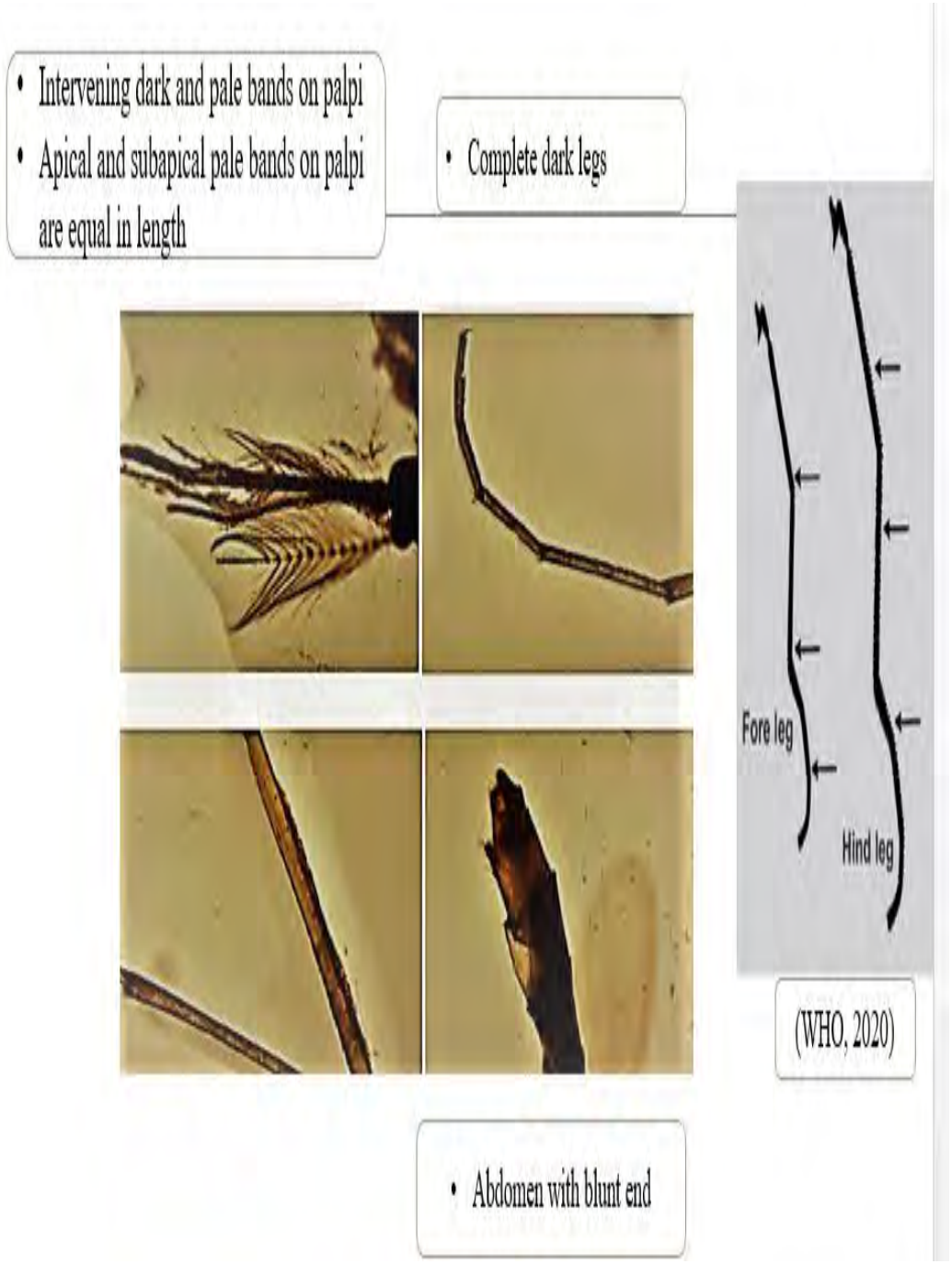


Figure 2.11: Identifying features of *An. aconitus*

10. There are scales with a clearly visible vertical white region. White scales cover the first flagellum segment. Palpi feature two equally wide stripes and are considerably thickened. Thorax doesn't have scales. Wings are discernible. *An. annularis* almost shares similar wing characteristics. Round and spherical white patches can be seen on the femur and tibia. The whitish segments of the hind tarsus. Dark hue of the abdomen. The dorsally scaled eighth abdominal segment is whitish. Cerci have scales of black colour. (Figure 2.12) ……. ***Anopheles splendidus koidzumi***

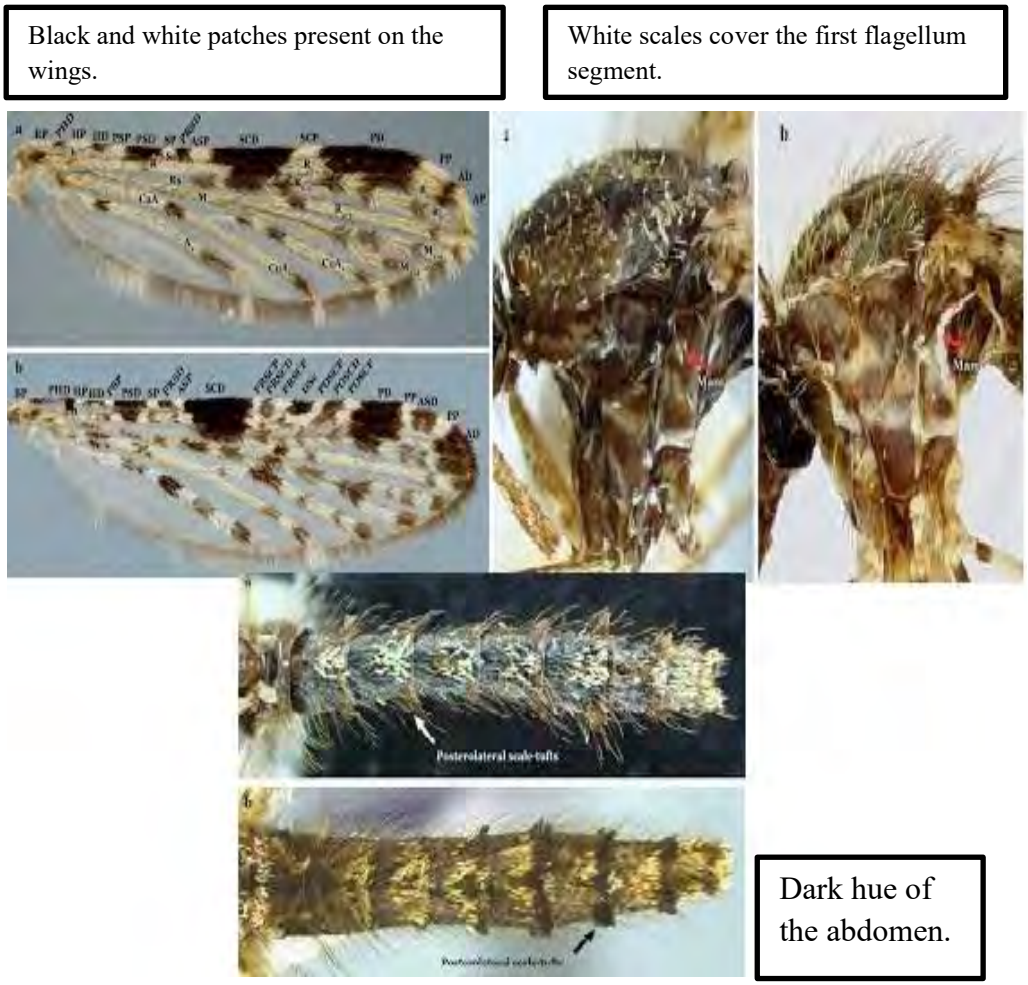


Figure 2.12: Identifying features of *An. splendidus koidzumi*

2.4.2: Subfamily *culicinae*

11. 8th terga and cerci without scales, blunt abdomen, presence of basal pale bands on terga, head light brown in color, thorax, abdomen, and proboscis darker (Figure 2.13) ……. *Culex quinquefasciatus*

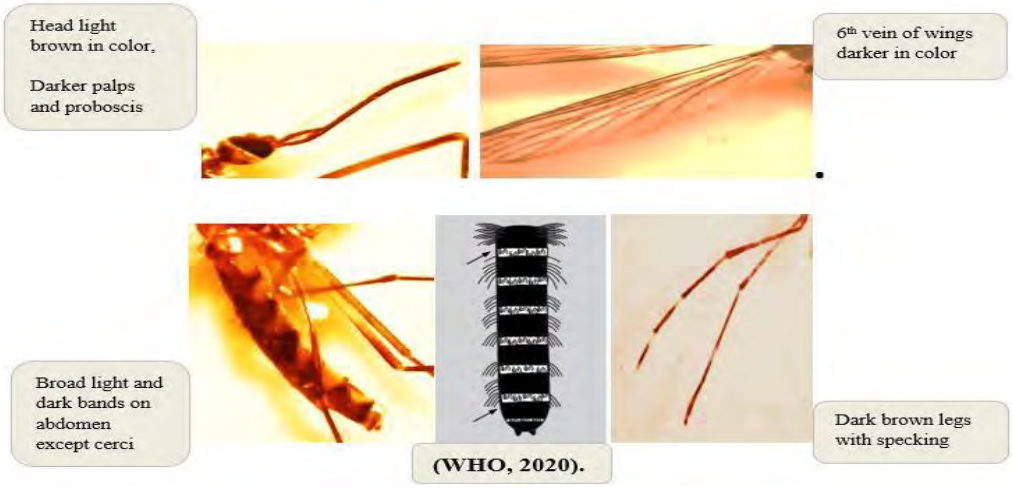


Figure 2.13: Identifying features of *Cx. quinquefasciatus.*

12. White spots on wings are present, wing 3.8-5.2mm, heavily speckled with dark and pale scales, abdominal terga speckled with pale bands (Figure 2.14) ……. *Culex bitaeneorhynchus*

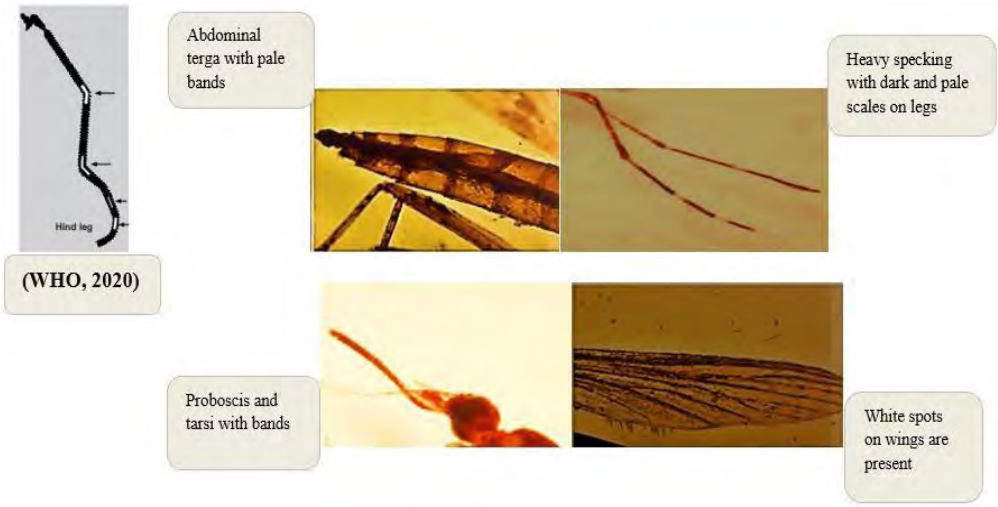


Figure 2.14: Identifying features of *Cx. bitaeneorhynchus.*

13. Narrow apical dark ring on hind femur, proboscis with accessory pale patches present (Figure 2.15).....*Culex tritaeniorhynchus*

Figure 2.15: Identifying features of *Cx. tritaeniorhynchus.*

14. Scales absent on cerci, small/ narrow pale bands, or absence of banding on abdomen, proboscis without pale band, dark in color (Figure 2.16) ……. *Culex fuscocephala*

Figure 2.16: Identifying features of *Cx. fuscocephala*

15. Dark tipped proboscis, abdominal terga with pale bands, Fore and middle femora not striped ordark in front, hind femur not pale from base to knee (Figure 2.17) ……. *Culex vagans*

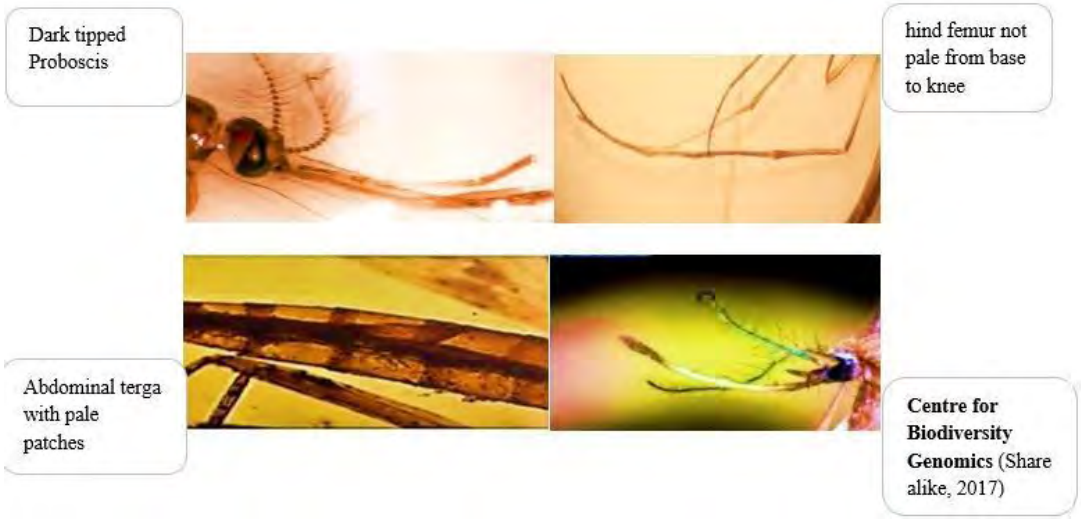


Figure 2.17: Identifying features of *Cx. vagans*

16. Dark pale scale area present on hind femur, speckling of pale scales on wings and femora (Figure 2.18) ……*Culex vishnui*

Figure 2.18: Identifying features of *Cx. vishnui*

17. Light brown in color, blunt abdomen, bifurcated proboscis with only dark scales, no distinctive markings on proboscis and legs, basal pale abdominal bands present (Figure 2.19) ……. *Culex pipiens*

Figure 2.19: Identifying features of *Cx. pipiens*

18. White spots on the wings are absent, wings with broad scales, complete dark leg, without bands, proboscis having alternate pale and dark bands (Figure 2.20) ……. *Culex gelidus*

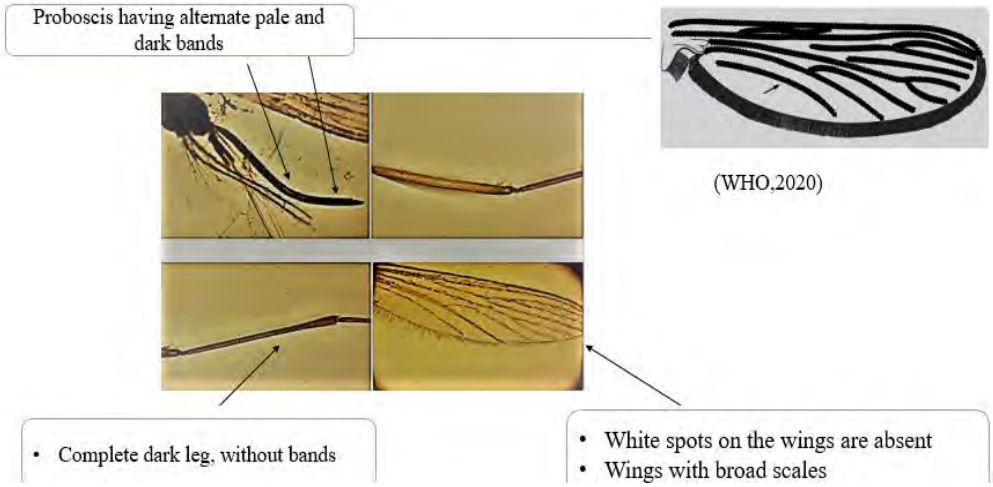


Figure 2.20: Identifying features of *Cx. gelidus*

19. Scales are absent on the wings, dark stripe on the anterior dorsal surface of hind femur (Figure 2.21) ……. *Culex fatigans*

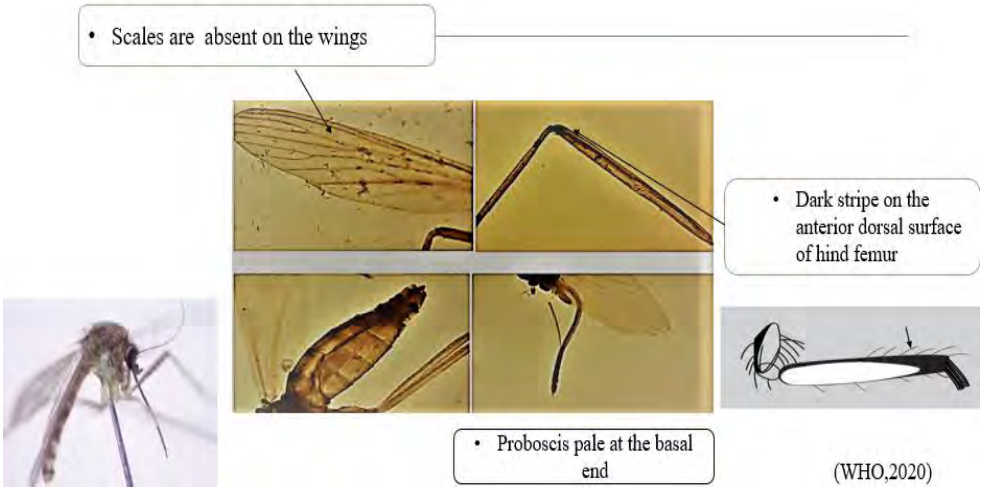


Figure 2.21: Identifying features of *Cx. fatigans*

20. Speckling of pale scales on the fore and mid femora, dark pale scaled area on hind femur, pale band on proboscis broad, as long as or longer than basal dark band, scales on prescutellar space, wings without white scales (Figure 2.22) ……*Culex raptor*

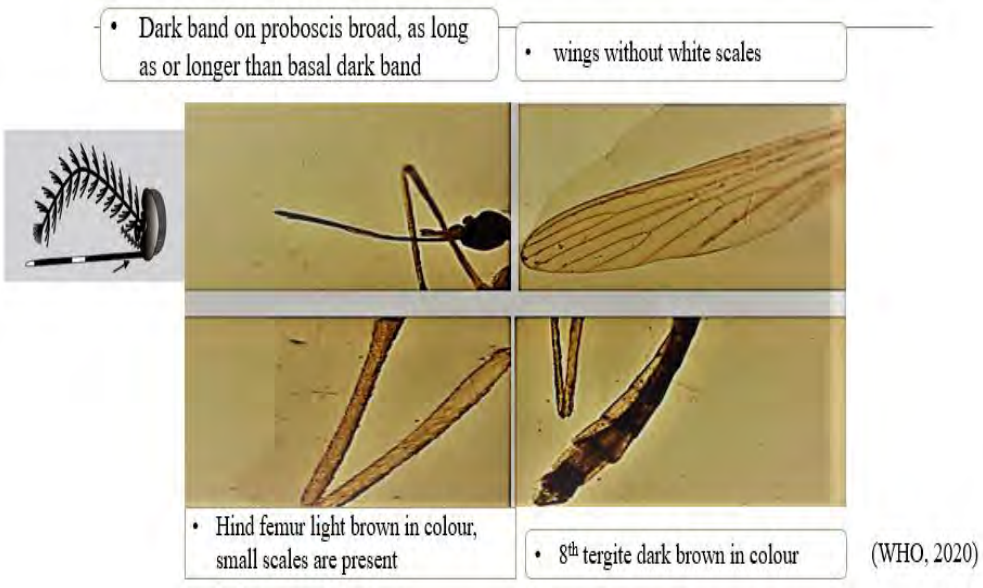


Figure 2.22: Identifying features of *Cx. raptor*

2.4.3: *Armigeres*

21. Proboscis and palpi brownish black in colour, palpi one third to the length of proboscis, dark scales on wings, brownish black abdomen, abdominal sternite 3rd and 4th segments with apically black band, claws on the middle leg are unequal in size (Figure 2.23) ……*Armigeres obturbans*

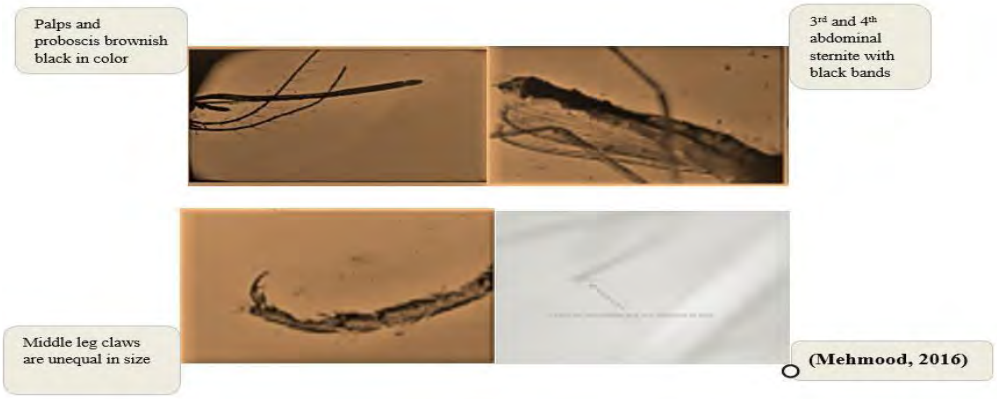


Figure 2.23: Identifying features of *Ar. obturbans*

22. Wings have few dark scales, same size of claws on the middle leg (Figure 2.24) ……*Armigeres kuchingensis*

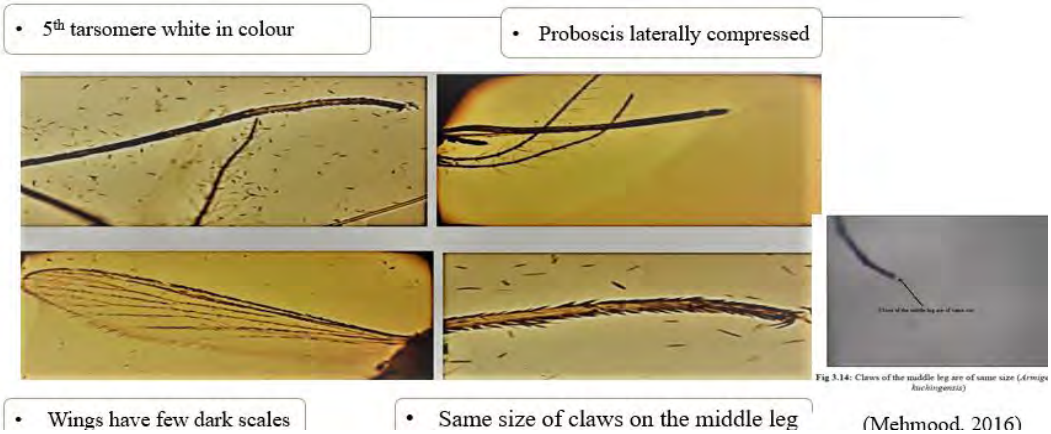


Figure 2.24: Identifying feature of Ar. *kuchingensis*

23. Proboscis curved downward and laterally compressed, terga with lateral pale patches notextending dorsally, legs with broad white band extending to or near apex (Figure 2.25) ……...*Armigeres subalbatus*

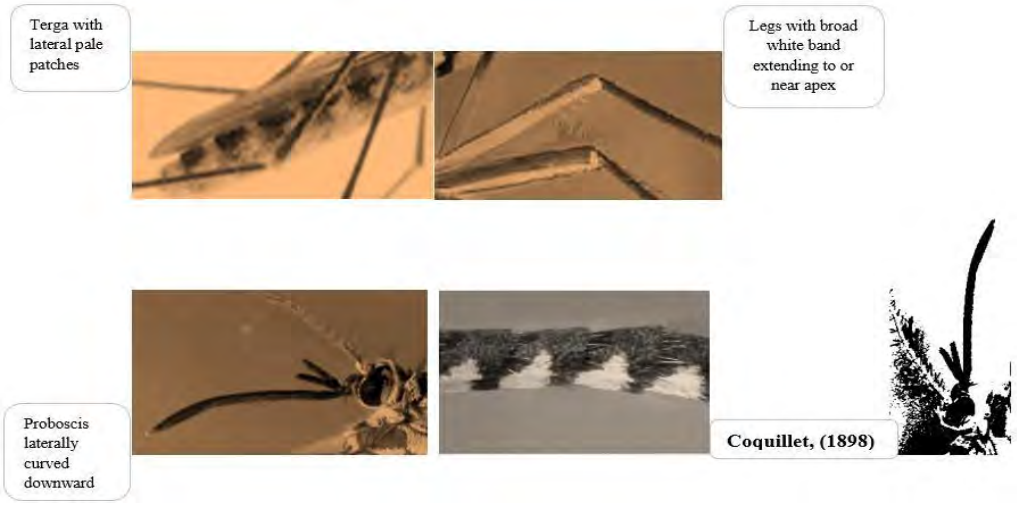


Figure 2.25: Identifying features of *Ar. subalbatus.*

24. Female proboscis is lateral compressed and slightly downturned at tip; male palpus is about the same length as proboscis; palpus is long, thin, and upturned; it is three-segmented, with the last two segments almost naked. Two lengthy rows of scales ventrally separating the eyes (Figure 2.26) ……. *Armigeres flavus*

Figure 2.26: Identifying features of *Ar. flavus*

2.4.4: *Aedes*

25. Dark colored body with typical white markings on the legs and lyre like markings on the thorax, Comb scales exhibiting a medial spine with stout, sub apical spines, proboscis is totally dark however palps are white tipped, dark scaled wings (Figure 2.27) …. .....*Aedes aegypti*

Figure 2.27: Identifying features of *Ae. aegypti.*

26. Identifiable by the palpus and tarsi's striking silver-white scales and prominent, lustrous black scales. The scutum (back) is black with an identifiable white stripe running down the middle from the dorsal surface of the head to the thorax, and it is free of sub apical spines (Figure 2.28) …… *Aedes albopictus*

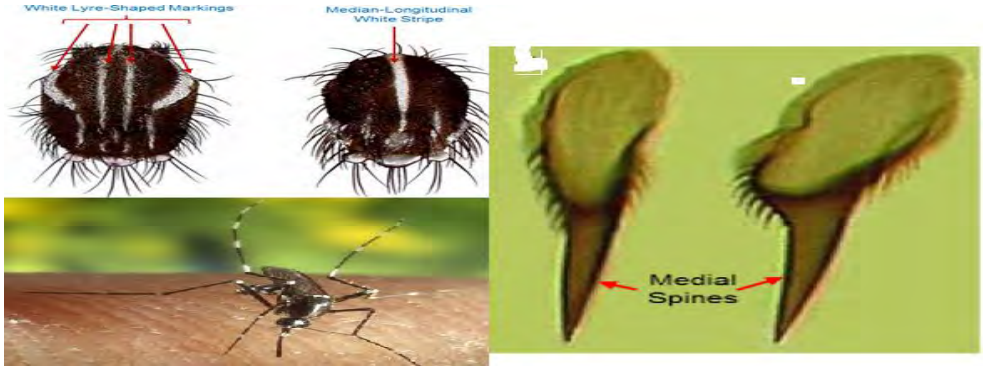


Figure 2.28: Identifying features of *Ae. albopictus*

2.4.5: *Mansonia*

27. Antenna length about equal to head width; head with prominent pointed projection under base of antenna. Clypeus bare, nearly as broad as long. Post spiracular setae present. Scales broad and asymmetrical, dark and pale scales intermixed (Figure 2.29) ……. *Ma. uniformis.*

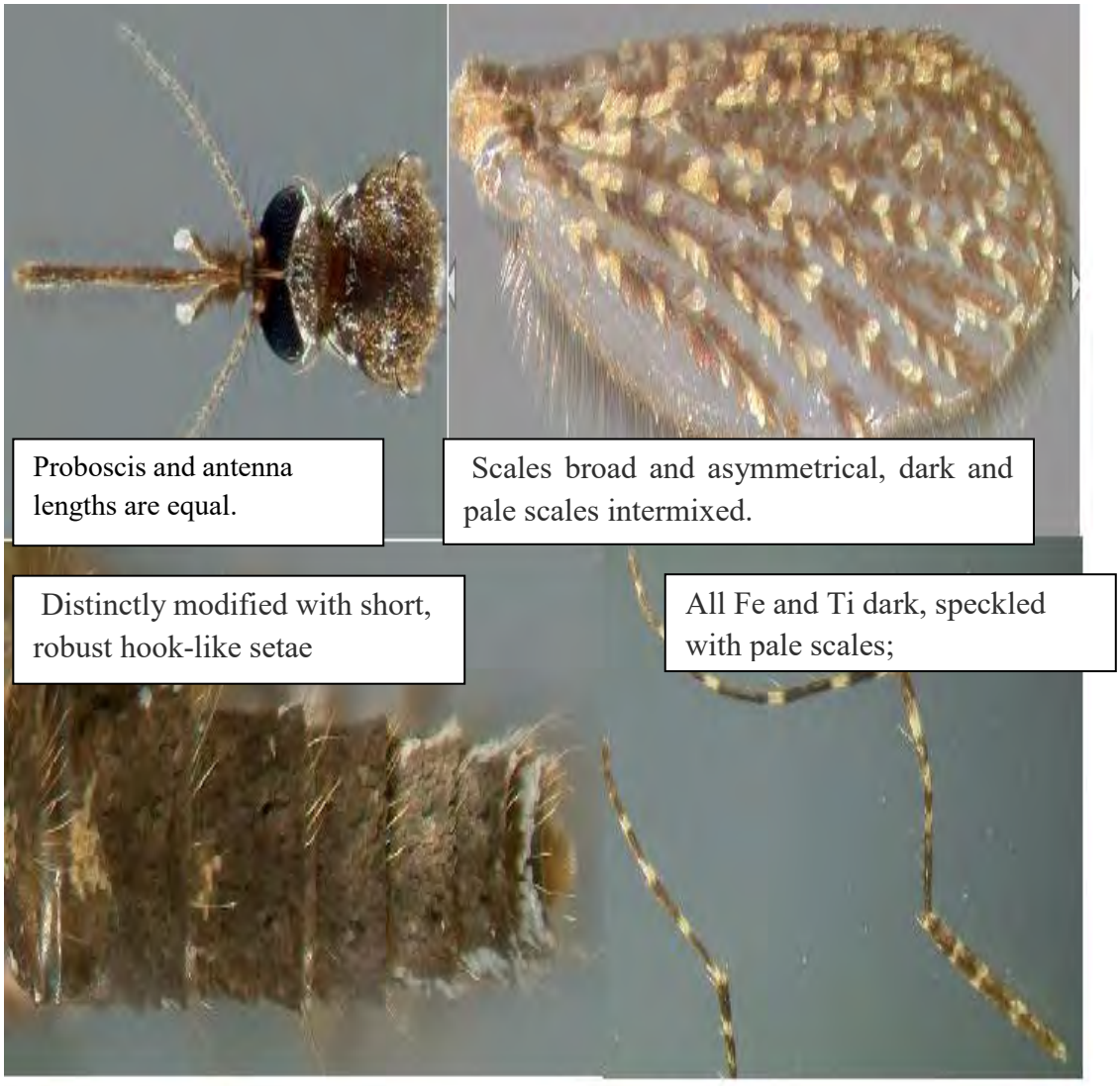


Figure 2.29: Identifying features of *Ma. uniformis*

2.5: MORPHOMETRIC MEASUREMENTS OF VARIOUS BODY PARTS OF MOSQUITOES SPECIES

2.5.1: *Anopheles annularis*

Table 2.6: Morphometric analysis of *An. annularis.*

Parameters (mm)	N	O. R	x̄	SD	SE	CV%
Full body length	6	3.2-5.3	4.2	1.3	0.9	32.1
Length of proboscis	6	1.9-2.7	2.3	0.5	0.4	22.3
Length of palpi	6	2.3-2.9	2.7	0.5	0.3	17.9
Length of antennae	6	1.7-2.2	2.0	0.4	0.3	21.1
Length of wings	6	2.9-3.9	3.5	0.7	0.5	20.1
Length of foreleg						
Femur	6	2.1-2.9	2.6	0.6	0.5	25.2
Tibia	6	1.8-3.1	2.4	0.9	0.7	36.8
Tarsus	6	2.9-3.9	3.4	0.7	0.5	20.1
Length of middle leg						
Femur	6	2.2-3.1	2.9	0.6	0.5	22.4
Tibia	6	1.9-2.9	2.5	0.7	0.5	27.8
Tarsus	6	3.1-3.8	3.4	0.6	0.5	17.0
Length of hind leg						
Femur	6	2.3-3.0	2.6	0.5	0.4	21.1
Tibia	6	2.1-3.1	2.5	0.7	0.5	27.1
Tarsus	6	3.1-4.3	3.7	0.8	0.6	22.9

Note: N=number of specimen, O. R=range, $\bar{X}$ =mean value, SD=standard deviation, SE=standarderror, C.V= coefficient of variance.

2.5.2: *Anopheles stephensi*

Table 2.7: Morphometric analysis of *An. stephensi.*

Parameters (mm)	N	O.R	x̄	SD	SE	CV%
Full body length	6	3.7-5.3	4.5	1.2	0.8	25.6
Length of proboscis	6	1.5-3.9	2.8	1.7	1.2	61.3
Length of palpi	6	1.6-4.1	2.8	1.7	1.2	61.9
Length of antennae	6	1.4-2.8	2.1	1.4	0.7	50.2

Length of wings	6	3.9-4.7	4.2	0.9	0.6	22.4
Length of foreleg						
Femur	6	1.9-3.0	2.5	0.8	0.6	32.7
Tibia	6	2.0-2.9	2.5	0.6	0.5	25.9
Tarsus	6	3.0-3.7	3.3	0.5	0.4	16.1
Length of middle leg						
Femur	6	2.1-3.0	2.5	0.5	0.4	23.5
Tibia	6	2.3-2.8	2.6	0.4	0.3	13.9
Tarsus	6	3.0-3.9	3.5	0.7	0.5	19.6
Length of hind leg						
Femur	6	2.1-2.9	2.5	0.6	0.4	23.9
Tibia	6	2.2-2.9	2.5	0.5	0.4	21.6
Tarsus	6	3.6-4.7	4.1	0.8	0.5	18.3

2.5.3: *Anopheles fluviatilis*

Table 2.8: Morphometric analysis of *An. fluviatilis*

Parameters (mm)	N	O.R	$\bar{x}$	SD	SE	CV%
Full body length	6	4.0-4.8	4.4	0.6	0.4	12.8
Length of proboscis	6	1.7-2.0	1.8	0.2	0.2	12.2
Length of palpi	6	1.5-1.9	1.7	0.3	0.2	17.1
Length of antennae	6	1.3-1.8	1.5	0.4	0.3	24.1
Length of wings	6	3.2-4.0	3.6	0.5	0.3	14.9
Length of foreleg						
Femur	6	2.1-3.1	2.1	1.0	0.7	4.7
Tibia	6	1.8-2.1	1.9	0.2	0.9	6.6
Tarsus	6	2.8-3.1	2.9	0.1	0.2	6.2
Length of middle leg						
Femur	6	2.1-2.5	2.2	0.3	0.2	14.2
Tibia	6	1.9-2.3	2.1	0.3	0.21	13.9
Tarsus	6	2.9-3.2	3.1	0.1	0.1	3.9
Length of hind leg						
Femur	6	2.1-2.5	2.3	0.3	0.2	11.1

Tibia	6	2.2-2.4	2.4	0.1	0.1	6.4
Tarsus	6	3.4-4.1	3.7	0.5	0.4	14.1

2.5.4: *Anopheles maculatus*

Table 2.9: Morphometric analysis of *An. maculatus.*

Parameters (mm)	N	O.R	x̄	SD	SE	CV%
Full body length	6	3.9-5.4	4.6	1.1	0.8	24.1
Length of proboscis	6	1.5-3.9	2.7	1.7	1.2	61.3
Length of palpi	6	1.6-3.9	2.7	1.6	1.2	60.1
Length of antennae	6	1.4-2.9	2.1	1.2	0.8	51.2
Length of wings	6	3.4-4.7	4.3	0.9	0.6	22.7
Length of foreleg						
Femur	6	1.9-3.1	2.4	0.8	0.6	32.2
Tibia	6	2.0-3.0	2.5	0.6	0.4	25.7
Tarsus	6	2.9-3.7	3.5	0.5	0.4	15.8
Length of middle leg						
Femur	6	2.1-2.9	2.5	0.6	0.4	23.3
Tibia	6	2.3-2.8	2.6	0.4	0.3	14.1
Tarsus	6	3.1-4.0	3.5	0.7	0.5	19.6
Length of hind leg						
Femur	6	2.1-2.9	2.5	0.6	0.4	23.6
Tibia	6	2.2-2.9	2.6	0.5	0.4	21.7
Tarsus	6	3.6-4.7	4.1	0.8	0.5	18.7

2.5.5: *Anopheles subpictus*

Table 2.10: Morphometric analysis of *An. subpictus.*

Parameters (mm)	N	O.R	x̄	SD	SE	CV%
Full body length	6	3.3-5.1	4.1	1.2	0.9	29.4
Length of proboscis	6	1.5-2.9	2.2	1.2	0.7	43.7
Length of palpi	6	1.7-3.1	2.4	0.9	0.6	37.3
Length of antennae	6	1.1-2.1	1.6	0.7	0.5	41.9
Length of wings	6	3.1-4.3	3.7	1.2	0.7	26.1
Length of foreleg						
Femur	6	1.9-2.1	1.9	0.1	0.6	4.2

Tibia	6	1.8-1.9	2.2	0.2	0.1	8.7
Tarsus	6	2.9-3.2	3.1	0.2	0.6	6.8
Length of middle leg						
Femur	6	2.1-1.9	1.9	0.2	0.4	1.2
Tibia	6	1.8-1.9	2.1	0.1	0.1	6.2
Tarsus	6	2.5-3.2	2.9	0.5	0.3	17.7
Length of hind leg						
Femur	6	2.1-2.2	2.1	0.2	0.1	7.6
Tibia	6	1.9-2.2	2.1	0..4	0.2	11.1
Tarsus	6	2.9-3.9	3.5	0.7	0.5	20.3

2.5.6: *Anopheles culicifacies*

Table 2.11: Morphometric analysis of *An. culicifacies.*

Parameters (mm)	**N**	**O.R**	$\bar{x}$	**SD**	**SE**	**CV%**
Full body length	6	3.3-5.1	4.2	1.2	0.9	29.3
Length of proboscis	6	1.6-2.5	2.1	0.6	0.5	31.7
Length of palpi	6	1.5-2.6	2.3	0.8	0.5	37.4
Length of antennae	6	1.3-2.0	1.7	0.5	0.3	28.6
Length of wings	6	3.3-4.8	4.1	1.1	0.8	26.9
Length of fore leg						
Femur	6	1.3-2.3	1.8	0.7	0.5	40.4
Tibia	6	1.3-2.3	1.8	0.7	0.5	39.9
Tarsals	6	2.4-3.6	3.1	0.8	0.6	27.5
Length of middle leg						
Femur	6	1.3-2.6	12.0	0.9	0.6	45.5
Tibia	6	1.3-2.6	1.9	0.9	0.7	47.6
Tarsals	6	2.3-3.8	3.1	1.0	0.7	33.3
Length of hind leg						
Femur	6	2.0-2.5	2.1	0.3	0.2	14.5
Tibia	6	1.9-2.8	2.4	0.6	0.4	23.1
Tarsals	6	3.2-4.3	3.8	0.8	0.6	20.8

2.5.7: *Anopheles sinensis*

Table 2.12: Morphometric measurements of *An. sinensis.*

Parameters (mm)	N	O.R	$\overline{X}$	SD	SE	CV%
Full body length	6	3.1-4.7	3.9	1.1	0.8	28.7
Length of proboscis	6	1.6-2.0	1.8	0.3	0.2	17.6
Length of palpi	6	1.5-2.5	1.9	0.7	0.5	34.7
Length of antennae	6	1.3-1.7	1.5	0.3	0.2	21.8
Length of wings	6	2.6-3.9	3.2	0.9	0.7	29.0
Length of fore leg						
Femur	6	1.9-2.1	2.1	0.2	0.1	7.8
Tibia	6	2.1-2.4	2.3	0.2	0.2	9.5
Tarsals	6	2.8-3.2	2.9	0.3	0.3	9.5
Length of middle leg						
Femur	6	2.2-2.9	2.4	0.6	0.4	25.1
Tibia	6	2.1-2.9	2.4	0.7	0.5	23.1
Tarsals	6	2.9-3.8	3.4	0.8	0.7	16.8
Length of hind leg						
Femur	6	2.0-2.9	2.5	0.6	0.4	24.6
Tibia	6	2.2-2.5	2.4	0.2	0.2	9.9
Tarsals	6	3.7-4.1	3.9	0.3	0.2	6.6

2.5.8. *Anopheles tesselatus*

Table 2.13: Morphometric measurements of *An. tesselatus.*

Parameters (mm)	N	O.R	$\overline{X}$	SD	SE	CV%
Full body length	6	3.6-4.1	3.8	0.7	0.3	9.5
Length of proboscis	6	1.6-2.0	1.8	0.3	0.2	16.6
Length of palpi	6	1.6-2.1	1.9	0.6	0.3	19.3
Length of antennae	6	1.3-1.8	1.5	0.4	0.4	22.9
Length of wings	6	3.3-3.6	3.5	0.1	0.1	4.2
Length of fore leg						
Femur	6	2.1-2.2	2.1	0.5	0.4	6.2
Tibia	6	1.9-2.0	1.9	0.3	0.2	1.7
Tarsals	6	2.2-2.8	2.5	0.4	0.3	14.5
Length of middle leg						
Femur	6	2.1-2.2	2.1	0.1	0.7	4.5
Tibia	6	1.9-2.0	1.9	0.5	0.8	0.4
Tarsals	6	3.0-3.3	3.2	0.2	0.2	7.6
Length of hind leg						
Femur	6	2.1-2.6	2.3	0.3	0.2	14.4

Tibia	6	1.9-2.4	2.2	0.3	0.2	14.2
Tarsals	6	3.9-4.5	4.2	0.4	0.3	10.

2.5.9: *Anopheles aconitus*

Table 2.14: Morphometric measurements of *An. aconitus*

Parameters (mm)	**N**	**O.R**	$\overline{X}$	**SD**	**SE**	**CV%**
Full body length	6	3.9-4.7	4.3	0.5	0.3	11.1
Length of proboscis	6	1.9-1.9	1.9	0.1	0.1	3.6
Length of palpi	6	1.8-2.1	1.9	0.3	0.5	7.2
Length of antennae	6	1.5-1.9	1.7	0.4	0.2	15.1
Length of wings	6	2.9-3.5	3.2	0.3	0.2	10.3
Length of fore leg						
Femur	6	1.8-2.1	1.9	0.1	0.1	6.9
Tibia	6	1.9-2.2	1.9	0.2	0.6	6.1
Tarsals	6	2.9-3.5	3.2	0.5	0.3	14.5
Length of middle leg						
Femur	6	1.9-2.1	1.9	0.1	0.1	5.3
Tibia	6	1.9-2.1	2.1	0.1	0.3	2.7
Tarsals	6	2.8-3.2	3.1	0.3	0.2	9.9
Length of hind leg						
Femur	6	1.9-2.1	1.9	0.2	0.1	0.9
Tibia	6	1.9-2.0	2.1	0.3	0.3	1.9
Tarsals	6	3.0-4.1	3.5	0.7	0.5	19.9

2.5.10: *Anopheles splendidus koidzum*

Table 2.15: Morphometric measurements of *Anopheles splendidus koidzum.*

Parameters (mm)	**N**	**O.R**	$\overline{X}$	**SD**	**SE**	**CV%**
Full body length	6	3.3-5.0	4.1	1.2	0.9	29.3
Length of proboscis	6	1.5-2.9	2.2	1.0	0.7	43.6
Length of palpi	6	1.7-3.0	2.4	0.9	0.6	37.2

Length of antennae	6	1.1-2.1	1.6	0.7	0.5	41.8
Length of wings	6	3.1-4.3	3.7	1.0	0.7	26.1
Length of fore leg						
Femur	6	1.9-2.0	1.9	0.1	0.1	4.2
Tibia	6	1.8-2.1	1.9	0.2	0.1	8.6
Tarsals	6	2.9-3.2	3.0	0.2	0.2	6.7
Length of middle leg						
Femur	6	2.1-2.3	2.0	0.2	0.1	1.1
Tibia	6	1.8-2.0	1.9	0.1	0.3	6.2
Tarsals	6	2.5-3.2	2.9	0.5	0.4	17.6
Length of hind leg						
Femur	6	2.1-2.2	2.1	0.2	0.7	7.5
Tibia	6	1.9-2.2	2.1	0.3	0.2	10.9
Tarsals	6	3.1-4.0	3.5	0.7	0.5	20.2

2.5.11: *Culex quinquefasciatus*

Table 2.16: Morphometric measurements of *Cx. quinquefasciatus.*

Parameters (mm)	**N**	**O.R**	$\overline{X}$	**SD**	**SE**	**CV%**
Full body length	6	3.2-4.2	3.7	0.7	0.5	19.3
Length of proboscis	6	2.0-2.8	2.4	0.5	0.4	21.2
Length of palpi	6	0.4-0.7	0.5	0.1	0.1	27.2
Length of antennae	6	1.3-1.6	1.4	0.2	0.2	15.9
Length of wings	6	2.5-2.7	2.6	0.1	0.1	5.6
Length of fore leg						
Femur	6	2.1-2.4	2.2	0.2	0.1	8.3
Tibia	6	2.0-2.2	2.1	0.2	0.1	9.7
Tarsals	6	2.4-3.0	2.7	0.4	0.3	16.3
Length of middle leg						
Femur	6	2.1-2.4	2.3	0.3	0.2	12.0
Tibia	6	2.0-2.3	2.2	0.2	0.2	11.1
Tarsals	6	2.7-3.0	2.9	0.2	0.2	8.0
Length of hind leg						

Femur	6	2.0-2.5	2.2	0.3	0.2	15.2
Tibia	6	2.3-2.5	2.4	0.2	0.1	7.5
Tarsals	6	30-3.8	3.4	0.6	0.4	16.9

2.5.12: *Culex tritaeniorhynchus*

Table 2.17: Morphometric measurements of *Cx. tritaeniorhynchus*

Parameters (mm)	**N**	**O.R**	$\overline{X}$	**SD**	**SE**	**CV%**
Full body length	6	3.1-5.6	4.4	1.8	1.2	40.4
Length of proboscis	6	1.8-3.7	2.8	1.4	1.0	49.0
Length of palpi	6	0.5-2.0	1.2	1.1	0.8	86.4
Length of antennae	6	1.3-3.3	2.3	1.4	1.0	62.5
Length of wings	6	2.6-4.6	3.6	1.4	1.0	40.0
Length of fore leg						
Femur	6	2.0-2.4	2.2	0.3	0.2	13.6
Tibia	6	1.9-2.1	2.0	0.2	0.1	7.9
Tarsals	6	2.7-3.0	2.8	0.2	0.2	8.2
Length of middle leg						
Femur	6	2.2-2.4	2.3	0.2	0.1	7.9
Tibia	6	1.9-2.4	2.1	0.4	0.3	18.2
Tarsals	6	2.8-3.6	3.2	0.5	0.4	16.0
Length of hind leg						
Femur	6	2.1-2.4	2.2	0.2	0.1	7.8
Tibia	6	2.4-2.4	2.4	0.1	0.1	1.8
Tarsals	6	2.9-3.5	3.2	0.4	0.3	12.6

2.5.13: *Culex bitaeniorhynchus*

Table 2.18: Morphometric measurements of *Cx. bitaeniorhynchus.*

Parameters (mm)	**N**	**O.R**	$\overline{X}$	**SD**	**SE**	**CV%**
Full body length	6	3.6-4.9	4.2	0.9	0.7	21.9
Length of proboscis	6	1.6-2.3	2.0	0.5	0.3	23.9
Length of palpi	6	0.2-1.4	0.8	0.8	0.6	100.4
Length of antennae	6	1.4-2.2	1.8	0.5	0.4	30.1
Length of wings	6	2.5-4.0	3.2	1.1	0.8	33.5
Length of fore leg						

Femur	6	2.2-2.5	2.3	0.2	0.2	10.0
Tibia	6	2.3-2.3	2.3	0.1	0.1	1.8
Tarsals	6	2.5-3.0	2.8	0.2	0.2	10.3
Length of middle leg						
Femur	6	2.2-2.5	2.3	0.3	0.1	7.6
Tibia	6	2.2-2.3	2.3	0.1	0.2	3.3
Tarsals	6	3.3-3.4	3.4	0.2	0.3	3.5
Length of hind leg						
Femur	6	2.1-2.3	2.2	0.1	0.1	4.4
Tibia	6	2.3-2.5	2.4	0.2	0.3	7.2
Tarsals	6	3.6-4.0	3.8	0.2	0.2	5.5

2.5.14: *Culex geli*dus

Table 2.19: Morphometric measurements of *Cx. gelidus.*

Parameters (mm)	**N**	**O.R**	$\overline{X}$	**SD**	**SE**	**CV%**
Full body length	6	3.4-4.9	4.2	1.1	0.7	25.3
Length of proboscis	6	2.0-2.9	2.4	0.6	0.4	26.0
Length of palpi	6	0.5-1.1	0.8	0.4	0.3	55.9
Length of antennae	6	1.7-2.8	2.3	0.8	0.6	37.4
Length of wings	6	2.4-3.3	2.9	0.6	0.4	22.0
Length of fore leg						
Femur	6	1.9-2.1	2.0	0.2	0.1	7.6
Tibia	6	2.1-2.1	2.1	0.1	0.4	0.6
Tarsals	6	2.9-3.1	3.0	0.2	0.3	5.6
Length of middle leg						
Femur	6	2.0-2.4	2.2	0.3	0.2	15.3
Tibia	6	2.0-2.3	2.2	0.4	0.3	11.6
Tarsals	6	3.0-3.5	3.3	0.5	0.4	11.9
Length of hind leg						
Femur	6	2.1-2.4	2.3	0.2	0.2	10.6
Tibia	6	2.1-2.3	2.2	0.5	0.1	7.3
Tarsals	6	3.3-4.0	3.7	0.4	0.3	11.4

2.5.15: *Culex fuscocephala*

Table 2.20: Morphometric measurements of *Cx. fuscocephala.*

Parameters (mm)	**N**	**O.R**	$\overline{X}$	**SD**	**SE**	**CV%**

Full body length	6	3.8-4.6	4.2	0.6	0.4	13.5
Length of proboscis	6	2.0-2.8	2.4	0.5	0.4	22.8
Length of palpi	6	0.4-1.0	0.7	0.4	0.3	61.2
Length of antennae	6	1.6-2.7	2.1	0.8	0.5	36.2
Length of wings	6	3.5-4.0	3.8	0.3	0.2	9.1
Length of fore leg						
Femur	6	1.8-2.0	1.9	0.1	0.3	7.1
Tibia	6	1.9-2.0	2.0	0.2	0.1	5.3
Tarsals	6	3.0-3.6	3.3	0.4	0.3	12.5
Length of middle leg						
Femur	6	1.9-2.0	1.9	0.1	0.8	6.0
Tibia	6	1.9-2.0	2.0	0.4	0.3	2.8
Tarsals	6	3.0-3.7	3.3	0.5	0.4	14.8
Length of hind leg						
Femur	6	1.9-2.1	2.0	0.2	0.1	7.9
Tibia	6	1.9-2.0	1.9	0.3	0.6	5.0
Tarsals	6	3.1-4.0	3.5	0.6	0.4	17.9

2.5.16: *Culex vishnui*

Table 2.21: Morphometric measurements of *Cx. vishnui.*

Parameters (mm)	**N**	**O.R**	$\overline{X}$	**SD**	**SE**	**CV%**
Full body length	6	3.9-6.0	4.9425	1.4	1.0	29.3
Length of proboscis	6	1.6-2.1	1.865	0.3	0.2	17.7
Length of palpi	6	0.5-1.6	1.0285	0.8	0.6	76.3
Length of antennae	6	1.7-2.3	1.9985	0.5	0.3	24.3
Length of wings	6	2.7-4.7	3.712	1.4	1.0	37.1
Length of fore leg						
Femur	6	2.0-2.2	2.1055	0.2	0.1	8.6
Tibia	6	2.0-2.3	2.1795	0.2	0.2	10.5
Tarsals	6	3.2-3.7	3.423	0.4	0.3	10.5
Length of middle leg						
Femur	6	2.0-2.5	2.244	0.3	0.2	13.4
Tibia	6	2.1-2.6	2.3445	0.3	0.3	14.0
Tarsals	6	3.2-3.8	3.5015	0.4	0.4	10.8
Length of hind leg						
Femur	6	2.1-2.5	2.282	0.3	0.2	13.3
Tibia	6	2.2-2.6	2.3835	0.4	0.3	12.2

Tarsals	6	4.0-4.6	4.246	0.4	0.3	10.6

2.5.17: *Culex fatigans*

Table 2.22: Morphometric measurements of *Cx. fatigans*.

Parameters (mm)	**N**	**O.R**	$\overline{X}$	**SD**	**SE**	**CV%**
Full body length	6	4.0-5.1	4.5	0.8	0.6	17.5
Length of proboscis	6	2.0-2.3	2.2	0.3	0.2	12.3
Length of palpi	6	0.5-1.1	0.8	0.4	0.3	50.5
Length of antennae	6	1.6-2.4	2.0	0.5	0.4	27.1
Length of wings	6	3.3-4.8	4.065	1.1	0.8	27.2
Length of fore leg						
Femur	6	1.9-2.1	2..0	0.1	0.1	6.1
Tibia	6	2.1-2.3	2.2	0.2	0.1	7.8
Tarsals	6	2.9-3.1	3.0	0.1	0.2	3.7
Length of middle leg						
Femur	6	2.0-2.1	2.1	0.1	0.079	5.4
Tibia	6	2.0-2.3	2.2	0.2	0.165	10.8
Tarsals	6	3.1-3.8	3.4	0.5	0.378	15.8
Length of hind leg						
Femur	6	1.8-2.2	2.0	0.3	0.1	12.4
Tibia	6	2.1-2.4	2.2	0.2	0.2	9.9
Tarsals	6	3.6-4.9	4.2	0.9	0.7	21.8

2.5.18: *Culex raptor*

Table 2.23: Morphometric measurements of *Cx. raptor.*

Parameters (mm)	**N**	**O.R**	$\overline{X}$	**SD**	**SE**	**CV%**
Full body length	6	4.8-5.9	5.4	0.8	0.6	15.0
Length of proboscis	6	2.0-2.9	2.4	0.6	0.4	24.6
Length of palpi	6	0.5-1.1	0.8	0.4	0.3	51.8
Length of antennae	6	1.8-2.7	2.2	0.6	0.4	27.3
Length of wings	6	3.5-4.6	4.0	0.7	0.5	18.6
Length of fore leg						
Femur	6	2.2-3.0	2.6	0.5	0.4	20.0
Tibia	6	2.3-3.0	2.7	0.5	0.3	18.0
Tarsals	6	3.0-4.2	3.6	0.8	0.6	22.5
Length of middle leg						

Femur	6	2.2-2.5	2.4	0.2	0.1	7.6
Tibia	6	2.4-2.6	2.5	0.3	0.1	5.8
Tarsals	6	3.4-4.5	4.0	0.8	0.6	20.0
Length of hind leg						
Femur	6	2.3-2.7	2.5	0.3	0.2	10.6
Tibia	6	2.3-2.7	2.6	0.4	0.2	12.0
Tarsals	6	4.0-4.9	4.4	0.6	0.4	14.2

2.5.19: *Culex vagans*

Table 2.24: Morphometric analysis of *Cx. vagans.*

Parameters (mm)	N	O.R	$\bar{x}$	SD	SE	CV%
Full body length	6	3.2-4.3	3.8	0.8	0.4	20.7
Length of proboscis	6	2.0-2.5	2.2	0.3	0.2	14.7
Length of palpi	6	0.6-2.0	1.3	1.0	0.5	75.5
Length of antennae	6	2.2-2.4	2.3	0.2	0.6	6.6
Length of wings	6	2.9-4.3	3.6	1.0	0.7	28.6
Length of foreleg						
Femur	6	2.1-2.4	2.3	0.2	0.1	8.8
Tibia	6	2.0-2.3	2.1	0.2	0.1	9.2
Tarsus	6	2.4-3.0	2.7	0.7	0.3	13.6
Length of middle leg						
Femur	6	2.1-2.5	2.3	0.2	0.2	12.4
Tibia	6	2.0-2.4	2.2	0.3	0.2	11.0
Tarsus	6	2.7-3.0	2.9	0.4	0.2	8.6
Length of hind leg						
Femur	6	2.0-2.5	2.2	0.3	0.2	15.5
Tibia	6	2.2-2.5	2.4	0.2	0.12	7.2
Tarsus	6	3.0-3.8	4.0	0.6	0.42	17.5

2.5.20: *Culex pipiens*

Table 2.25: Morphometric analysis of *Cx. pipiens.*

Parameters (mm)	N	O.R	$\overline{X}$	SD	SE	CV%
Full body length	6	3.2-4.2	3.7	0.7	0.5	19.3

Length of proboscis	6	2.2-2.3	2.2	0.1	0.1	3.8
Length of palpi	6	0.6-2.0	1.3	1.1	0.7	80.4
Length of antennae	6	1.3-1.6	1.4	0.2	0.2	16.6
Length of wings	6	3.5-4.0	3.8	0.3	0.2	8.9
Length of foreleg						
Femur	6	2.1-2.4	2.2	0.2	0.1	8.4
Tibia	6	2.0-2.2	2.1	0.2	0.1	10.0
Tarsus	6	2.1-3.0	2.7	0.4	0.3	16.4
Length of middle leg						
Femur	6	2.1-2.4	2.2	0.3	0.2	12.2
Tibia	6	2.0-2.4	2.2	0.2	0.2	11.3
Tarsus	6	2.7-3.0	2.9	0.2	0.2	8.4
Length of hind leg						
Femur	6	2.0-2.5	2.2	0.3	0.2	15.3
Tibia	6	2.3-2.5	2.4	0.2	0.1	7.4
Tarsus	6	3.0-3.8	4.0	0.6	0.4	17.2

2.5.21*: Aedes aegypti*

Table 2.26: Morphometric analysis of *Ae. aegypti.*

Parameters (mm)	N	O.R	$\overline{X}$	SD	SE	CV%
Full body length	6	1.8-2.6	3.0	0.6	0.5	9.3
Length of proboscis	6	1.9-2.1	2.0	0.2	0.1	8.2
Length of palpi	6	0.5-0.5	0.5	0.1	0.2	0.3
Length of antennae	6	1.8-2.0	1.9	0.1	0.1	8.0
Length of wings	6	2.7-2.7	2.7	0.2	0.3	1.8
Length of foreleg						
Femur	6	1.8-1.9	1.8	0.3	0.2	1.9
Tibia	6	1.9-1.9	1.9	0.2	0.4	0.3
Tarsus	6	2.271-2.4	2.4	0.1	0.1	4.8
Length of middle leg						
Femur	6	1.9-2.2	2.1	0.3	0.2	12.9

Tibia	6	1.9-2.1	2.0	0.1	0.1	7.5
Tarsus	6	2.7-2.8	2.7	0.1	0.2	0.3
Length of hind leg						
Femur	6	1.7-1.8	1.7	0.1	0.4	0.2
Tibia	6	1.8-2.5	2.2	0.5	0.7	0.4
Tarsus	6	3.5-3.9	3.7	0.3	6.9	0.2

2.5.22: *Aedes albopictus*

Table 2.27: Morphometric measurements of *Ae. albopictus.*

Parameters (mm)	**N**	**O.R**	$\overline{X}$	**SD**	**SE**	**CV%**
Full body length	6	4.0-4.8	4.4	0.6	0.4	12.7
Length of proboscis	6	1.7-2.0	1.8	0.2	0.2	12.2
Length of palpi	6	1.5-1.9	1.7	0.1	0.2	17.0
Length of antennae	6	1.3-1.8	1.5	0.4	0.3	23.8
Length of wings	6	3.2-4.0	3.6	0.5	0.1	14.9
Length of fore leg						
Femur	6	2.0-2.1	2.0	0.1	0.1	4.6
Tibia	6	1.8-2.0	1.9	0.2	0.2	6.4
Tarsals	6	2.8-3.0	2.9	0.6	0.1	5.7
Length of middle leg						
Femur	6	2.0-2.5	2.2	0.3	0.2	13.9
Tibia	6	1.9-2.3	2.1	0.4	0.2	13.8
Tarsals	6	3.0-3.2	3.1	0.1	0.5	3.9
Length of hind leg						
Femur	6	2.1-2.5	2.3	0.5	0.2	11.0
Tibia	6	2.2-2.4	2.3	0.2	0.1	6.3
Tarsals	6	3.4-4.1	3.7	0.5	0.4	14.1

2.5.23: *Armigeres subalbatus*

Table 2.28: Morphometric analysis of *Ar. subalbatus.*

Parameters (mm)	N	O.R	$\bar{x}$	SD	SE	CV%

Full body length	6	3.6-4.9	4.3	0.6	0.4	14.7
Length of proboscis	6	2.0-2.6	2.3	0.3	0.2	14.6
Length of palpi	6	0.3-1.0	0.6	0.3	0.2	50.5
Length of antennae	6	2.0-3.0	2.5	0.5	0.4	20.4
Length of wings	6	2.9-4.1	3.5	0.6	0.4	17.3
Length of foreleg						
Femur	6	1.7-2.0	1.9	0.1	0.1	6.7
Tibia	6	1.9-2.0	2.0	0.1	0.4	3.2
Tarsus	6	3.0-3.2	3.1	0.1	0.3	3.8
Length of middle leg						
Femur	6	1.7-1.9	1.8	0.1	0.1	5.9
Tibia	6	1.8-2.0	1.9	0.2	0.1	4.5
Tarsus	6	2.9-3.3	3.1	0.2	0.2	7.5
Length of hind leg						
Femur	6	1.8-2.0	1.9	0.3	0.1	5.1
Tibia	6	2.0-2.1	2.0	0.1	0.1	3.7
Tarsus	6	4.0-4.1	3.9	0.2	0.2	5.6

2.5.24: *Armigeres obturbans*

Table 2.29: Morphometric analysis of *Ar. obturbans*

Parameters (mm)	N	O.R	$\bar{x}$	SD	SE	CV%
Full body length	6	4.0-6.0	5.0	1.40	1.0	28.2
Length of proboscis	6	2.1-4.0	3.0	1.3	0.9	42.2
Length of palpi	6	0.6-2.0	1.3	1.0	0.7	77.1
Length of antennae	6	2.0-3.5	2.7	1.1	0.7	38.8
Length of wings	6	2.9-4.9	3.9	1.4	1.0	35.4
Length of foreleg						

Femur	6	1.3-2.8	2.0	1.0	0.7	50.0
Tibia	6	1.5-2.7	2.1	0.8	0.6	38.0
Tarsus	6	2.8-3.7	3.2	0.6	0.5	20.0
Length of middle leg						
Femur	6	1.4-2.9	2.1	1.0	0.7	49.0
Tibia	6	1.5-3.0	2.2	1.1	0.8	48.2
Tarsus	6	3.0-4.0	3.5	0.7	0.5	20.0
Length of hind leg						
Femur	6	1.5-2.8	2.1	0.9	0.6	42.2
Tibia	6	1.7-2.9	2.3	0.9	0.6	37.6
Tarsus	6	3.0-4.1	3.5	0.8	0.7	22.0

2.5.25: *Ar. kuchingensis*

Table 2.30: Morphometric analysis of *Ar. kuchingensis.*

Parameters (mm)	N	O.R	$\bar{x}$	SD	SE	CV%
Full body length	6	3.6-4.9	4.3	0.9	0.7	21.9
Length of proboscis	6	2.0-2.7	2.3	0.5	0.4	21.4
Length of palpi	6	0.3-1.0	0.7	0.5	0.3	71.7
Length of antennae	6	2.0-3.0	2.5	0.7	0.5	29.1
Length of wings	6	2.9-4.1	3.5	0.9	0.6	24.4
Length of fore leg						
Femur	6	1.7-2.1	1.9	0.2	0.1	9.6
Tibia	6	1.9-2.0	2.1	0.1	0.2	4.5
Tarsus	6	2.9-3.2	3.1	0.2	0.3	5.3
Length of middle leg						
Femur	6	1.7-1.9	1.8	0.2	0.1	8.4
Tibia	6	1.8-2.0	1.9	0.1	0.2	6.6
Tarsus	6	2.9-3.4	3.1	0.3	0.2	10.7
Length of hind leg						

Femur	6	1.8-2.0	1.9	0.3	0.1	7.2
Tibia	6	2.0-2.1	2.0	0.1	0.2	5.3
Tarsus	6	3.7-4.1	3.9	0.3	0.3	7.9

2.5.26: *Armigeres flavus*

Table 2.31: Morphometric measurements of *Ar. flavus.*

Parameters (mm)	**N**	**O.R**	$\overline{X}$	**SD**	**SE**	**CV**
Full body length	6	3.7-5.1	4.3	0.9	0.7	21.9
Length of proboscis	6	2.1-2.7	2.3	0.5	0.4	21.3
Length of palpi	6	0.3-1.0	0.7	0.6	0.3	71.4
Length of antennae	6	1.9-3.0	2.5	0.7	0.5	28.9
Length of wings	6	2.9-4.1	3.5	0.8	0.6	24.3
Length of fore leg						
Femur	6	1.7-2.1	1.9	0.2	0.1	9.4
Tibia	6	1.9-2.0	2.0	0.1	0.1	4.3
Tarsals	6	3.1-3.2	3.1	0.2	0.2	5.2
Length of middle leg						
Femur	6	1.7-1.9	1.8	0.2	0.4	8.4
Tibia	6	1.8-2.1	1.9	0.1	0.3	6.6
Tarsals	6	2.9-3.4	3.1	0.3	0.2	10.6
Length of hind leg						
Femur	6	1.8-2.1	1.9	0.1	0.6	7.3
Tibia	6	2.0-2.1	2.0	0.1	0.2	5.1
Tarsals	6	3.7-4.1	3.9	0.3	0.2	7.8

2.5.27*: Mansonia uniformis*

Table 2.32: Morphometric measurements of *Ma. uniformis.*

Parameters (mm)	**N**	**O.R**	$\overline{X}$	**SD**	**SE**	**CV %**
Full body length	6	3.2-5.1	4.0	1.3	0.9	32.0
Length of proboscis	6	1.9-2.7	2.3	0.5	0.4	22.2
Length of palpi	6	2.3-3.1	2.7	0.5	0.3	18.0
Length of antennae	6	1.7-2.3	2.0	0.4	0.3	21.1
Length of wings	6	3.0-4.0	3.5	0.7	0.5	20.1
Length of fore leg						

Femur	6	2.1-3.1	2.5	0.6	0.4	24.9
Tibia	6	1.8-3.1	2.4	0.9	0.6	36.8
Tarsals	6	2.9-3.8	3.4	0.7	0.5	20.4
Length of middle leg						
Femur	6	2.2-3.0	2.6	0.6	0.4	22.4
Tibia	6	1.9-2.9	2.4	0.7	0.5	27.8
Tarsals	6	3.0-3.8	3.3	0.8	0.4	17.1
Length of hind leg						
Femur	6	2.2-3.0	2.6	0.5	0.4	21.1
Tibia	6	2.0-3.1	2.5	0.7	0.5	27.0
Tarsals	6	3.1-4.2	3.7	0.8	0.6	22.9

2.6: Relative abundance and Distribution status of mosquito species

2.6.1: Punjab

In terms of relative abundance 18 species were dominant, 4 subdominant and 5 species were satellite while in terms of distribution 3 species were constant, 1 frequent, 4 infrequent and 5 species were sporadic.

The highest number of mosquitoes was 275 and they belong to *Cx. pipiens* with relative abundance of 25.39% followed by *Cx. quinquefasciatus* with relative abundance 22.25%, *Ar. subalbatus* with relative abundance 16.34% and *Ar. obturbans* having relative abundance 11.08%. The rest of the species represent relatively less number and *Cx. bitaeniorhynchus* represents the lowest relative abundance of 1.29%.

Table 2.33: Relative abundance and distribution status of mosquitoes recovered from eleven districts of Punjab.

Species	Abundance	Relative abundance	Relative abundance status	Distribution status
Cx. quinquefasciatus	241	22.25	Dominant	Constant
Cx. tritaeniorhynchus	211	20.15	Dominant	Constant
Cx. vagans	95	10.23	Dominant	Frequent
Cx. vishnui	31	2.86	Satellite	Sporadic
Cx. fatigans	14	1.29	Satellite	Sporadic
Cx. raptor	56	5.2	Dominant	Infrequent

Cx. pipiens	122	7.6	Dominant	Frequent
Cx. gelidus	33	3.04	Subdominant	Infrequent
Cx. bitaeniorhynchus	275	25.39	Dominant	Constant
Cx. fuscocephala	33	3.04	Subdominant	Sporadic
An. culicifacies	187	18.45	Dominant	Constant
An. annularis	25	2.3	Satellite	Infrequent
An. stephensi	132	8.7	Dominant	Frequent
An. subpictus	145	13.54	Dominant	Constant
An. maculatus	21	1.93	Satellite	Sporadic
An. sinensis	122	11.47	Dominant	Frequent
An. fluviatilis	110	10.87	Dominant	Frequent
An. splendidus koidzum	98	9.65	Dominant	Frequent
An. tesselatus	47	4.33	Subdominant	Infrequent
An. aconitus	43	3.97	Subdominant	Infrequent
Ar. flavus	75	7.21	Dominant	Frequent
Ar. subalbatus	177	16.34	Dominant	Frequent
Ar. obturbans	120	11.08	Dominant	Constant
Ar. kuchingensis	84	7.24	Dominant	Frequent
Ae. aegypti	164	8.37	Dominant	Frequent
Ae. albopictus	175	17.24	Dominant	Constant
Ma. uniformis	23	2.12	Satellite	Sporadic

2.7: Molecular identification of mosquitoes

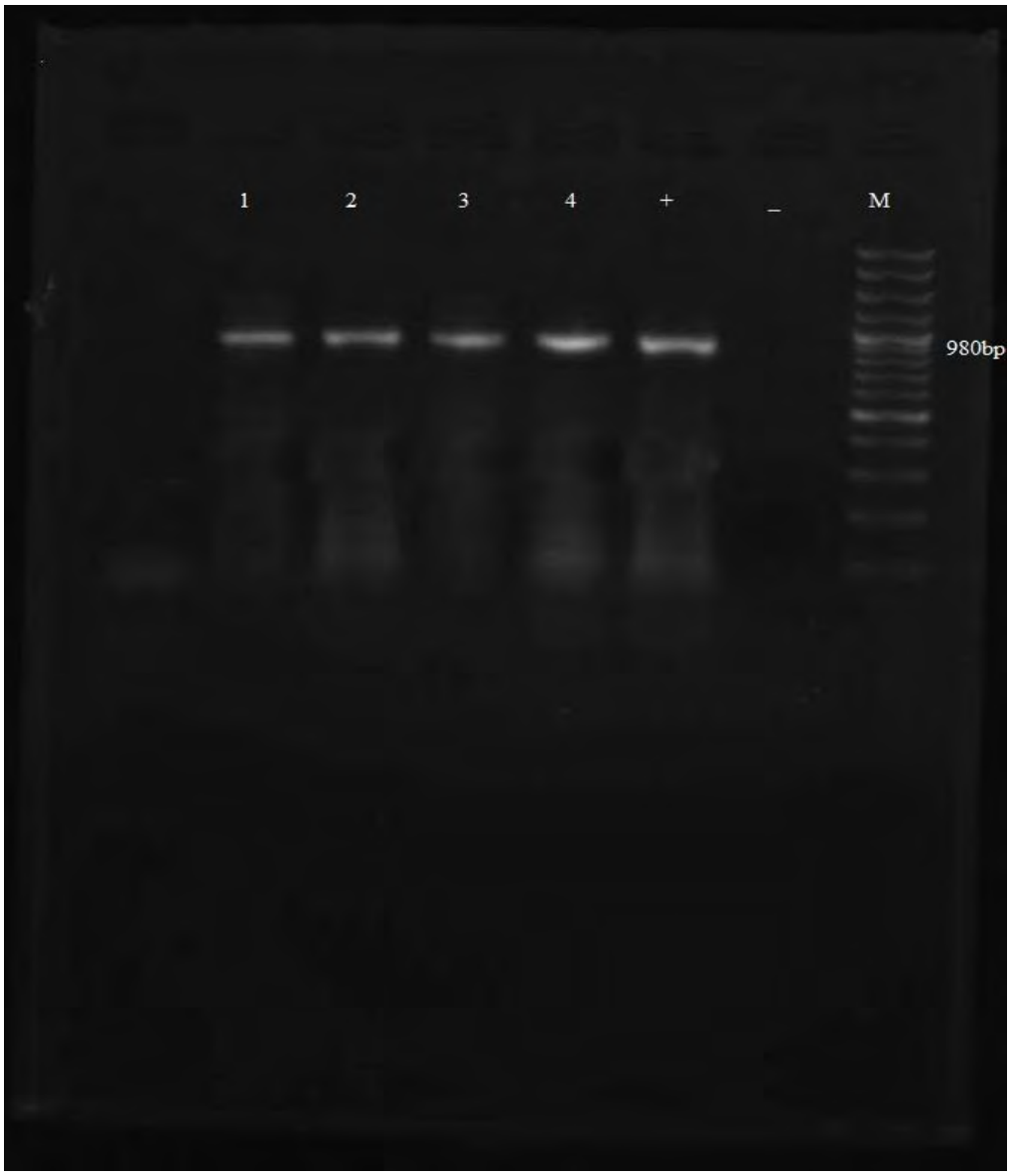


Figure 2.30: The PCR with the specific primers CO1 resulted in the amplification giving approximately 980bp amplification band size when visualized under UV trans illuminator. Lane M: 100bp DNA ladder. +ve standard MN919391, MN919392, MN919393, MN919394 *Anopheles gambiae*, Lane -ve: distill water and Lane 1-4: samples from CO1 amplified genes.

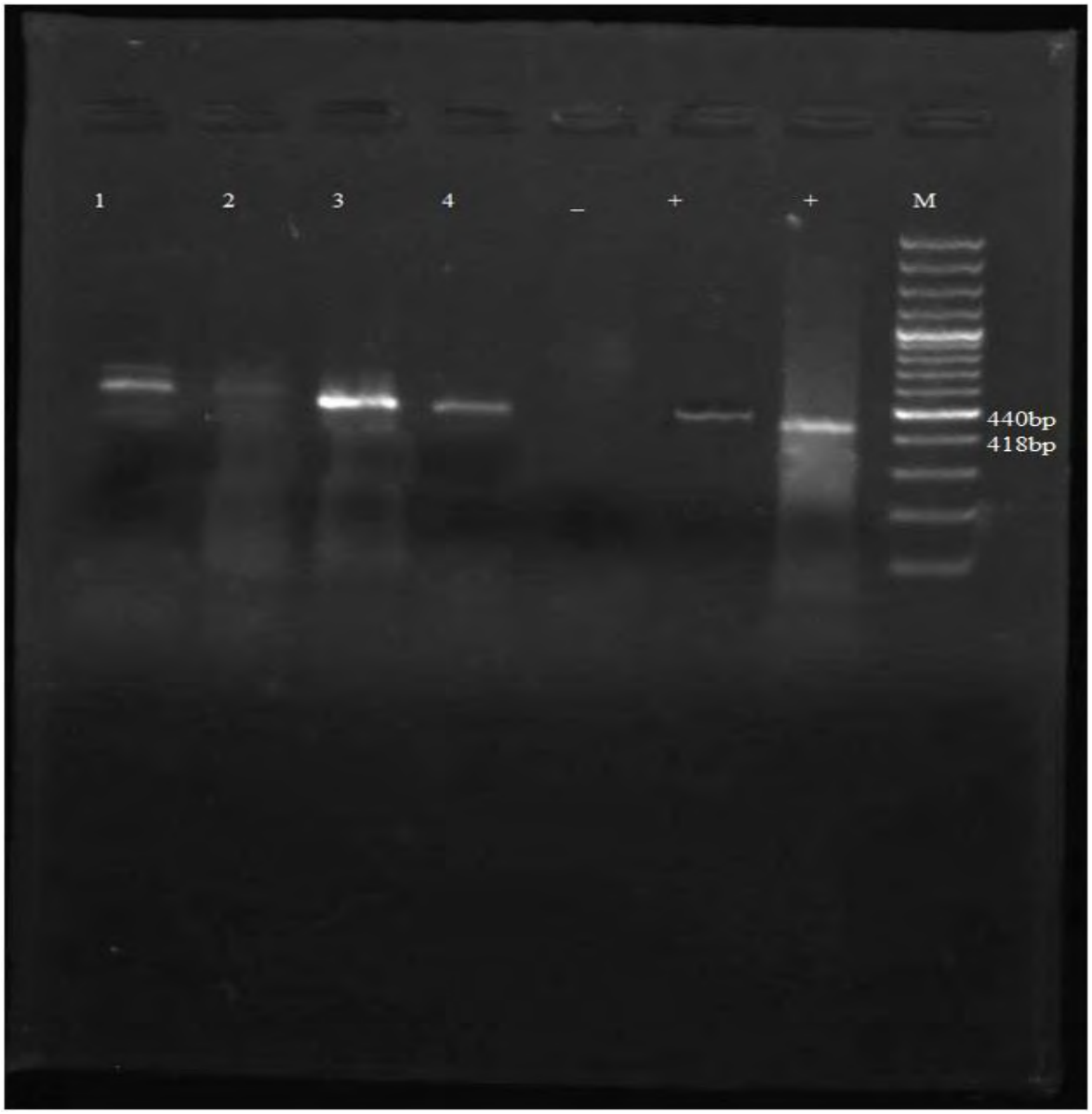


Figure 2.31: The PCR with the specific primers CO1 resulted in the amplification giving approximately 418 and 440bp amplification band size when visualized under UV trans illuminator for *Aedes aegypti* and *Aedes albopictus,* Lane M: 100bp DNA ladder. +ve standard MN909288, MN909289, MN909290, MN909291 for *Aedes albopictus* while in second lane + MN906911, MN906912, MN906913 for *Aedes aegypti*, Lane -ve: distill water and Lane 1-4: samples from CO1 amplified genes.

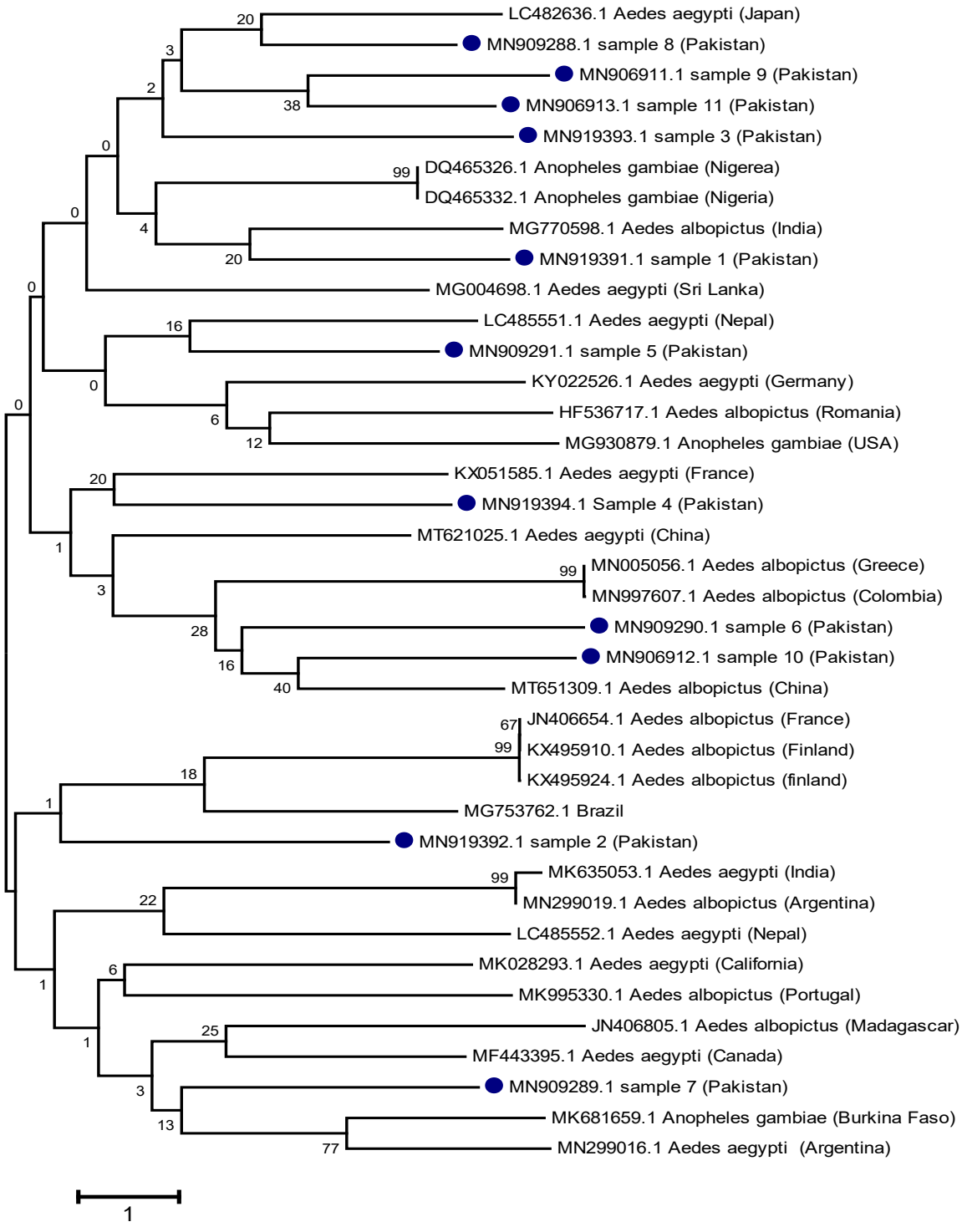


Figure 2.32: Evolutionary relationships of taxa.

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 113.21161130 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 38 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 228 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

DISCUSSION

Mosquitoes belong to the order Diptera, sub order Nematocera, and the family Culicidae and these insects serve as numerous disease vectors. Because of its geography and environment, Pakistan is a breeding ground for mosquitoes, which are the carriers of diseases including malaria, dengue fever, yellow fever, and others that can be fatal (Ashfaq *et al.,* 2014). With the exception of the permanently frozen zones in Antarctica, mosquitoes are widely distributed worldwide (Bibi, 2019). Additionally, they could to a lesser extent spread bacterial diseases. The Culicidae family includes mosquitoes with medical value. The three most prevalent genera of mosquito vectors are *Anopheles*, *Aedes*, and *Culex* (Tandina *et al*., 2014). Mosquitoes are significant due to their function as carriers of diseases and parasites, as well as their employment as laboratory animals for experiments on biochemistry, physiology, and behaviour (Clemens, 2011).

Species of mosquitoes vary in various regions of the world. Neotropical region hosts 31% of the known species, followed by Oriental region (30%), Afrotropical region (22%), and Australasian region with 22% (Foley *et al*., 2007). The least number of species are found in the Nearctic (5 % of the total). A wide variety of species from the Palearctic, Oriental, and Ethiopian areas can be found in Pakistan (Usman *et al*., 2017). There are 134 mosquito species in Pakistan, all of which are members of the Anophelinae and Culicinae subfamilies. One of the subtropical nations where vector-borne diseases are a problem is Pakistan. Like other Asian nations, Pakistan is experiencing considerable climatic change that favors epidemics of illnesses carried by mosquitoes (Manzoor *et al*., 2020)

Research on body forms has helped the biological sciences better comprehend systematics, taxonomy, and evolution (Stella and Palloma, 2017). A proven, affordable, and precise way of identifying mosquitoes is morphometric technique. It can be used to distinguish cryptic species, sibling species, and females of some species that are challenging to identify using other methods, epidemiologically significant vector mosquitoes, and more. Identification of mosquito species is essential for determining epidemiological patterns of disease transmission, which are related to the quantity,

infectivity, and vector competence and ability of the vector mosquitoes. Additionally, complexes of species that share physical similarities, such *Cx. pipiens* and *An. gambiae*, may play different epidemiological functions, making accurate identification of the species in these complexes essential for creating an effective vector mosquito control programmes (Wilke *et al*., 2016).

The usual method for describing species and identifying problematic species is through morphology. Technology has made it possible to identify sibling/cryptic species complexes by revealing the polymorphism of several traditional taxonomic and systematic traits. The phylogeny and systematics of *Anophelines* have been altered as a result of the cytogenetic and molecular descriptions of such challenging and conventional species (Chhilar, 2014). It has been demonstrated that the method of geometric morphometric for mosquito classification is trustworthy. They enable quick and precise identification of mosquito species, even closely related species and genera. Morphometric identification may be helpful when molecular identification is not feasible due to a lack of morphological competence (Martinet *et al*., 2021).

The earliest attempt at describing the Culicidae fauna in Pakistan was performed by Aslam Khan, who recorded 134 mosquito species from the nation, including 89 species from East Pakistan (now Bangladesh) and 91 species from West Pakistan. Pakistan's mosquito fauna included three *Culex* species and one *Anopheles* species between 1934 and 1971. He later found 16 native mosquito species in Pakistan and 17 were found during a survey in the Charsadda district (Aslamkhan, 1972).

There are 66 *Culex* and 34 *Anopheles* species in Pakistan, out of the 252 *Culex* and 43 *Anopheles* species that have been identified as existing on the Indian subcontinent (Khan *et al*., 2015). In Peshawar, Ali and Rasheed identified 9 species of mosquitoes from the genera *Anopheles* and *Culex* (Ali and Rasheed, 2009). *Anopheles*, *Aedes*, *Culex*, *Armigeres*, and *Culiseta* are among the five genera represented by the fifteen species that have been documented from Swat (Usman *et al*., 2017). Fifteen species have been identified in the districts of Malakand and Swat Ranizai (Ali *et al*., 2013). Sixteen more species were reported from Swat in addition to these (Ilahi and Suleman, 2013).

Mosquitoes species from Murree Hills have been recorded as 9 Culicine and 4 *Anopheles* (Qasim *et al*., 2014). There are 29 different types of mosquitoes in the Changa Manga Lahore area of Lahore (Aslamkhan and Salman, 1969). Three *Culex* species and one *Anopheles* species were added to Pakistan's mosquito fauna between 1934 and 1971 (Khan, 1971).

Anopheles has nine species, followed by *Culex* five, *Armigeres* five, and *Mansonia* one species (Ali *et al*., 2015). *Anopheles*, *Culex*, *Aedes*, *Culiseta*, and *Armigeres* were among the fifteen mosquito species that were discovered during the first investigation of mosquito distribution in southern KP (Kohat Hangu). While the other three genera each had one species, *Culex* and *Anopheles* each had six species (Ali *et al*., 2013). The mosquito fauna surveyed in Swat, Pakistan, had eight *Anopheles* species, seven *Culex*, four *Aedes*, one each of *Culiseta* and *Armigeres* species, and others (Ilahi & Suleman 2013). Five mosquito species from three genera (2 *Culex*, 2 *Anopheles*, and 1 *Aedes*) were found in Malir Karachi (Kamimura *et al*., 1986).

A survey of seven villages in the Lahore region using four collection techniques (indoor-resting, outdoor-resting, bovid biting, and light traps), recording 43 mosquitoes species, including 12 *Aedes* species, nine *Anopheles* species, 16 *Culex* species, and one each of *Coquilletidia*, *Culiseta*, *Minomya*, *Mansonia*, *Orthopodomya*, and *Uranotaenia* species (Reisen 1978). Among the fifteen species recorded from Swat belong to five genera— *Anopheles*, *Aedes*, *Culex*, *Armigeres*, and *Culiseta*. In the Narowal district, there are 11 species of mosquitoes that fall within the *Anopheles*, *Culex*, *Armigeres*, and *Aedes* genera (Rasool *et al*., 2015).

In my research work, many mosquito species were identified using a stereoscopic microscope. A number of criteria were used to correctly identify mosquito species. Complete body, proboscis, palpi, wings, forelegs, middle leg, and hind leg length are the following parameters were used in morphometric analysis: Morphometric measurements of the body parts of twenty-seven mosquitoes collected from different districts of Punjab are given in the tables (2.5-2.31).

Twenty-seven distinct species from eleven Punjab districts (Northern (Rawalpindi, Gujar Khan, Gujrat, Chakwal, Gujranwala and Lahore) and Southern (Dera Ghazi khan, Rajanpur, Rahim yar Khan, Multan, and Bahawalpur) were found to be part of the current study. These species belonged to the five genera (*Anopheles (An. culicifacies, An. stephensi, An. sinensis, An. subpictus, An. maculatus, An. splendidus koidzum,* and *An. aconitus*, *Culex (Cx. quinquefasciatus, Cx. raptor, Cx. tritaeniorhynchus, Cx. bitaeniorhynchus, Cx. vagans Cx. vishnui, Cx. fatigans, Cx.pipiens, Cx. gelidus,* and *Cx. fuscocephala,*), *Aedes (Ae. aegypti and Ae. albopictus), and Armigeres* (*Ar. kuchingiensis, Ar. subalbatus Ar. subalbatus, and Ar. flavus),* and *Mansonia (Ma. uniformis).*

Eleven species were identified in earlier studies done in Rawalpindi and Islamabad. 13 species of mosquitoes from five genera i.e. *Anopheles*, *Culex*, *Armigeres*, *Aedes,* and *Culiseta* were identified in the Murree Hills. These included *An. theobaldi*, *An. maculatus*, *An. stephansi*, *An. fluviatilis*, *Cx. nilgiricus*, *Cx. vagans*, *Cx. fatigans* (Qasim *et al*., 2014). *Anopheles, Culex, Aedes*, *Mansonia*, and *Armigeres* are only a few of the five genera found in the Punjab region, where the current study is being conducted. According to the findings, several species are exactly like those in Murree Hills, suggesting that the ecological conditions are similar.

Aedes albopictus and *Aedes aegypti* which appears to be the principal vector of recent dengue virus epidemics, was recorded mostly in District Lahore, Gujar khan and Multan, to determine the vector of dengue diseases (Jabeen *et al*., 2021). A study was carried out in district Lahore to evaluate the biodiversity of mosquito species. *An. barriensis, Cx. pipiens, Cx.vishnui, Cx. pseudovishnui, Cx. fatigans, Cx. barraudi, Cx. .fuscocephala, Cx. epidesmis, Ar. subalbatus, Ae. aegypti,* and *Ae. micropterus* are among the eleven species identified from the four genera *Anopheles, Culex, Armigeres* and *Aedes* (Baboo *et al*., 2021). The most common species in our study was *Cx. quinquefasciatus*, which is consistent with findings from past research in Swat ((Ilahi and Suleman 2013), but earlier research in Punjab suggested that *Cx. tritaeniorhynchus* predominated (Reisen, 1978).

The prevalent *Aedes* species in Punjab was *Ae. albopictus*, however it was not seen at higher elevations (1100 m) in Swat. *Ae. lineatopennis* was interestingly discovered to be the most prevalent species of *Aedes* by Reisen *et al*. (1982), indicating a change in species composition through time. *Ae. albopictus* was only found in Pakistan at locations with an elevation of less than 700 m, although being found at altitudes as high as 1200m on La Reunion.

The results of this study show that some species found in Lahore and Rawalpindi are equivalent to those found in Dera Ghazi Khan, suggesting that the two locations have similar geographic and environmental characteristics. The investigation of the species in these locations is being done in the current study for the first time. In the current study, the relative abundance and distribution status were also noted. In terms of relative abundance, there were 18 dominant, 4 subdominant, and 5 satellite species. In terms of distribution, there were 3 constant, 1 frequent, 4 rare, and 5 sporadic species. The species with the highest relative abundance, *Cx. pipiens* (275%), followed by *Cx. quinquefasciatus* (22.25%), *Ar. subalbatus* (16.34%), and *Ar. obturbans* (11.08%). The remaining species are considerably rarer, with *Cx. bitaeniorhynchus* having the lowest relative abundance (1.29%) among them.

Our findings are consistent with a recent study on *Cx. quinquefasciatus* from Malaysia (Low *et al*., 2013), that claimed that this species had a low COI diversity. Sharma *et al*. (2010), however, revealed a substantial diversity in 16S rRNA for the same species from India. This discrepancy can provide credence to the continuing discussion of animal mitochondrial and nuclear discordance (Toews and Brelsford, 2012). Prior research on *Ae. aegypti* and *Ae. albopictus* revealed significant genetic diversity (Kamgang *et al*., 2011), offering crucial hints on population linkages and origins and insights into their function in the transmission of dengue (Mousson *et al*., 2005).

Both species displayed significant barcode variation inside Pakistan, but when research took into account each species' whole distribution, *Ae. albopictus* was less varied than *Ae. aegypti*, a finding consistent with Mousson *et al.* (2005). According to the findings of our survey, *Ae. albopictus* was more widely dispersed and prevalent in Punjab than *Ae. aegypti*. *Ae. aegypti* larvae predominated (65%) in samples from water-pots

inside of homes in Lahore during 2011, according to Akhtar *et al*. (2012) Although the current dominance of *Ae. albopictus* in Pakistan suggests a global trend towards *Ae. albopictus* spread and a reduction of *Ae. aegypti* (Bagny *et al*., 2009), the influence of sampling method on the variation in results cannot be completely disregarded (Raharimalala *et al*., 2012).

Despite being the primary human malaria vectors, Anopheline mosquitoes (Culicidae, Anophelinae) are poorly understood phylogenetically (Krzywinski *et al*., 2001b). Six subgenera of the worldwide 437 species *of Anopheles* have been identified: the Old World Cellia, the Neotropical Kerteszia, the Nyssorhynchus, the Lophopodornyia, and the Stethornyia (Harbach , 2004). *An. stephensi, An. culicifacies*, *An. splendidus, An. pulcherrimus, An. annularis, An. maculatus, An. nigerrimus, An. subpictus, An. superpictus, An. fluviatilis, and An. tarkhudi* are among the 11 anophelines species found in Khyber Pakhtunkhwa province in Pakistan (Suleman, 1993). There are 24 anophelines species overall (Mahmood *et al*., 1984). Most of the species belong to subgenus *Cellia*. The majority of Pakistani mosquitoes belong to the subgenus Cellia, which displayed a monophyletic lineage (bootstrap value: 97). The findings are consistent with the most recent evolutionary tree for the relationships between Anophelines as described by Harbach in 2004 and in another research (Sallum *et al*., 2000). The present research considered molecular systematics of one *Anopheles* species (*An. gambiae)* and two *Aedes* species (*Ae. agypti* and *Ae. albopictus*).

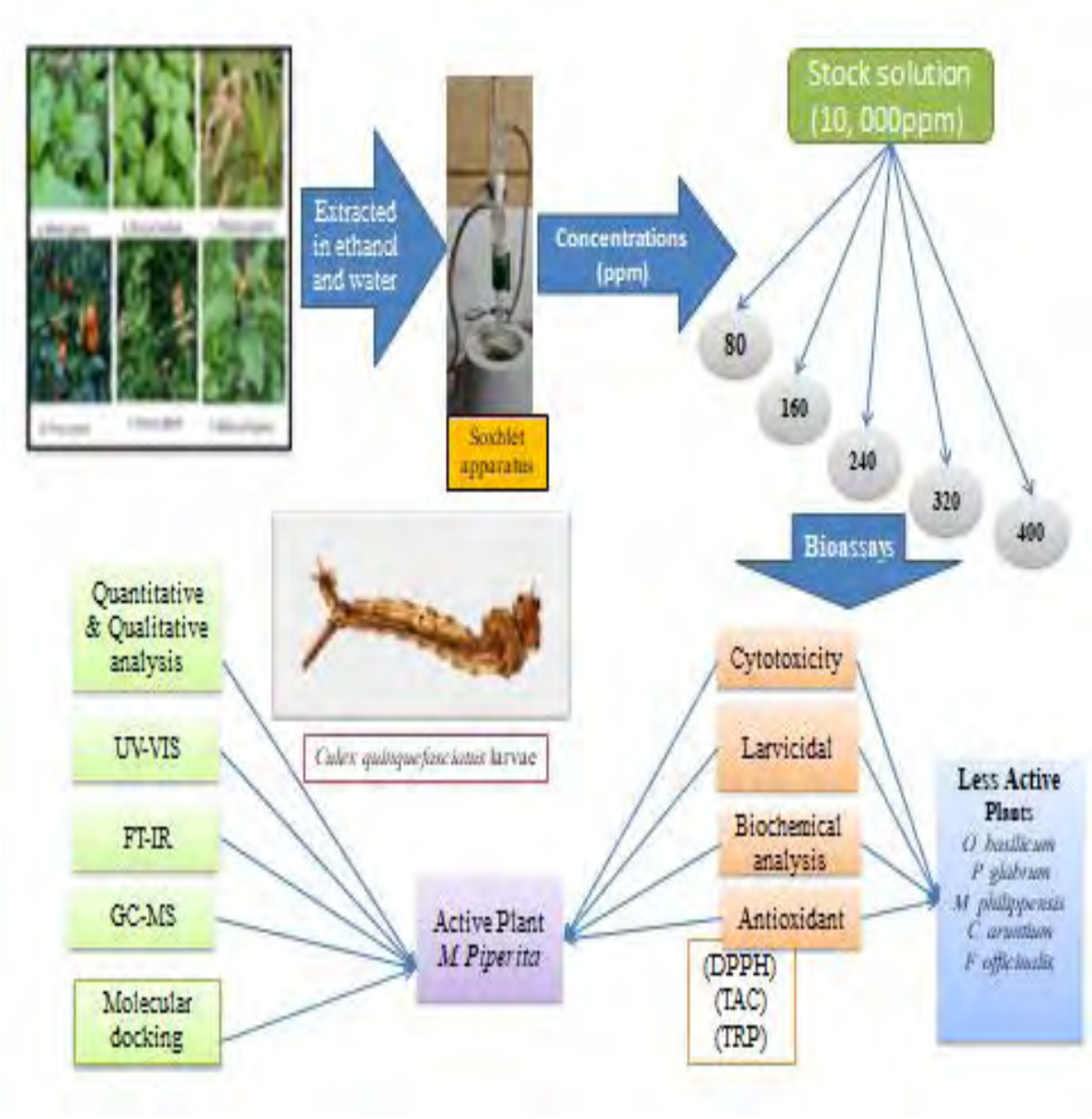
The mosquito species from Punjab were described in the recent study. Five genera, namely *Anopheles*, *Culex*, *Armigeres*, *Aedes*, and *Mansonia*, make up the mosquito fauna. The results of this study allow us to draw the conclusion that the distribution and diversity of Punjab's mosquitoes fauna are being influenced by a global climatic shift along with widespread development. As a result, there is always a chance that diseases carried by mosquitoes would manifest. In Punjab, it was also discovered what species of mosquito were prevalent and where they were distributed. In Pakistan, there is limited knowledge of the molecular diversity of mosquitoes. There will probably be many new mosquito species found in the nation. More and more entomological and field level surveys are needed for that aim. Strong biometric research is needed to

establish reliable morphological identification up to the species level. The current work showed how mitochondrial CO1 genes can be used to infer evolutionary relationship among *Anopheles* and *Aedes* species. Morphometric and molecular study is a useful and reliable process for identifying *Anopheles* and *Aedes* species.

CHAPTER 3

SCREENING OF PLANTS FOR THEIR LARVICIDAL POTENTIAL, ANTIOXIDANT ACTIVITY, CYTOTOXICITY, AND PHYTOCHEMICAL ANALYSIS USING UV-VIS, FT-IR, GC-MS, AND MOLECULAR DOCKING

GRAPHCAL ABSTRACT



ABSTRACT

Mosquitoes are native of tropical and subtropical regions and are a vector of many diseases concerning public health e.g. malaria, filariasis, chikungunya, and dengue fever, etc. Although numerous pesticides are available but due to resistance and environmental hazardance more eco-friendly, chemicals are needed for insects control. The objective of the current study is the characterization, molecular docking and evaluation of plants extracts to be used as larvicides against *Culex quinquefasciatus* larvae. Two solvents (water and ethanol) were used to prepare the extracts from shaded dried leaves of six medicinal plants (*Ocimum basilicum, Polygonum glabrum, Mentha piperita, Mallotus philippensis, Citrus aruntium, and Fumaria officinalis)*. The cytotoxic test was assessed through brine shrimp lethality bioactivity. The brine shrimp was used to evaluate the toxicity of plant extracts. The ethanolic and aqueous leaves extract of *M. piperita* was prepared using the Soxhlet apparatus and made five concentrations multiple of 80ppm. Each concentration was applied for its larvicidal efficacy setting an experiment (in triplicate) in plastic containers of 1000ml with extracts, 30 larvae of all four instar stages separately, and fed with dog biscuits along with controls. Observations were taken after each 12hrs till 72. The LC_{50} and LC_{90} values were calculated by Probit analysis. DPPH radical scavenging, ferric reducing antioxidant, and total antioxidant capacity methods were used for the estimation of antioxidant activity of leaves extracts. The extract of *M. piperita* was characterized by preliminary qualitative and quantitative phytochemicals, UV-VIS spectroscopy, FT-IR, and GC-MS analysis by standard protocols. Out of all the plants tested, the ethanolic extract of *M. piperita* exhibited the highest larvicidal efficacy against 4th instar mosquito larvae (LC_{50}=208.976ppm). Biochemical analysis of dead mosquito larvae treated with ethanolic *M. piperita* extract showed the presence of significantly fewer values of proteins, carbohydrates, cholesterol, triglycerides and high-density lipid as compared with control. *M. piperita* and *O. basilicum leaves extract* showed the maximum total antioxidant (125.4 ± 3.5, 115.6±2.8), ferric reducing (378.1± 2.5, 350.2±2.2), and DPPH radical scavenging activity (85.43± 0.22, 78.33± 0.42) . All six experimented plants indicated cytotoxicity in a protective array >550ppm

but *F. officinalis* (LC_{50}=569.31ppm). The qualitative phytochemical analysis demonstrated the presence of organic compounds (e.g. phenols, saponins, tannins, volatile oil, alkaloids, flavonoids, carbohydrates, and flavonoids) in maximum number present in *M. piperita* as compared to the others five experimented plants. The quantitative phytochemical analysis revealed the presence of the highest total phenolic (6.44±0.0018 µgGAE/mgDW) and flavonoid contents (8.47±0.0017 µgQA/mg DW) in *M. piperita*. Two absorption peaks were recorded by UV-VIS spectroscopy at 209.509 and 282.814nm having absorption of 2.338 and 0.796 respectively. FT-IR confirmed the presence of alcohols, alkanes, aldehyde, aromatic rings, ether linkage, ester, and halo-compounds. GC-MS analysis revealed the presence of ten compounds with a high percentage of t-Butyl hydrogen phthalate (13.99%), 2H-Pyran-2,4(3H)-dione,3-ethyl-5,5-dimethyl -6-phenyl (13.15%) and 1,2-Benzenedicarboxylic acid, mono-(2-ethylhexyl) ester (12.45%). Among all the compounds, 1,2-Benzenedicarboxylic acid and 3-ethyl-5,5-dimethyl -6-phenyl bound well to the NS3 protease domain with PDB ID: 2FOM. The results revealed that *M. piperita* exhibited maximum larvicidal potential out of seven plants experimented with and can be used for the isolation and characterization of chemicals to be used for formulating insecticides, larvicides, or insect repellents.

INTRODUCTION

Insects are a very important part of the biodiversity of terrestrial and aquatic ecosystems (Marquardt, 2005). Among arthropods, class insecta is of great importance to public health (Khasnis and Nettleman, 2005).

Mosquitoes serve as a vector of many diseases transmit in humans and animals worldwide such as malaria, zika virus, Japanese encephalitis, yellow fever, dengue fever, chikungunya, and filariasis causes great decease in humid and sub-humid areas mainly by four genera (*Culex*, *Anopheles*, *Mansonia*, and *Aedes*) of mosquitoes (Arivoli *et al*., 2011). Thus, both vectors and vector-borne diseases have become thought-provoking complications that have social and economic impacts (Raveen *et al*., 2014).

WHO has stated the mosquitoes as "public enemy number one" (WHO, 1996). Out of 3601 species and subspecies worldwide, 104 have been reported from Pakistan and Bangladesh (Tang *et al*., 2007). Mosquitoes has total 42 genera and 140 subgenera but in Pakistan only 65 species belonging to 06 genera (*Anopheles* (44), *Culex* (9), *Aedes* (8), *Culiseta* (2), *Armigeres* (1), and *Mansonia* (1) of mosquitoes are reported (Harbach, 2007). In more than 100 countries mosquitoes are responsible for infecting more than 700 million people every year, almost 20% of the world's population are in danger of getting infections of mosquito borne-diseases (Becker *et al*., 2010). Several species of mosquitoes frequently contribute to deficiency and social weakness in tropical countries related to genera *Anopheles*, *Aedes,* and *Culex* are the vectors for various diseases causing agents. There are two well-known subfamilies of mosquitoes, the *Anophelinae* and *Culicidae* (Asiry *et al*., 2017).

Culex mosquito is probably the most abundant house mosquito in towns and cities of tropical countries. *Culex* mosquitoes develop in standing water, such as polluted ponds, marshes, tanks, street gutters, tin cans, barrels, ornamental ponds, puddles, creeks, ditches, etc. (Matthys *et al*., 2006). In Pakistan, during the summer months, a high level of population buildup of *Cx. quinquefasciatus* has been recorded in various cities (Chiang *et al.,* 2012). In Pakistan, *Cx. quinquefasciatus* is the most prevalent mosquito species (Ashfaq *et al.*, 2014).

Three different species of microscopic, thread-like worms are responsible for the parasitic disease lymphatic filariasis. Depending on the region, a variety of mosquitoes can spread the parasite. *Anopheles* is the most prevalent vector in Africa and *Cx. quinquefasciatus* in Americas. The Middle East and countries of the Eastern Mediterranean are among the regions of the world where this illness is prevalent (Gupta and Dikshit, 2010). It is also a potential vector of the viruses *Dirofilaria immitis,* West Nile virus (WNV), rift valley fever virus, avian pox, and protozoa like Plasmodium relictum, which causes bird malaria (Manguin and Boet, 2011). Additionally, it can transmit the Japanese Encephalitis Virus (JEV), St. Louis Encephalitis Virus (SLEV), Reticuloendotheliosis Virus, Murray Valley Encephalitis, and Reovirus Type 3 (Sakthivadivel *et al*., 2016).

Culex quinquefasciatus may also cause protozoan, viral, parasitic, and helminthic diseases. In 73 countries of Pacific Island, Africa, Southeast Asia, and India, 120 million people are affected by lymphatic filariasis each year, while one to two million deaths are universally reported from malaria (Bagavan *et al*., 2011). This causes both high levels of morbidity and mortality along with a socioeconomic disturbance in developing countries (Kamaraj *et al.,* 2011).

Culex quinquefasciatus acts as an important "urban bridge vector" which bridges different reservoir/amplifier hosts to humans because of its encounter with different vertebrates. It also creates an ecological bridge between urban and rural areas owing to its presence and adaptability in diverse ecological niches. They emerged as a vector because of the adaptive fitness, ecological plasticity, invasive behavior, host specificity, and high reproductive potential along with expanded immune gene variety at the genetic level. This mosquito possesses the necessary potential to initiate and facilitate disease transmission by establishing an effective vector-host transmission cycle for diverse pathogens in different environments (Bhattacharya *et al.,* 2006).

The mismanagement of pesticides in farming and public health programs elevated numerous health and environment-connected complications like conflict to insecticides, a manifestation of pests, harmful to humans, non-target species, and contamination of the

environment. Because of overcrowded cities and unsanitary circumstances, Pakistan is at high threat of great epidemics (Zhu and Zeng, 2006).

Biological control at the larval stage of mosquitoes is one of the appropriate methods which deal with an economical, easy-to-use, and ecological friendly process with mosquito control. Effective frequent use of controlling agents has interrupted natural biological control organizations and led to epidemics of insect species presenting pesticide resistance. It has also activated unwanted effects, including harmful to non-target organisms, cultivated environment, and human health anxieties (Lee *et al*., 2001). Vector control is the main factor in a disease control program. The control of mosquitoes at the larval stage is essentially and capable of integrated mosquito management (Tennyson *et al*., 2011).

Moreover, the use of synthetic insecticides in public health and agriculture programs is not biodegradable and has caused ecological imbalances (Rawani *et al.,* 2012). Several insecticides are prepared for the control of mosquitoes but due to their widespread use have developed resistance, so search for alternative control strategy is necessary (Markouk *et al.,* 2000; Fazal *et al.,* 2014). Larviciding is an effective method of decreasing mosquito densities in their breeding places earlier they develop into adults. Larviciding mostly depends on the usage of synthetic chemical insecticide organophosphates (*e.g.* temephos, fenthion, chlorpyrifos,) insect growing regulators (*e.g.* diflubenzuron, methoprene). Various of which are selective and have slight or no harmful effect on non-target organisms and the environment (Pontes *et al*., 2007**;** Kino *et al*., 2008).

Plants are the source of active compounds for the production of innovative remedies (Kumar and Singh, 2011). Many pathologically important plants can produce from which the up-to-date drugs are produced. The occurrence of bioactive compounds makes plants, a good applicant for drug manufacturing (Mahmood *et al*., 2012). In developing countries, widespread medicines are used because of their low cost. The traditional therapists due to that some of their remedies are more active as compared to the recent medicines (Chadwick and Marsh, 2008).

For centuries, medicinal plants have been used as a medicine for human diseases as they are environmentally friendly and contain secondary metabolites and bioactive components of beneficial value (Aouinty *et al*., 2006). One of the best ways to control a disease is to destroy its vectors eggs or larvae as they are motionless at that stage of their life cycle (Tennyson *et al.,* 2012). Natural pesticides and chemicals are less toxic and are biodegradable e.g. *Euphorbiaceae* family members are of vast importance due to their medicinal values (Ozcan and Chalchat, 2008). These family members contain alkaloids, flavonoids, and other secondary metabolites. It is reported that *Euphorbia hirta* and *Croton bonplandianum* have the potential to be used as an ideal environmental approach for the control of vectors e.g., *Ae. stephensi* (Perumal *et al.,* 2013).

Plant chemicals are observed as secondary metabolites because they are grown in all portions of the plant body, bark, leaves, stems, root, flower, fruits, seeds, etc., i.e. every character of the plant body may contain active constituents (Ugochukwu *et al*., 2013). This chemical effort with nutrients and fibers form a cohesive voice of the defense system against several ailments and stress conditions. These chemical constituents are known as secondary metabolites. The best signs of these bioactive groups of plants are phenolic, alkaloids, tannins, terpenoids, saponins, steroid, and flavonoids compounds (Santhi, 2011).

The Relationship between the phytocomponents and the bioactivity of the plant is necessary to identify for the production of compounds with precise activities to treat many health disorders and chronic infection as well (Yadav *et al*., 2014). The significance of the therapeutic plant in medicine development is recognized to us and humans have used them for diverse diseases from the foundation of human history (Elango *et al.,* 2009). A traditional folk usage from wild plants has always directed researchers to examine innovative medicines to improve healthy life for humans and animals (Ghosh *et al*., 2012).

Phytochemicals derived from plant sources can act as larvicides, insect growth regulators, repellents, and ovipositor attractants. Many plants derived chemicals play an important role in interruption vectors like mosquito transmission of the individual and as well as at the community level (Govindarajan *et al.,* 2008; Kumar and Singh, 2011).

Citrus aurantium belongs to the family Rutaceae known as bitter orange or marmalade orange. In agricultural yields, *Citrus* fruits are of the utmost vital because of their nutritional value and distinctive flavor. Several members of the *Citrus* genus are widely recognized for their therapeutic, biological, and pharmacological properties comprising antimicrobial, antioxidant, anticancer, anti-inflammatory, and hypoglycemic activities (Ladaniya, 2008; Hamdan *et al*., 2013). . *C. aurantium* peel is frequently used in marmalade and dry peel is used in diverse kinds of food and drinks. Orange flower water is used to flavor sweets, the essential oil is used in perfumes, and flowers are used in tea (Kiple and Ornelas, 2000). The studies related to biological effects of *C. aurantium* showed potential mosquitoes repellent, larvicidal and insecticidal activities of this plant (Cetin *et al*., 2006; Sumroiphon *et al*., 2006; Yoon *et al*., 2009).

Basil (*Ocimum basilicum*) belongs to the family Lamiaceae may be either annual or perennial. This plant is normally used as an insect repellent and has larvicidal activity against houseflies, blue bottle flies, and mosquitoes (Chatterjee and Pakrashi, 1997**;** El Ghazali *et al*., 1998). The plant leaves are a rich source of essential oil found to be useful for the treatment of common colds, muscular spasms, constipation, cough, headaches, warts, kidney problems, vomiting, stress, and as first aid treatment for snake bites and wasp stings (Vlase *et al*., 2014). In addition to the medicinal properties of the plant, it is best known for its fragrance and is then frequently used in different varieties of perfumes and cosmetics. It is also used as a food preservative and flavoring agent in ice creams, condiments, non-alcoholic beverages, and other foods. Due to the rich source of beneficial phytochemicals, it is often referred to as the "king of herbs" (Makri and Kintzios, 2008). The chemical description of *O. basilicum* extracts exhibited the existence of tannins, flavonoids, and saponins (Lorenzi and Matos, 2002). It has antioxidant, antibacterial, and antimicrobial activity (Kaya *et al*., 2008).

Mallotus philippensis locally known as Kamla belongs to the family Euphorbiaceae. It is a large genus of small trees and common perennial shrubs spread generally in the tropical and subtropical areas of the World (Widén and Puri, 1980). The powder of this plant has many medicinal properties to relieve cough, constipation, wounds, ulcers, treatment of bronchitis, abdominal diseases, spleen enlargement, and

antiparasitic (Gangwar *et al*., 2014), etc., also applied externally for skin disorders such as scabies and cutaneous troubles. It has an antifilarial, antibacterial, antioxidant activity, anti-inflammatory, and immune-regulatory activity (Kumar *et al*., 2006). Medicinal plants are one of the best sources of obtaining antimicrobial drugs (Sofowora, 1996).

Polygonum glabrum (polygonaceae) is commonly known as dense flower knotweed is a semi-aquatic perennial plant (Shankar and Mishra, 2012). The surface of the leaves is without hairs and covers many stomata complexes. This wetland plant has bunches of beautiful pink flowers which generally fascinate butterflies and insects (Khan *et al*., 2014). Conventionally, the rootstock is used for the usage of jaundice and piles. A decoction of the leaves and seeds is used as cardiotonic, astringent, and anthelmintic. Peels from the stem are used for treating rheumatism. Pharmacologically, this plant has been effectively reported for several studies (Palani *et al*., 2014).

Mentha piperita belongs to the family Labiatae is an herbaceous plant of small size with greenish feathery leaves. The genus of *Mentha* comprises more than 20 species, mostly perennial herbs growing wildly in damp places throughout the temperate regions of Europe and Australia. Worldwide *M. piperita* leaves are used in various forms of preparations such as tea preparation, alcohol extracts, tincture, or as oil (Nair, 2001). It contains volatile oils, resins, and flavonoids (İşcan *et al*., 2002). In vitro, several studies have shown that *M. piperita* has antibacterial effects, a strong antioxidant, antinociceptive, anti-inflammatory, antitumor action, and antiallergenic potential (Edris, 2007**;** Bassolé *et al*., 2010**).**

Fumaria officinal commonly called Fumitory or Earth smoke belongs to Family Papaveraceae. It has antimicrobial activity (Ahmad *et al*., 2015) and antioxidant activity (Sengul *et al*., 2009). These are annual weeds, growing wildly in plains and lower hills of India, Pakistan, Afghanistan, Turkey, Iran, and Central Asia (Singh *et al*., 2013). In herbal therapy, fumitory, a dried or live blooming plant, is used to treat digestive and biliary tract diseases (Shakya *et al*., 2016). To alleviate dyspepsia, treat constipation, and avoid vomiting, the plant can be administered internally combined with honey. It is used as a blood purifier for skin diseases and applied externally in leukoderma and as a fomentation for swollen joints. The dried plant is also used as an anthelmintic, diuretic,

and diaphoretic and, in combination with black pepper, for jaundice (Khan and Qureshi, 2013).

Crude extracts of many plants are infectious devotion since of their biomonitoring properties (Holley *et al.,* 2005). Traditional treatments usually involve the use of unrefined medications such tinctures, teas, powders, and other herbal concoctions. A particular plant variety can be utilized to treat a particular disease (VanVuuren and Naidoo, 2010). Plant extracts deliver an important range of new medicinal agents (Bozine *et al.,* 2006). Modern analytical spectroscopic techniques like ultraviolet and visible spectroscopy, fourier transform infrared spectroscopy (FT-IR) and gas chromatography-mass spectrometry needs a vital role in the identification and characterization of bioactive components having antimicrobial, antioxidant, antifungal, antibacterial, insecticidal, antitumor, and antiproliferative activities (Tranchida, 2004).

Molecular docking is the computer-aided drug strategy method in which two molecules fit together in 3D space. It is the main tool in structural biology and for the discovery of antilarval compounds. To understand the protein-ligand interactions, molecular docking methods are usually used in the modern drug design process. For achieving biological functions, the three-dimensional structure of the protein-ligand complex could be helped as a significant basis for understanding the way proteins interact with one another. On the molecular level, molecular docking and virtual screening-based studies have become an important part of many modern structure-based drugs innovation efforts. Therefore, knowledge of the protein and ligand interactions with the specific drugs may provide a noteworthy understanding of the binding interactions and relativity of the drug (Taha *et al*., 2015).

Hence the present study was designed to evaluate the larvicidal efficacy of ethanolic and aqueous leaves extract of plants like *C. aurantium, O. basilicum, M. philippensis, P. glabrum, M. piperita* and *F. officinal* against *Cx. quinquefasciatus* (*C. fatigans*) larvae to identify the most effective plant, their characterization, and molecular docking. Then this plant will be subjected to isolation and identification of the bioactive compounds present in them. Therefore, natural plant products can be replaced by synthetic insecticides to prevent health hazards.

MATERIALS AND METHODS

3.1: Collection of plants:

Leaves of six plants *M. piperita* L., *O. basilicum* L., *P. glabrum* L., *M. philippensis* L., *C. aruntium* L., *F. officinalis* L. were collected locally within the vicinity of Quaid-i-Azam University, Islamabad Pakistan and identified by using the key (Nasir and Ali, 1977). These plants were selected based on their pharmacological properties and also on their traditional uses.

3.2: Plants selected for Bioassays

Plants selected for antilarval activity are listed in figure 3.1 and table 3.1

Figure 3.1: Plants selected for antilarval bioassays.

Table 3.1: General description of the experimented plants.

Plant species / Botanical name	**Local name**	**Family**	**Plant parts used**	**Biological activity**	**References**
Mentha piperita	Peppermint	Lamiaceae	Fresh leaves	Antibacterial, Antioxidant, Antimicrobial, Antiproliferative.	Singh *et al*., 2015
Ocimium basilicum	Basil	Lamiaceae	Fresh leaves	Antimicrobial Activity, Antifungal activity, Antibacterial activity, insect repelling activity.	Hussain *et al*., 2008
Polygonum glabrum	knotweed, knotgrass	Polygonaceae.	Fresh leaves	Antioxidant, Antidepressant, Anti-inflammatory activity.	Said *et al*., 2015
Mallotus philippensis	kamala tree or red kamala or kumkum tree	Euphorbiaceae	Fresh leaves	Antioxidant, Antimicrobial, Antifungal, Antibacterial, antiplasmodial.	Moorthy *et al*., 2007
Citrus aruntium	Bitter orange, sour orange.	Rutaceae	Fresh leaves	Antibacterial, Antimicrobial, Antimutagenic, Antioxidant, free radical scavenging activity.	Kim *et al*., 1997
Fumaria officinalis	common fumitory, drug fumitory or earth smoke.	Papaveraceae	Fresh leaves	Antioxidant, Antimicrobial, Antibacterial.	Sengul *et al*., 2009

3.3: Preparation of plants extracts

The leaves of these plants were splashed with tap water to get rid of dust particles and impurities. Leaves remained desiccated in the shade for 2 weeks at room temperature ranging from 27 to 37°C and were crushed mechanically with the support of an electrical blender (Daigger Scientific USA). 40 and 60 mesh reserved material was carefully selected. Thirty-gram powdered material of each plant was eluted in 300ml of two different solvents i.e. ethanol and water for 6 to 8hrs (two cycles per hour) in a Soxhlet extraction apparatus (Shanghai Heqi®, China). The filtrates were sieved through Whatman filter paper No.1. The dried-out filtrates were collected by evaporating the solvent using a rotary vacuum evaporator (R-300, Rotavapor®, Germany), stored at 4ºC in airtight bottles to prepare stock solution larvicidal bioassay. For making stock solution, 1g of crude extract was dissolved in 100ml of distilled water. From the stock solution, different concentrations (80, 160, 240, 320, and 400ppm) were prepared by applying a formula as described by Jayal *et al.* (2010).

$$C_1V_1=C_2V_2$$

Where,

C_1= Stock solution conc. (ppm),

V_1= Required volume

C_2 =Required Conc.

V_2= given volume

3.4: Larvae collection and bioassay

Larvae of *Cx. quinquefasciatus* were collected from natural ponds in Quaid-i-Azam University, Islamabad, Pakistan in the pre-monsoon season with the help of the dipping method (Robert *et al.,* 2002). Larvae were identified according to the key of Corbel *et al*. (2007) and saved in plastic containers containing tap water with net casing cultures of larvae were preserved at 26±2°C and 70-80% RH under dark and light conditions. The I^{st}, 2^{nd}, 3^{rd}, and 4^{th} instars larvae were detached and saved in a 1000ml plastic container. Thirty larvae were released with the help of a dropper in each container containing 200ml water with different concentrations (80, 160, 240, 320, and 400ppm) of plants extract. A control (distilled water only) was also set up with each concentration.

The larvae were nourished with a crushed mix of dog biscuits and yeast in 3:1 and each container was enclosed with a mosquito net. Along with one control (having no extract), the experiments were replicated three times for each concentration. The average larval mortality was recorded after each 24hrs till 72hrs. The morphological changes were detected under a Trinocular microscope (Optika® 500, Italy) in experimented and control larvae. The larvae were considered dead if they indicated no symbol of the movement.

3.5: Biochemical analysis of experimental larvae

After 48hrs of exposure, dead larvae from each concentration were removed, eroded with saline solution, dried out, and were weighed with electric balance. With the aid of a homogenizer, sucrose solution (0.25M) was used to homogenize 90mg of each sample. At 13000rpm, the homogenate was centrifuged for 15-20min. To determine carbohydrate, protein, and lipid concentrations, the supernatant was collected and stored at -20℃ for later use. Proteins were assessed by Lowry's method, taking bovine serum albumin (BSA) as standard (Lowry *et al*., 1951), carbohydrates were evaluated by the Phenol-Sulphuric acid method and used glucose as standard (Masuko *et al.,* 2005), lipid values (Cholesterol, triglyceride and high-density lipid) were measured with the help of biochemistry analyzer (Motenu®, China).

3.6: Antioxidant Assay

3.6.1: Free Radical Scavenging Assay (DPPH assay)

The free radical scavenging activity was exhibited by the discoloration of the purple-coloured DPPH solution (9.2 mg/100 ml methanol). 20ul aliquot (4mg/ml DMSO) was shifted to the 96 well microplates and then add DPPH reagent (180ul) to each well comprising the sample. The final concentration of the sample was 400µg/ml. Incubate the mixture for 60mins at 37C° in dark. Ascorbic acid (1mg/ml) was used as positive control while DMSO was used as a negative control. Absorbance was measured at 517nm by a microplate reader. The drop-in absorbance of the mixture containing DPPH reagent that indicates the existence of radical scavenging properties in the sample. At initial concentration, the samples which possessed more than 70% scavenging activity were further observed by using three times serial dilution to evaluate LC_{50} value (Rashid *et al*., 2017). Percent radical scavenging activity was calculated using the following formula;

Radical scavenging (%) = [(A0-A1/ A0) × 100%]

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample extracts. Each sample antioxidant activity was expressed in terms of IC_{50}. IC_{50} values expressed as µg AAE/mg of extracts.

3.6.2: Total Reducing Power (TRP)

The reducing power test was attained by taking 200µl of each sample (4mg/ml DMSO stock solution) and 400µl each of 0.2M phosphate buffer (0.2 M/l, pH 6.6) and 1% (w/v) K3Fe (CN)6 (potassium ferricyanide). Incubate the mixture for 50mins at 30C° and then add 400µl of 10% (w/v) TCA (trichloroacetic acid) solution. At 3000rpm, centrifuge the mixture for 10mins.150µl of aliquot was taken from supernatants and transferred to the 96 well microplates. Finally, 50ul $FeCl_3$ (0.1% w/v) solution was mixed into each well. At 700nm, absorbance was recorded. Ascorbic acid (1mg/ml DMSO) was used as positive control while DMSO was used as a negative control. The assay was run three times and the reducing power of the sample was revealed as ug ascorbic acid equivalent (AAE) per mg DW (Rashid *et al*., 2017).

3.6.3: Total Antioxidant Capacity (TAC)

A reagent solution consisting of ammonium molybdate (4mM), sodium phosphate (28mM), sulfuric acid (0.6M) was used and 100ul of plant extract (4mg/ml DMSO) were added with 1ml of the reagent solution. After incubation, for 90min at 90C° and then it was cooled at room temperature. At 645nm, absorbance was recorded by a microplate reader. Ascorbic acid (1mg/ml DMSO) was used as positive control while DMSO was used as a negative control. The test was run three times and the resultant TAC was showed as ug ascorbic acid equivalent (AAE) per mg DW (Rashid *et al*., 2017).

3.7: Cytotoxicity assay

The bioassay of brine shrimp lethality method was used to determine the toxicity estimation of particular plants. Concisely the brine shrimp were hatched in a conically shaped vessel (1L), with the help of brine shrimp eggs occupied with artificial seawater (38g sea salt/L, pH 8.5 using 1N NaOH) with a continuous source of oxygen for 2 days at an ambient temperature of 23±1°C. Later emerging, the nauplii were collected and used

for bioactivity. The trials of stock solution were established by mixing the essential quantity of extracts in an exact volume of 0.5% dimethyl sulfoxide and seawater. For the screening of the toxicity of plants extracts (*M. piperita*, *O. basilicum*, *P. glabrum, M. philippensis, C. aruntium, F. officinalis*), stock solutions were prepared and transferred to clean vials with selected concentrations (80, 160, 240, 320, and 400ppm) of extractives, the assay was carried out in triplicate and final volume up to 10ml by using seawater. In each test tube, active shrimps were released with the support of a Pasteur pipette. To check the test technique, the control group was added and consequences were achieved due to the cytotoxic activity of the test agent. The tubes were observed with the help of a magnifying lens after 24hrs, and the number of survived nauplii in every vial was calculated and interpretations were noted for each vial. Tricaine methanesulfonate was used as a control (El Badry *et al.,* 2015).

3.8: Characterization of most effective plant

3.8.1: Preliminary qualitative phytochemical analysis:

Preliminary phytochemical tests are performed on ethanolic extract of *M. piperita* with standard methods for various secondary metabolites.

Table 3.2: Summary of different qualitative phytochemical tests conducted for the analysis of *M. piperita.*

Sr. No	Secondary Metabolite	Name of test	Methodology	Results	References
1.	Alkaloids	Mayer ‘s test	2ml conc. HCl + 2ml of extract + few drops of Mayer ‘s reagent	White precipitate or green color	(Harborne, 1984)
		Hager ‘s test	1ml of extract + few drops of dilute HCL + 1ml of Hager ‘s reagent	Yellow ppt.	(Harborne, 1984
		Wagner ‘s test	1ml of extract + 1ml of Wagner’s reagent	Reddish brown ppt.	(Parekh *et al*., 2006).
2.	Carbohydrates	Molisch ‘s test	1ml extract + 5ml dist. H_2O + 1 drop of Molisch ‘s reagent + 1ml H_2SO_4	Reddish violet ring at the junction	(Harborne, 1984)
		Fehling ‘s test	1ml of filtrate + 5-8 drops of Fehling ‘s solution + heated on a water bath for half an hour.	Brick red precipitation color	(Akinyemi *et al*., 2005)
3.	Flavonoids	Alkaline reagent test	2ml of 2% NaOH + 2ml of extract + dil. HCl	Yellow solution with NaOH, Turns colorless with dil. HCl	**(**Onwukaeme *et al*., 2007).
		$FeCl_3$ test	Few drops of $FeCl_3$ + 1ml of extract	Blackish red ppt.	(Harborne, 1984)

4.	Cardiac glycosides	Liebermann-Burchard's test	2ml of acetic acid + 2ml of Chloroform + 2ml of extract + mixture was then cooled + added a few drops of conc. H_2SO_4	Green color	(Sofowora, 1993)
		Salkowski' s test	2ml of dil. H_2SO_4 + 2ml of extract	Reddish brown color	(Sofowora, 1993)
		Keller - Kiliani test	2ml filtrate + 1ml glacial acetic acid + 1ml + 1ml ferric chloride + 1ml conc. H_2SO_4	Green blue coloration	(Parekh and Chanda, 2007).
5.	Saponins	Frothing test / Foam test	1ml filtrate + 4ml dist. H_2O +mixed well and shaken vigorously	Foam formation	(Parekh and Chanda, 2007).
		Lead acetate test	1ml filtrate + 1ml ammonia solution + 1ml lead acetate + mixed well and shaken vigorously	Black green ppt. or drop green foam	(Harborne, 1984)
6.	Tannins	$FeCl_3$ test	2ml of 5% $FeCl_3$ + 1ml extract	Greenish black or dark blue color	(Sofowora, 1993)
		Alkaline reagent test	1ml of 1N NaOH + 1ml extract	Appearance of yellow to red color	(Harborne, 1984)
		Braymer 's test	2ml of extract + 2ml H_2O + 2-3 drops of $FeCl_3$ (5%)	Green ppt.	(Harborne, 1984

7.	Sterol	Salkowski ‘s test	5ml of Chloroform + 2ml of plant extract + 1ml of conc.H_2SO_4	Reddish brown color	(Harborne, 1984
8.	Quinones		1ml conc. H_2SO_4 + 1ml extract	Red color	(Harborne, 1984
9.	Terpenoid	Salkowski ‘s test	5ml extract + 2ml Chloroform + 3ml conc. H_2SO_4	Reddish brown color	(Edeoga *et al.,* 2005).
10.	Phenol	Ellagic acid test	Few drops of 5% glacial acetic acid + 1ml extract + few drops of 5% $NaNO_2$	Muddy brown color	(Harborne, 1984
			1ml extract + 1ml lead acetate	White ppt.	(Harborne, 1984)
11.	Amino Acid	Ninhydrin test	1ml extract + few drops of Ninhydrin reagent	Purple color	(Harborne, 1984)
		Xanthoproteic test	1ml extract + 1ml conc. H_2SO_4	White ppt.	(Harborne, 1984)
12.	Volatile oil		2ml extract + 0.1ml dil. NaOH + dil. HCl + shake the solution	Formation of white ppt.	**(**Dahiru *et al*., 2006).
13.	Starch		1ml extract + few drops of iodine solution	Color change	(Harborne, 1984)
14.	Cellulose		1ml extract + few drops of iodine solution + few drops of conc. H_2SO_4	Brown red color	(Harborne, 1984)

15.	Anthocyanin and Betacyanin	NaOH test	1ml of 2N NaOH + 2ml of extract + heated at 100 ·C for about 5 min.	Bluish green color **(Anthocyanin)** Yellow color **(Betacyanin)**	(Harborne, 1984)
16.	Anthraquinones	Borntrager ‘s test	3ml extract + 3ml Benzene + 5 ml NH_3 (10 %)	Pink, Violet or red coloration in Ammonical Layer	(Onwukaeme *et al*., 2007).
17.	Coumarins		1ml of 10 % NaOH + 1ml of extract	Yellow color	(Sofowora, 1993)
18.	Phlobatannins	Precipitate test	1ml extract + 1ml HCl (10%) + heat	Red ppt.	(Edeoga *et al*., 2005).
19.	Steroid and Phytosteroid		1ml extract + equal volume of Chloroform + few drops of conc. H_2SO_4	Brown color **(Steroids)** Bluish –brown ring **(Phytosteroid)**	**(**Edeoga *et al.,* 2005).
20.	Leucoanthocyanin		5ml extract + 5ml Isoamyl alcohol	Organic layer into red	(Harborne, 1984)

3.8.2: Quantitative phytochemical analysis

Table 3.3: Summary of different quantitative phytochemical tests conducted for the analysis of *M. piperita.*

Sr. No	Secondary metabolite	Methodology	Absorbance	Reference
1	Alkaloids	1ml filtrate + 5ml of 60% H_2SO_4 + after 5min mix 5ml of 0.5% formaldehyde + allowed to stand for 3hr.	Absorbance was read at 565 nm.	Ekwueme *et al*., 2015
2	Tannins	0.5ml of sample extract + 3.7ml of distilled water + 0.25ml of Folin Phenol reagent + 0.5ml of 35% sodium carbonate solution.	Absorbance was measured at 725nm.	Prabhavathi *et al*., 2016
3	Flavonoids	5m l filtrate + 5ml dilute ammonia + shaken for 5min and upper layer was collected.	Absorbance was read at 490nm.	Ekwueme *et al*., 2015
4	Terpenoids	2.5ml filtrate + 2.5ml of 5% aqueous phosphomolybdic acid solution +2.5ml of concentrated H_2SO_4+ mixed. The mixture was left to stand for 30min and then made up to 12.5ml with ethanol.	Absorbance was taken at 700nm.	Ekwueme *et al*., 2015
5	Proteins	100µl of the sample extract + 3ml of Bradford's reagent + incubate in the dark for 5min.	Absorbance was measured at 595nm	Prabhavathi *et al*., 2016
6	Steroids	2ml filtrate + 2ml of chromagen solution + solution left to stand for 30min.	Absorbance was taken at 550nm.	Ekwueme *et al*., 2015
7	Cardiac Glycosides	1ml filtrate + 4ml of alkaline pirate solution + mixture was boiled for 5min and allowed to cool.	Absorbance was read at 490nm.	Ekwueme *et al*., 2015
8	Reducing sugar	1ml filtrate + 1ml alkaline copper reagent + mixture was boiled for 5min and allowed to cool + 1ml of phosphomolybdic acid reagent + 2ml of distilled water.	Absorbance was measured at 420nm.	Ekwueme *et al*., 2015

9	Carbohydrate	1ml of sample solution + 1ml of 5% phenol + 5ml of concentrated Sulphuric acid + mix well + leave for 10min.	Measure the absorbance at 488nm.	Prabhavathi *et al*., 2016
10	Saponins	10g dried fine particles, plant sample + 50ml of 20% aqueous ethanol + heated (55˚C) on a water bath for 4hrs with continuous stirring + filtered and the residue re-extracted with another 100ml of 20% ethanol + extract was further reduced to 20ml over a hot water bath (90˚C) + conc. extract was transferred into a 250ml separating funnel +10ml diethyl ether + shaken vigorously. Ether layer was discarded and the aqueous layer was collected + purification was repeated +30ml n-butanol + combined n-butanol extract was washed twice + 5ml 5% aqueous sodium chloride +remaining solution was heated in a water bath + After evaporation, the samples were dried in the oven and weighed.		Ajuru *et al*., 2017
11	Phenols	5ml filtrate+ 0.5ml Folin calculates reagent + allowed to stand for 30min + 2ml of 20% sodium carbonate was added.	Absorbance measured at 650nm.	Ekwueme *et al*., 2015

3.8.3: UV-VIS Spectroscopy analysis:

The extract was examined under UV and visible light for proximate analysis. The extract was centrifuged at 300rpm for 10min and filtered through Whatman No.1 filter paper. The sample is diluted to 1:10 with the same solvent. The extract was scanned under UV and visible light in the wavelength ranging from 200-800nm using a UV visible spectrophotometer (Perkin Elmer, USA Model: Lambda 950), and the characteristic peaks were detected (Karpagasundari and Kulothungan, 2014).

3.8.4: Fourier-transform infrared spectroscopy:

FT-IR analysis of the plant extract was obtained using Shimadzu FT-IR–8400s Fourier transform infrared spectrophotometer, Japan using Bruker, Germany Vertex 70 infrared spectrometer which was used to detect the characteristic peaks and their functional groups. The dried extract powder (10mg) was compressed in 100mg of KBr pellet to prepare the transparent sample disc. The IR scan was carried out in the wavenumber region of 400-4000^{cm-1}. The peak values of FT-IR were recorded (Pramila *et al.,* 2012).

3.8.5: **Gas chromatography-mass spectrometry:**

A chromatographic system consisting of a Shimadzu GC-201, auto-injector AOC-20i, autosampler AOC-20s, and a gas chromatograph equipped with a QP2010ultra mass-selective detector was used for GC-MS analysis (Shimadzu). With a maximum temperature capability of 325°C, a fused-silica capillary column DB-5MS (0.25mm 30m 0.25m) was employed for screening. Electron impact (EI) mass spectra were used to confirm the structure. For peak identification and quantification, 70eV EI ionization was used with a 1.2kv electron multiplier setup in full scan operation mode (m/z = 50 to 350).

Perfluorotributylamine (PFTBA) with mass m/z 69, 219, and 502 was used to auto-tune the mass-selective detector. The chromatograph was set to split injection system mode, with a purge flow of 3.0 ml/min after the injection. The injection volume was 1.0 l, with a 10:1 split ratio. Highly pure helium gas was employed as the carrier gas, with a flow rate of 1.0 ml/min. Temperatures were set at 250°C for the injector and 290°C for the interface. The initial temperature of the column was 100°C. After injection, the temperature was held at 100°C for 0.5min, and then increased at a rate

of 24°C/min to 280°C for 3 min, for a total run time of 11 min (Visveshwari *et al*., 2017).

3.9: Identification of Phytoconstituents

The National Institute of Standards and Technology (NIST) database having more than 62000 patterns was used for the interpretation of the mass spectrum. The comparison between spectra of known and unknown compounds, from the NIST library, helps in the interpretation of the mass spectrum and in the determination of the name, chemical formula, chemical structure, and molecular weight of identifying compounds (Rukshana *et al.,* 2017).

3.10: Molecular docking

3.10.1: Building the Receptor

Ligand molecules were retrieved from PubChem database. From the protein data bank (PDB), the 3D structure of the receptor has been copied and adjusted. The compounds were retrieved in 3D SDF format. According to the accessible parameters involved, stabilizing charges, elimination of the water molecules from the cavity, generation of the side chains and filling in the missing residues, etc. It is biologically active and stable after modification of the receptor. The NS3 protease domain structure with PDB ID: 2FOM was recovered from the protein data bank (Li *et al*., 2004).

3.10.2: Identification of the active site

The active site was recognized after building the receptor. One of the active sites was designated because the receptor has many sites. Many water molecules and hetero- atoms were removed. Against possible drug targets, the MOE is computer-generated screening software for drug designing and is used to display lead compounds and study the binding affinity of test compounds with protein NS3 in the form of E- value. A three-dimensional grid with coordinates (x, y, and z) was set to provide maximum area for compounds binding and further default parameters availed for MOE docking **(**Seyoum *et al*., 2002).

3.10.3: Ligand Preparation

Ligands can be acquired from many databases like ZINC; PubChem can be drawn with the help of tools like Chemsketch **(**Weigel *et al*., 2018).

3.10.4: Docking

The ligand is docked onto the receptor and the interactions were checked. The scoring function generates scores depending on which ligand with the best fit was selected. Docked compounds screened against 1, 2-Benzenedicarboxylic acid and 3-ethyl-5, 5-dimethyl -6-phenyl of *Cx. quinquefasciatus* **(**Yusufzai *et al*., 2018).

3.11: Statistical analysis

Mean mortality of mosquito larvae for each plant extracts was compared and statistically significant differences were determined by one-way ANOVA with Tukey's multiple comparison tests using "R language". Results with a P value less than 0.05 were considered to be significant and less than 0.05 was considered strongly significant. Furthermore, Probit analysis (Finney, 1971) was performed to calculate LC_{50}, LC_{90}, 95% confidence limits (upper and lower), and chi-square was calculated by using Minitab.

RESULTS

3.1: Antilarval activity of plants extracts against mosquito larvae

The antilarval activity of leaf extracts of six plants in ethanol and water solvent against all instars larvae were assessed. In fifteen days experiment, five different concentrations (80, 160, 240, 320, 400ppm) were used and the mean mortality of four instars larvae of mosquitoes was calculated. All concentrations were originated to have significant impact on larvae mortality as compared with control (F (*df)* = 520.9(5); P< 0.05) in hours dependent manner, that also differed significantly from each other (F (*df)* = 480.4(5); P< 0.05). Also, all plants exposed differential antilarval activity in the dose and time-dependent manner (F (*df*) =460.8 (5); P<0.05). Mortalities increased with an increase in the concentrations of the ethanolic and aqueous plants extract.

The effect of time (hours), concentrations, and plants on the mean mortality of all instars larvae is denoted in **figure (3.2-3.17)**. Among the tested plants, *M. piperita* was found most effective with a significant (P<0.05) increase in mortality of instars larvae (93%) at the end of the experiment. However, *O. basilicum, P. glabrum,* and *M. philippensis* also significantly (P<0.05) reduced larvae population with 78%, 62%, and 42% mortality. *While C. aruntium* and *F. officinalis* have a non-significant effect on larvae mortality with less than 31% in the larvae population. Further Chi-square test, the level of significance of plants treated with all instar's larvae and their LC_{50} and LC_{90} values are represented in **(Table 2).** The water extracts also produced similar results with non -significant differences from ethanolic extracts of the same plants as compared to control **(Figure 3.2-3.17).**

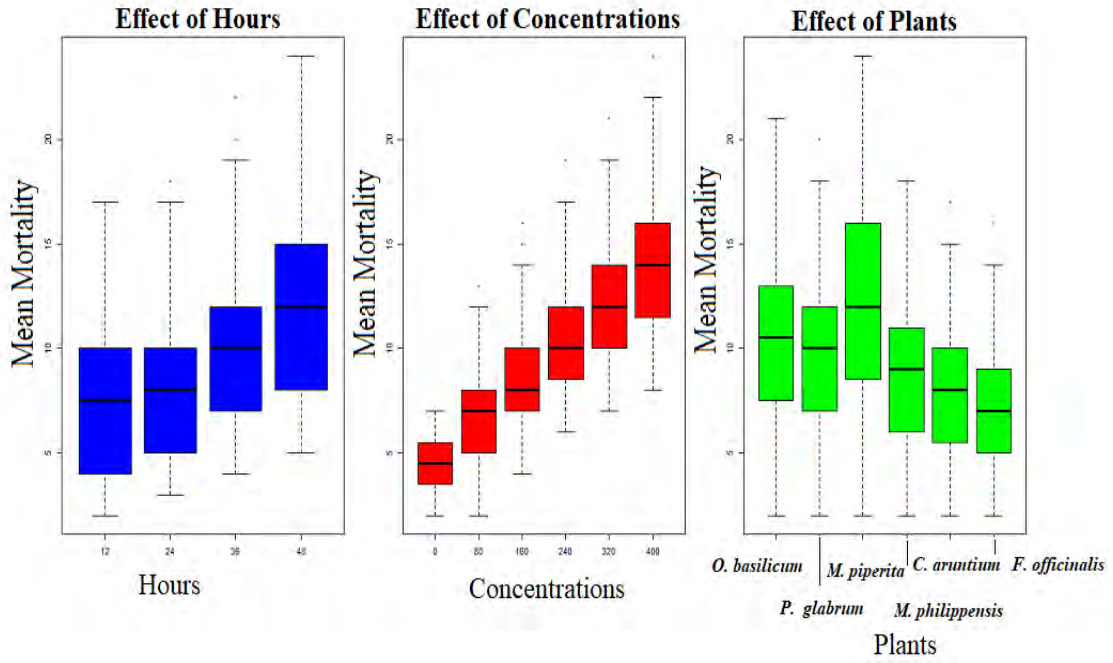


Figure 3.2: A box plot showing effect of hours, concentrations and plants on average mortality of I[st] instar larvae in ethanol solvent.

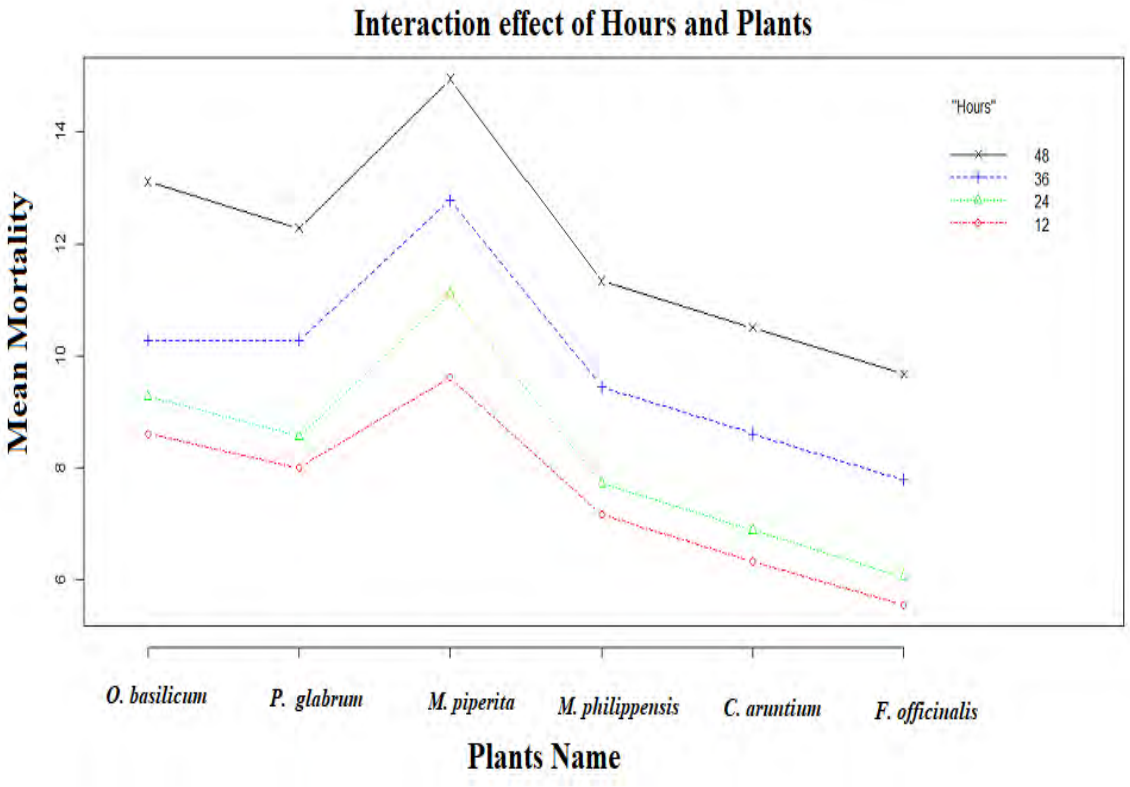


Figure 3.3: Interaction effects of hours and plants on average mortality of I[st] instar larvae.

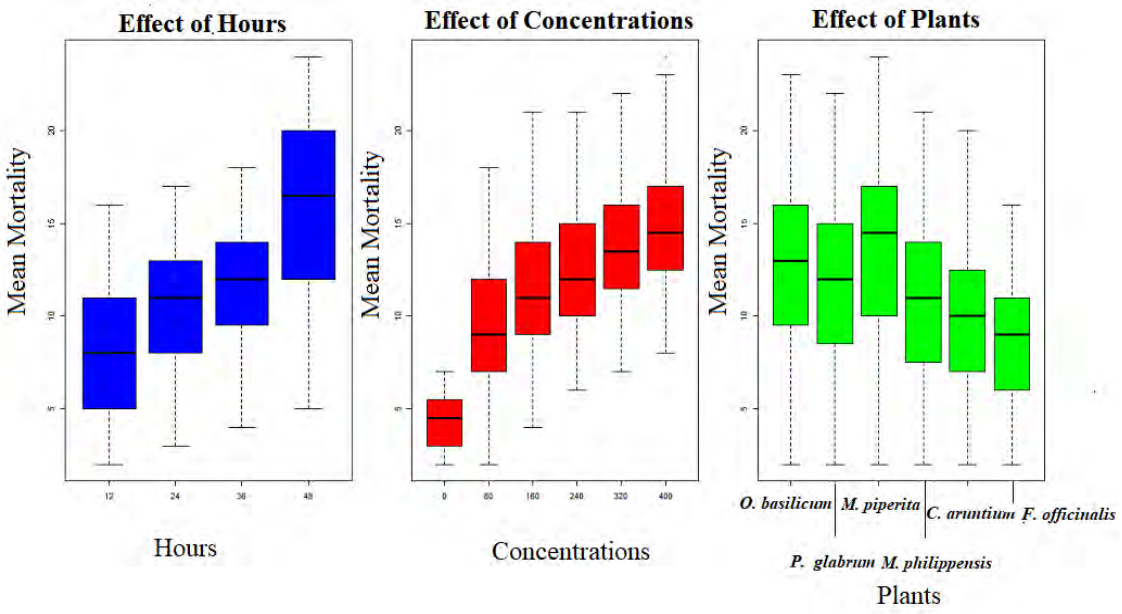


Figure 3.4: A box plot showing effect of hours, concentrations and plants on average mortality of 2nd instar larvae in ethanol solvent.

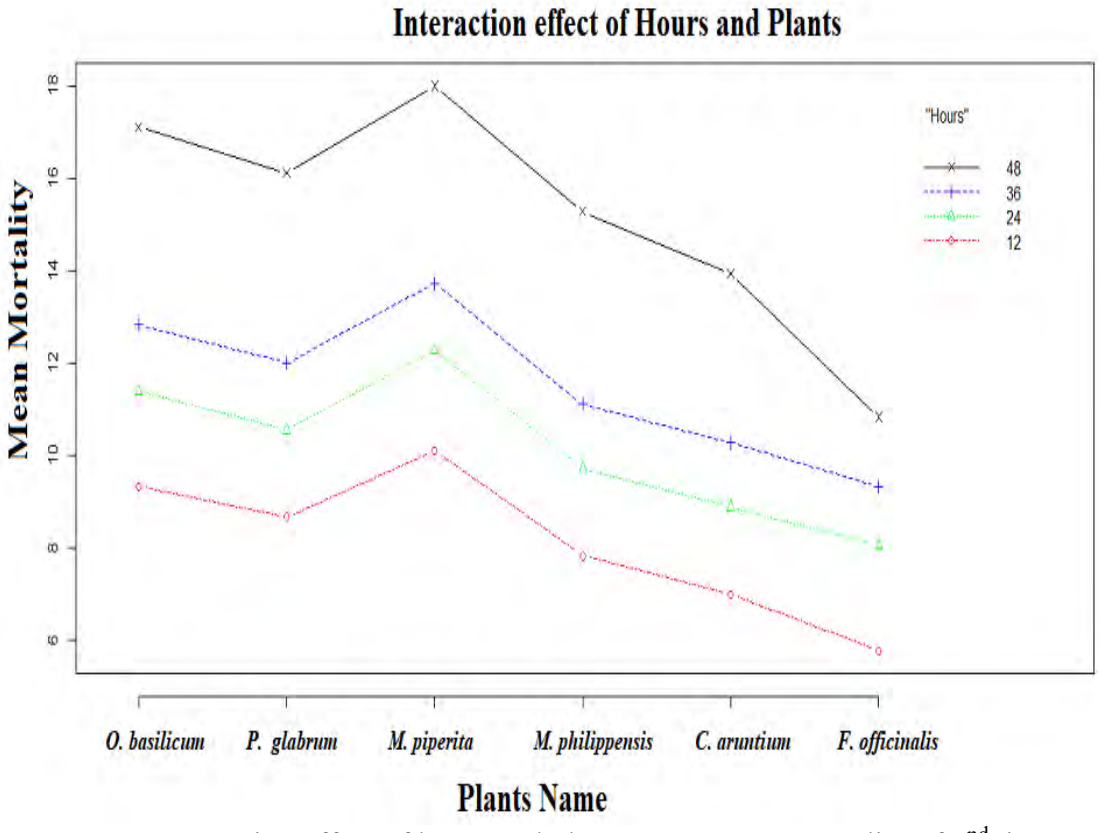


Figure 3.5: Interaction effect of hours and plants on average mortality of 2nd instar larvae.

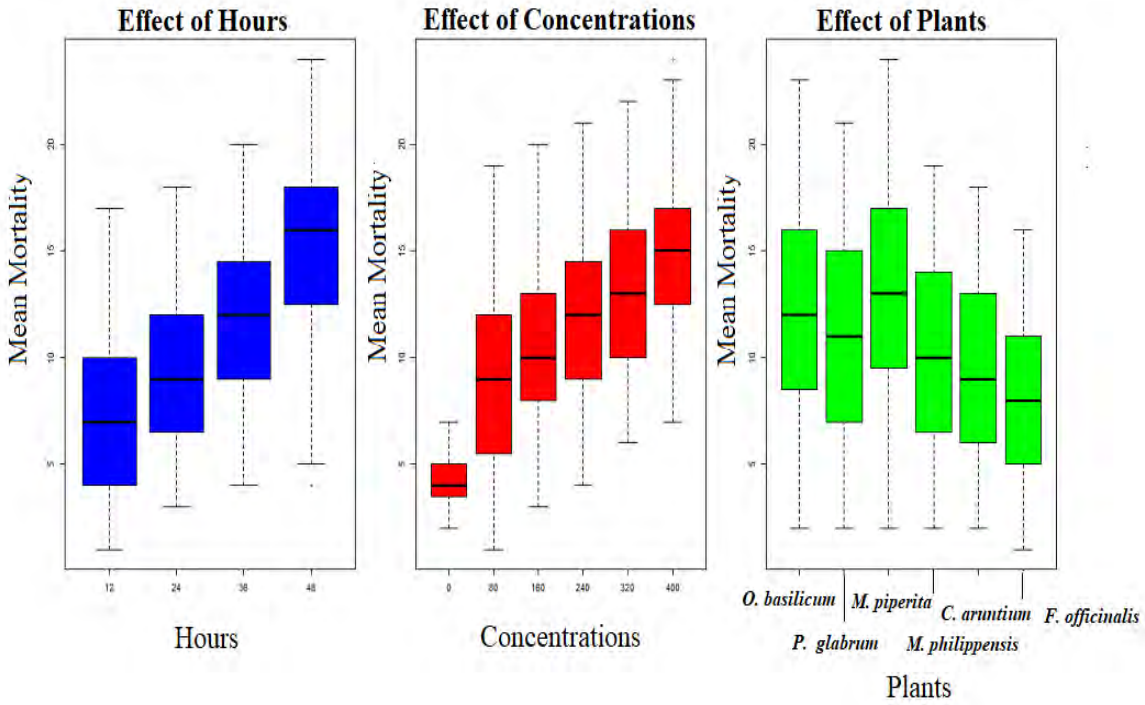


Figure 3.6: A box plot showing effect of hours, concentrations and plants on average mortality of 3rd instar larvae in ethanol solvent.

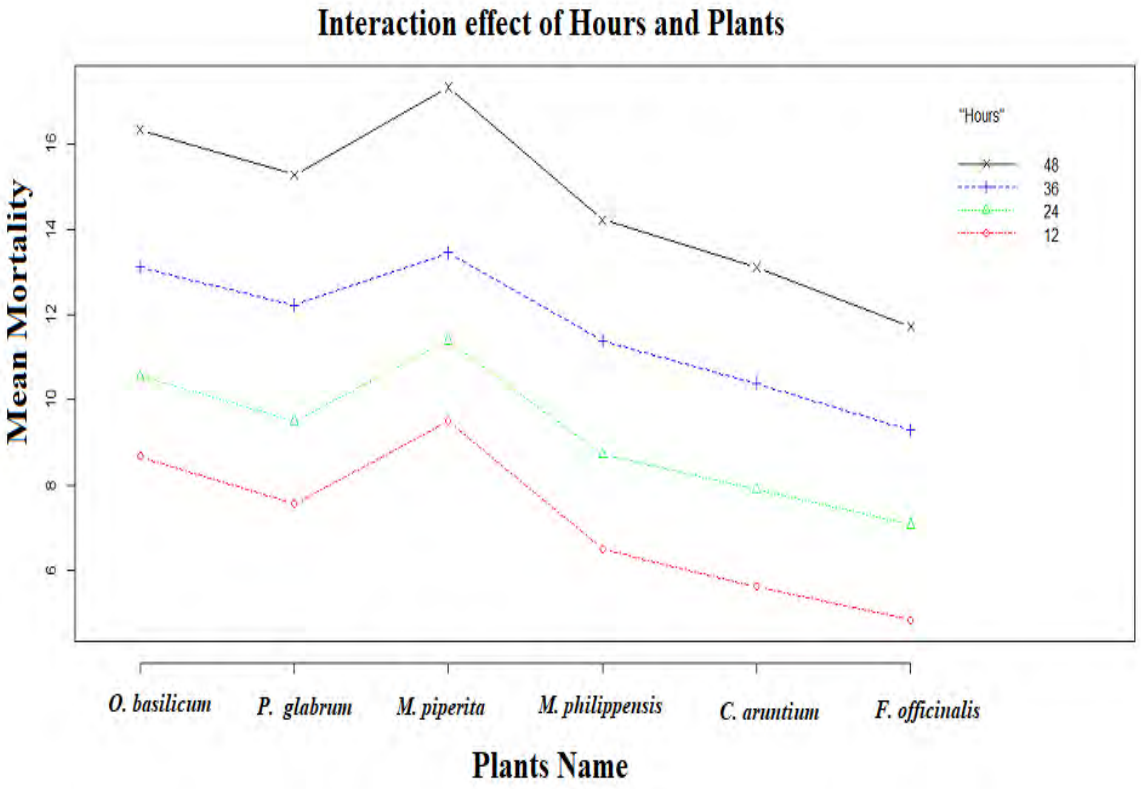


Figure 3.7: Interaction effect of hours and plants on average mortality of 3rd instar larvae.

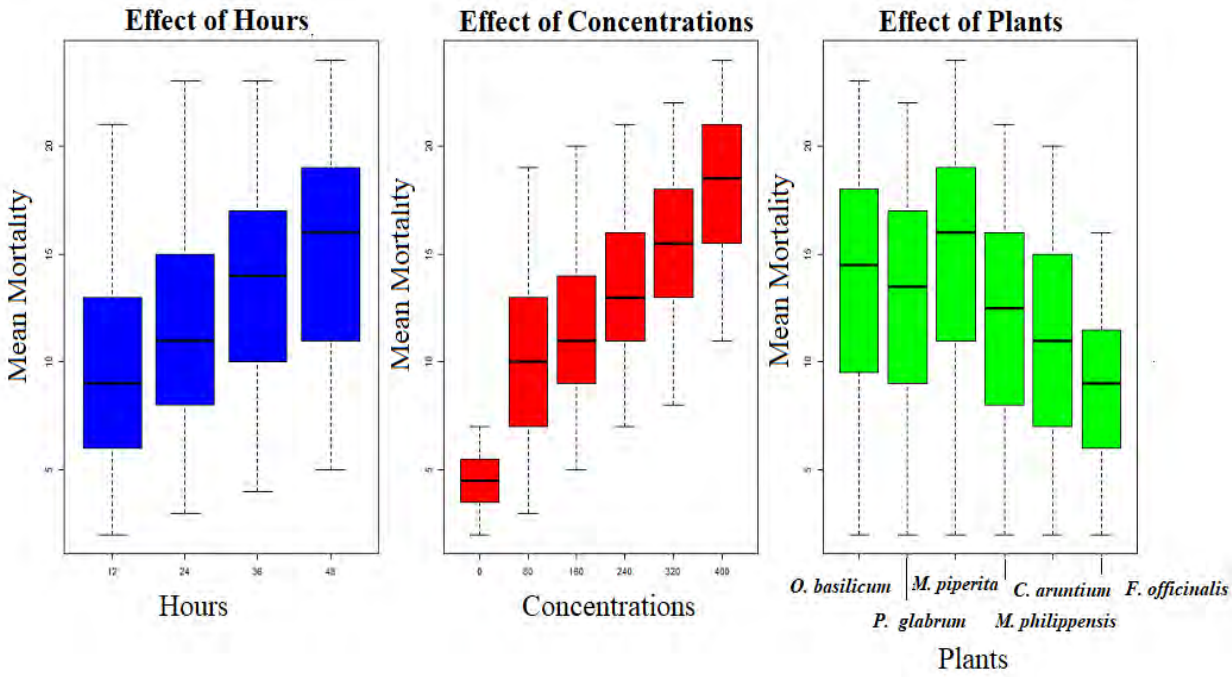


Figure 3.8: A box plot showing effect of hours, concentrations and plants on average mortality of 4th instar larvae in ethanol solvent.

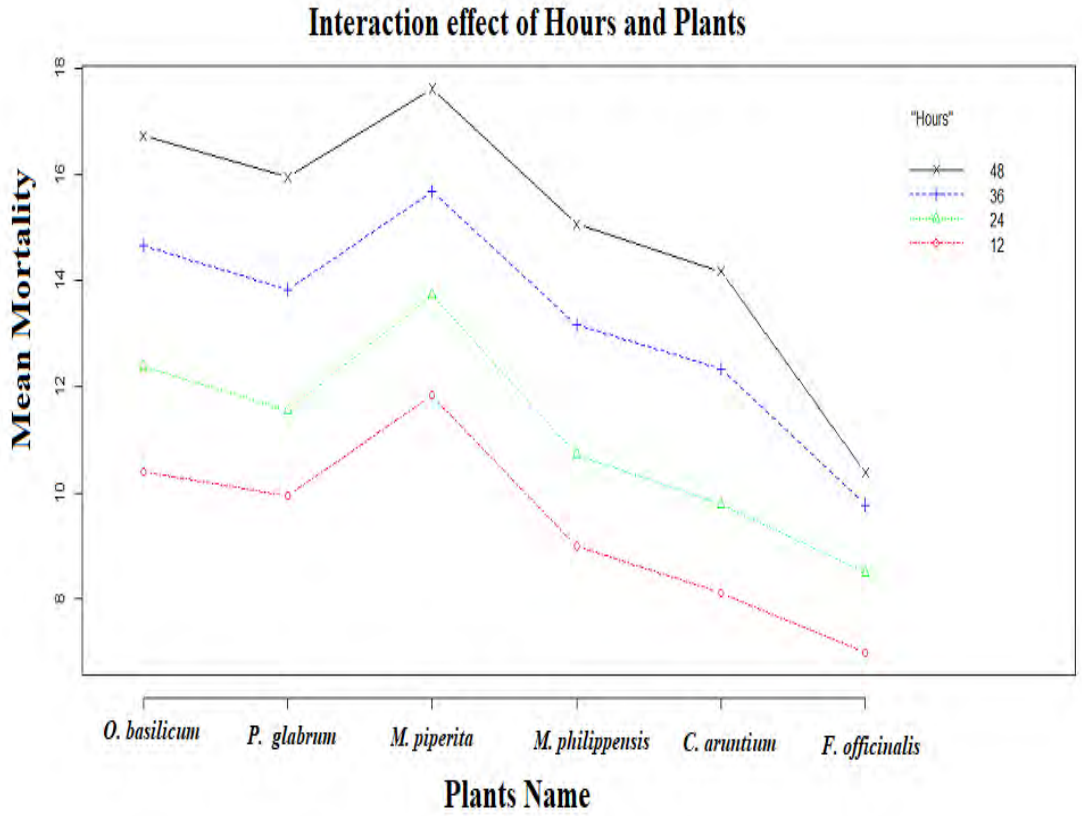


Figure 3.9: Interaction effect of hours and plants on average mortality of 4th instar larvae.

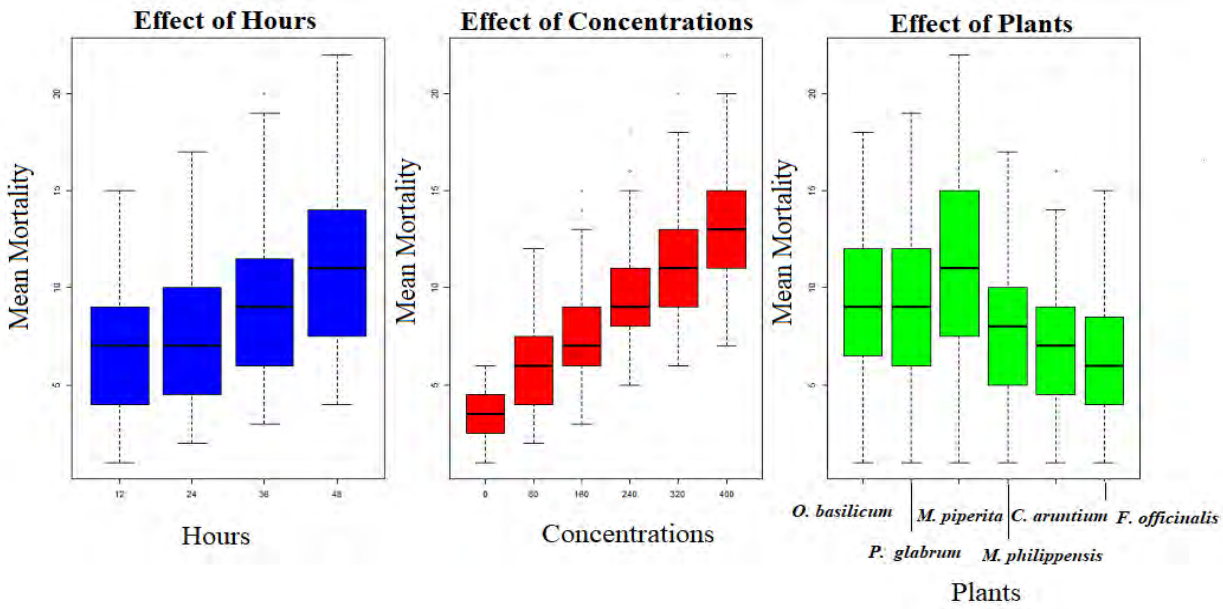


Figure 3.10: A box plot showing effect of hours, concentrations and plants on average mortality of I[st] instar larvae in water solvent.

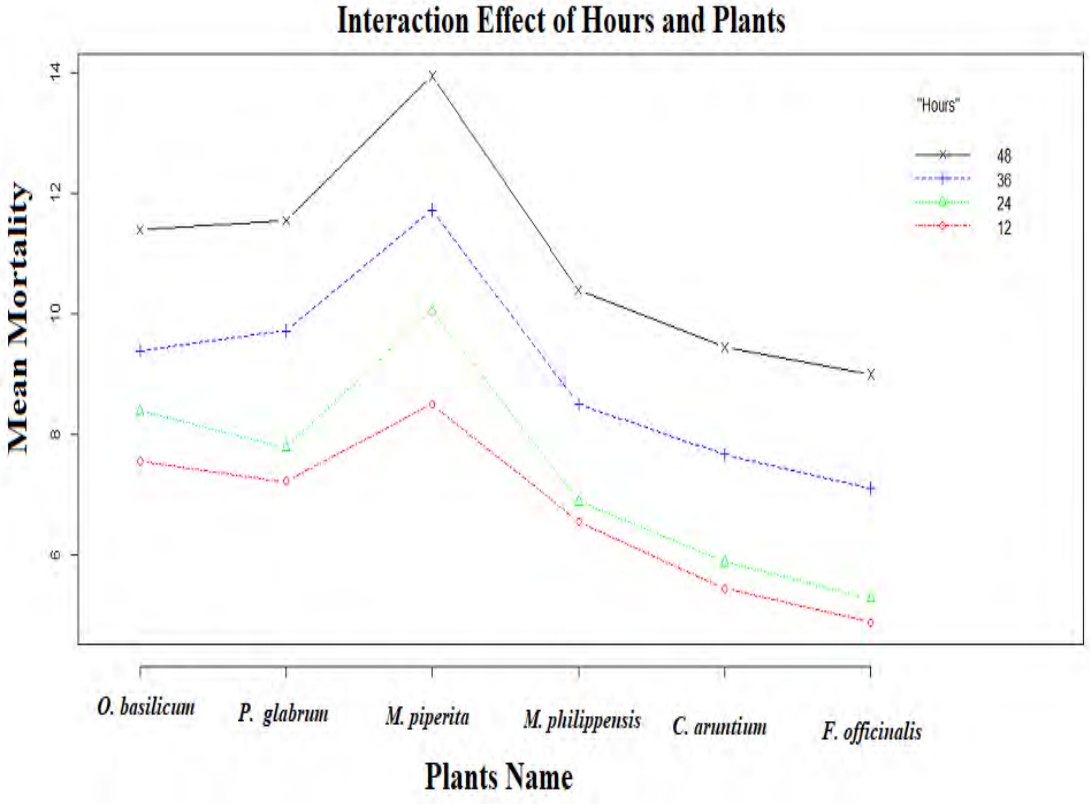


Figure 3.11: Interaction effects of hours and plants on average mortality of I[st] instar larvae.

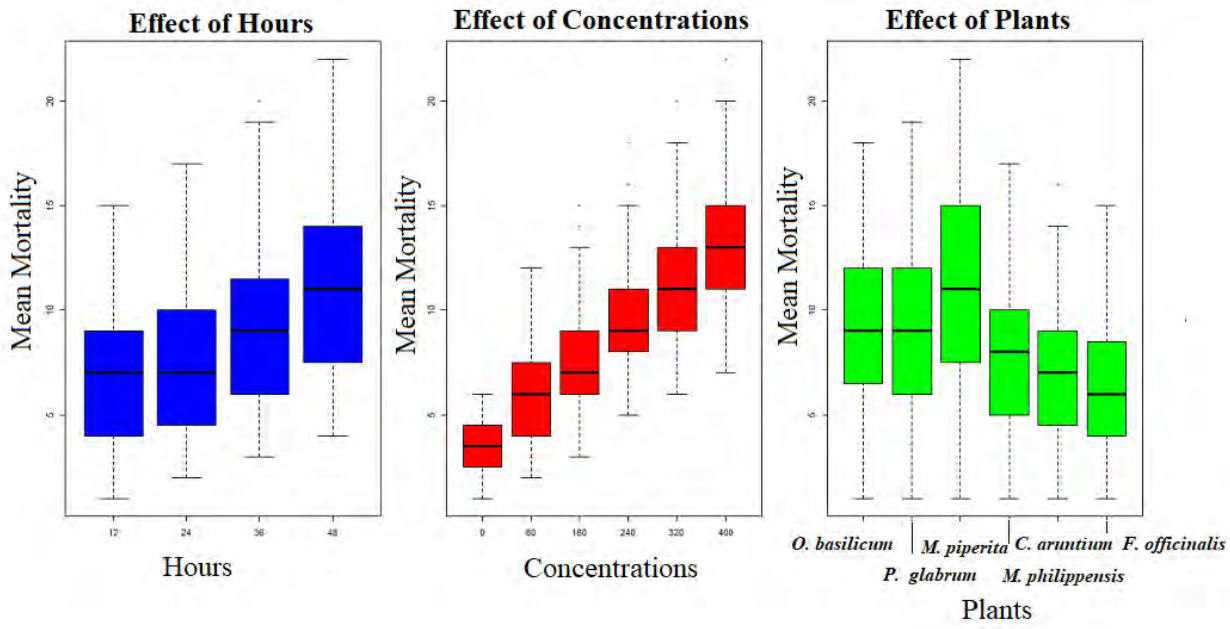


Fig.ure 3.12: A box plot showing effect of hours, concentrations and plants on average mortality of 2nd instar larvae in water solvent**.**

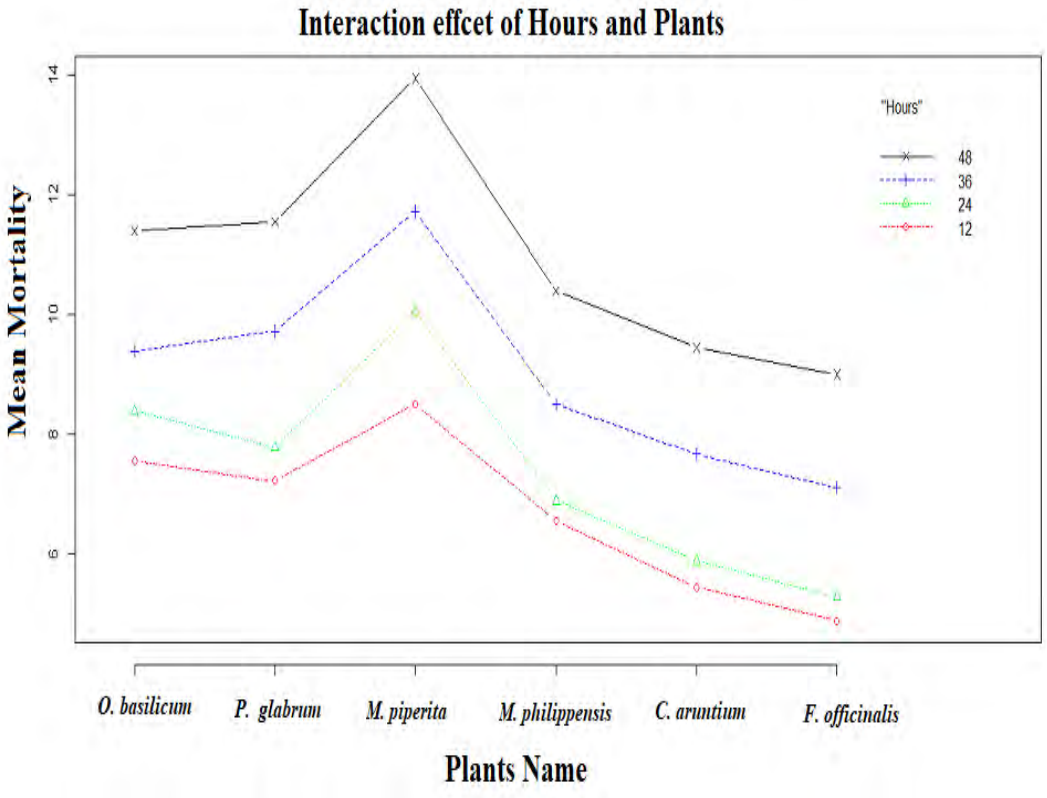


Figure 3.13: Interaction effects of hours and plants on average mortality of 2nd instar larvae.

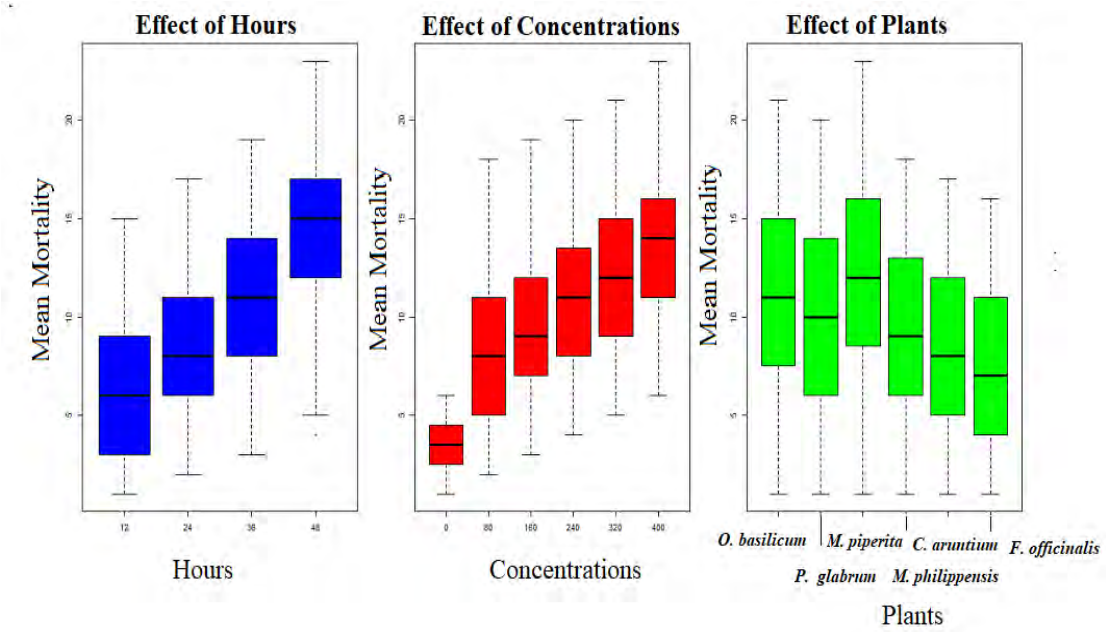


Figure 3.14: A box plot showing effect of hours, concentrations and plants on average mortality of 3rd instar larvae in water solvent.

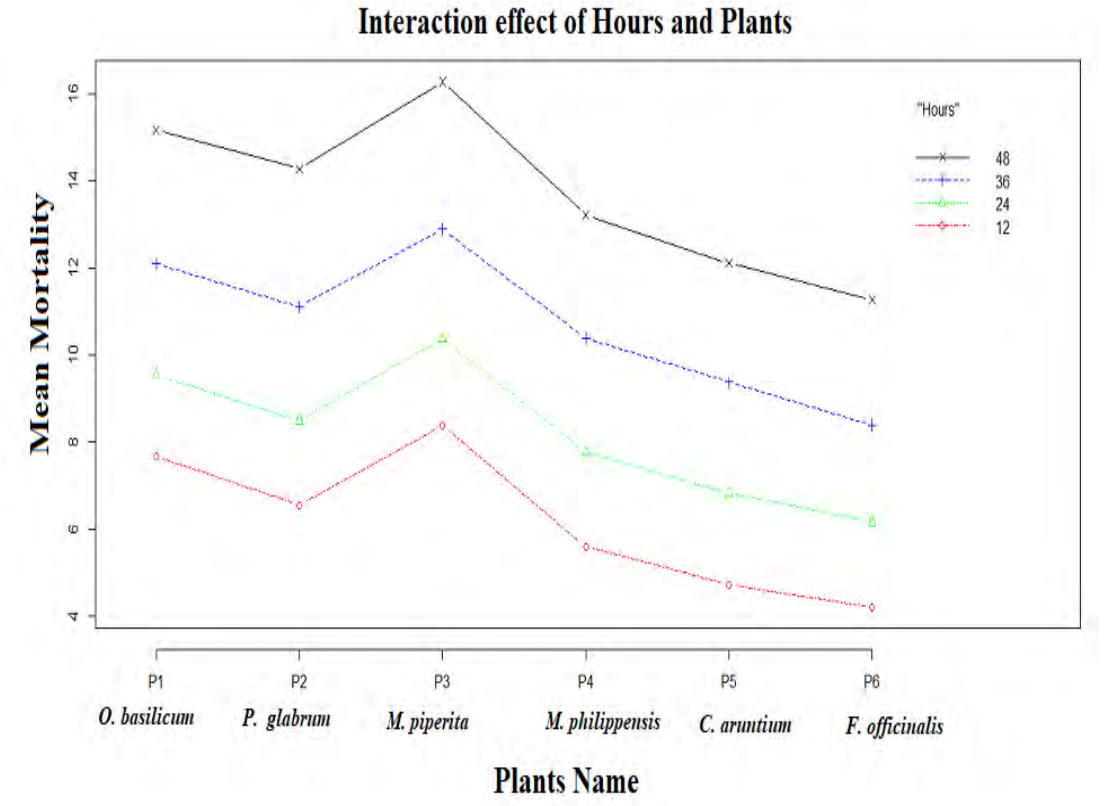


Figure 3.15: Interaction effects of hours and plants on average mortality of 3rd instar larvae.

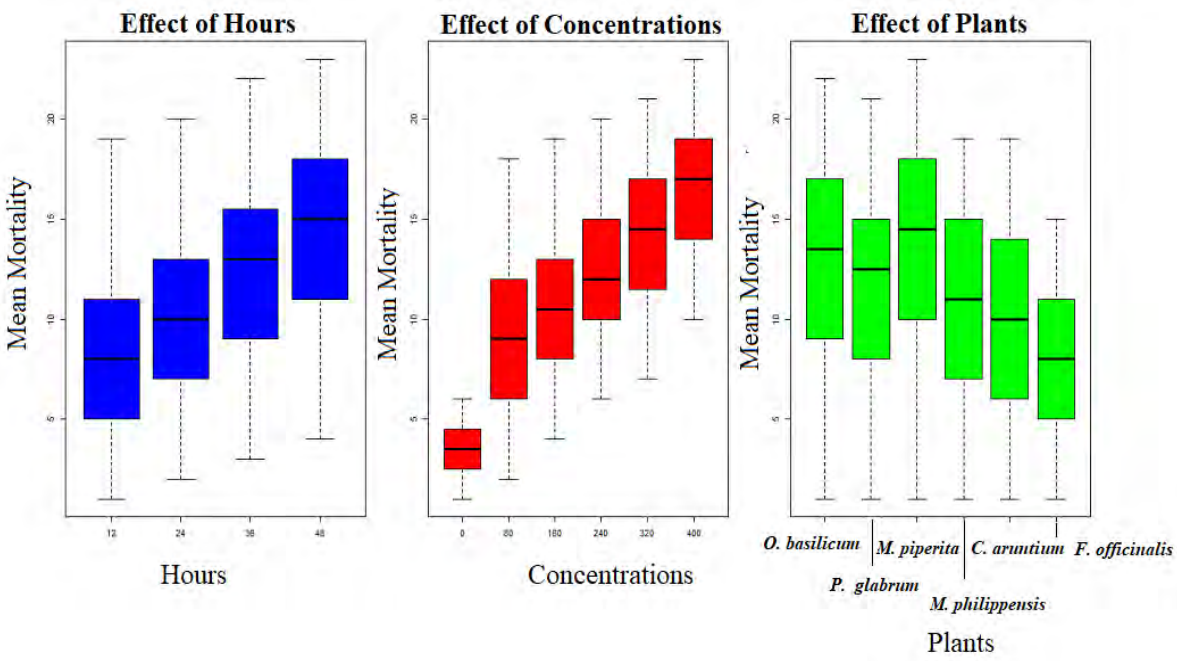


Figure 3.16: A box plot showing effect of hours, concentrations and plants on average mortality of 4th instar larvae in water solvent.

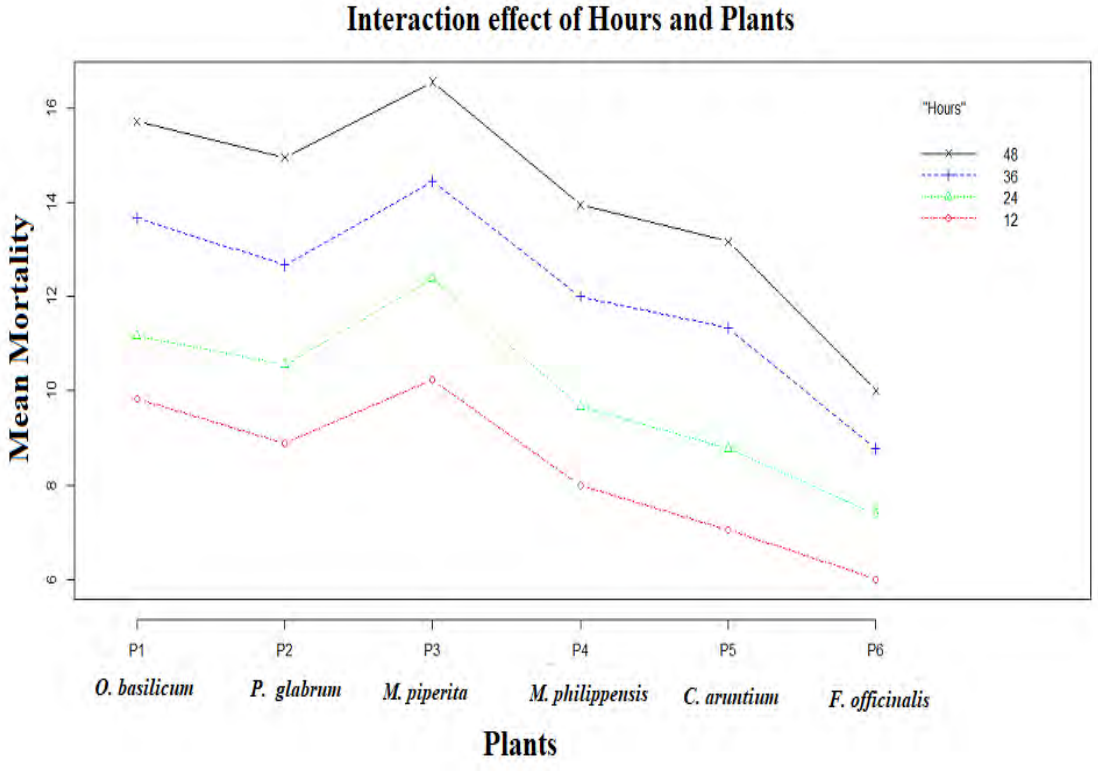


Figure 3.17: Interaction effects of hours and plants on average mortality of 4th instar larvae

3.2. BIOCHEMICAL TESTS:

The biochemical tests (lipids: (triglyceride, cholesterol and high-density lipid) proteins, carbohydrates) accomplished against all instars larvae treated with all plants extract in ethanol and water solvent was significantly lesser than control ($p<0.05$) **(Figure 3.18-3.37).**

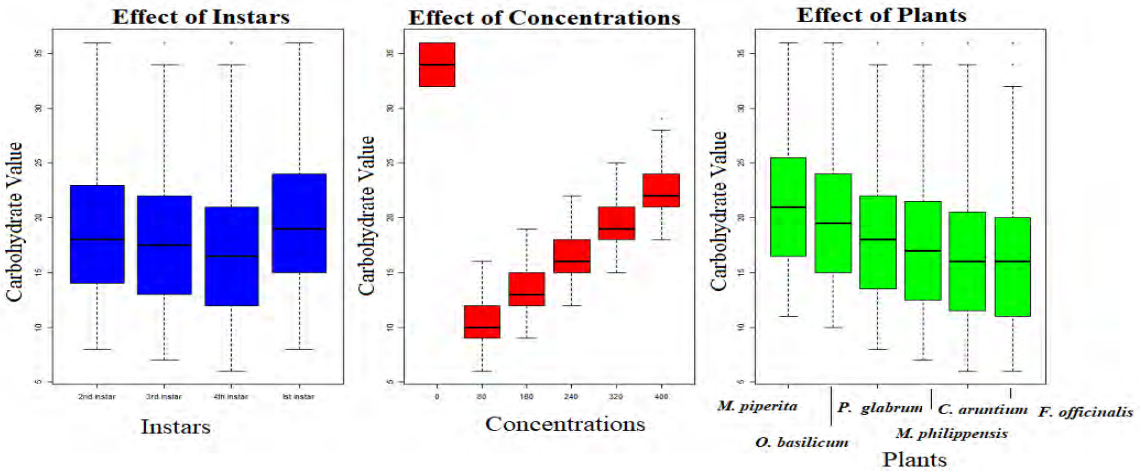


Figure 3.18: A box plot showing effect of instars, concentrations and plants on carbohydrate values in ethanol solvent.

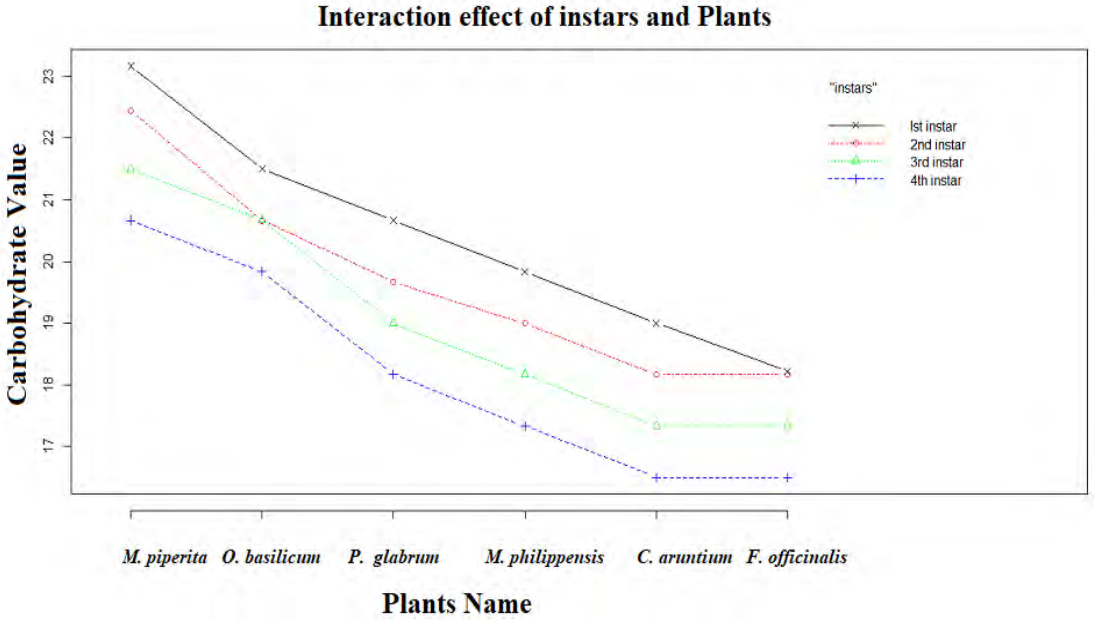


Figure 3.19: Interaction effects of instars and plants on carbohydrate value.

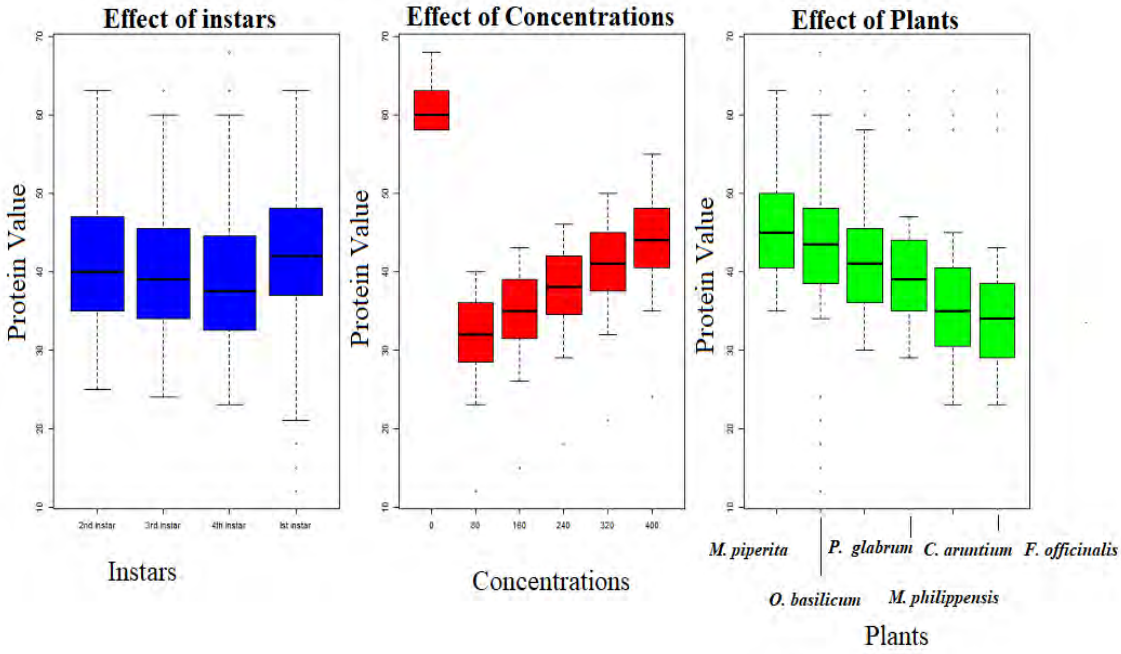


Figure 3.20: A box plot showing effect of instars, concentrations and plants on protein value in ethanol solvent.

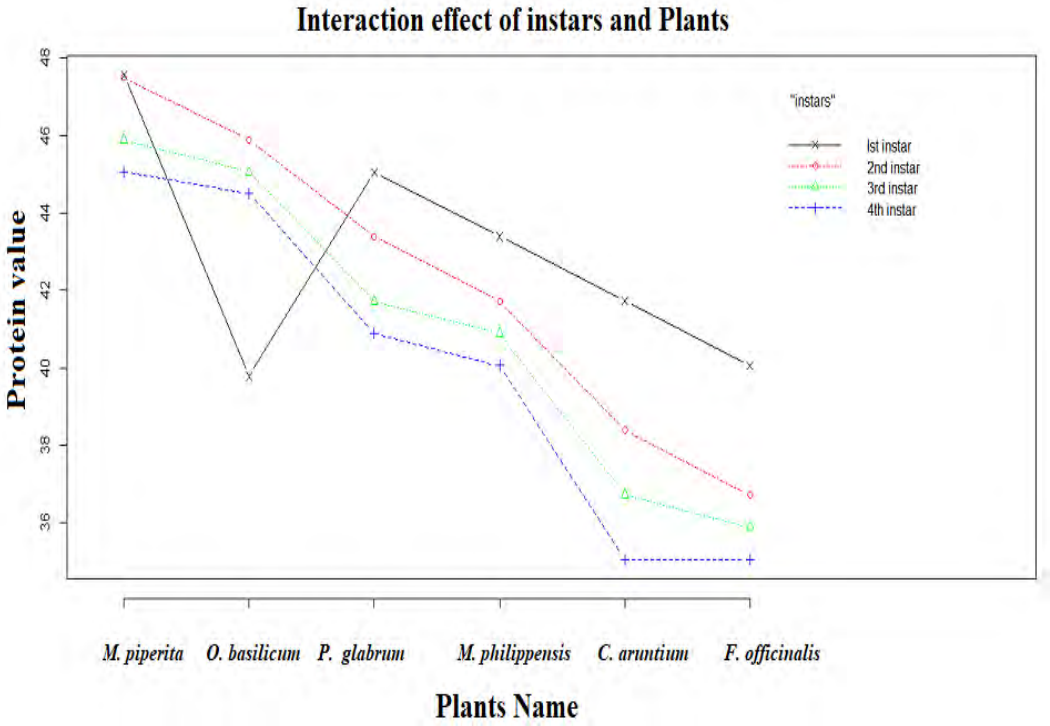


Figure 3.21: Interaction effects of instars and plants on protein value.

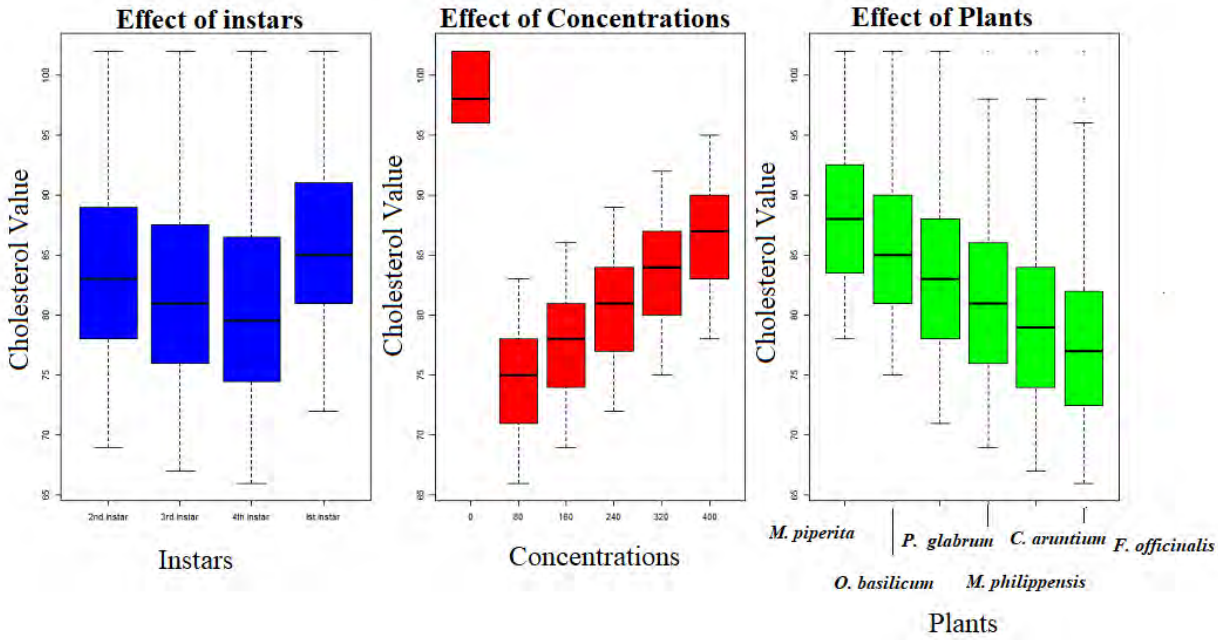


Figure 3.22: A box plot showing effect of instars, concentrations and plants on cholesterol values in ethanol solvent.

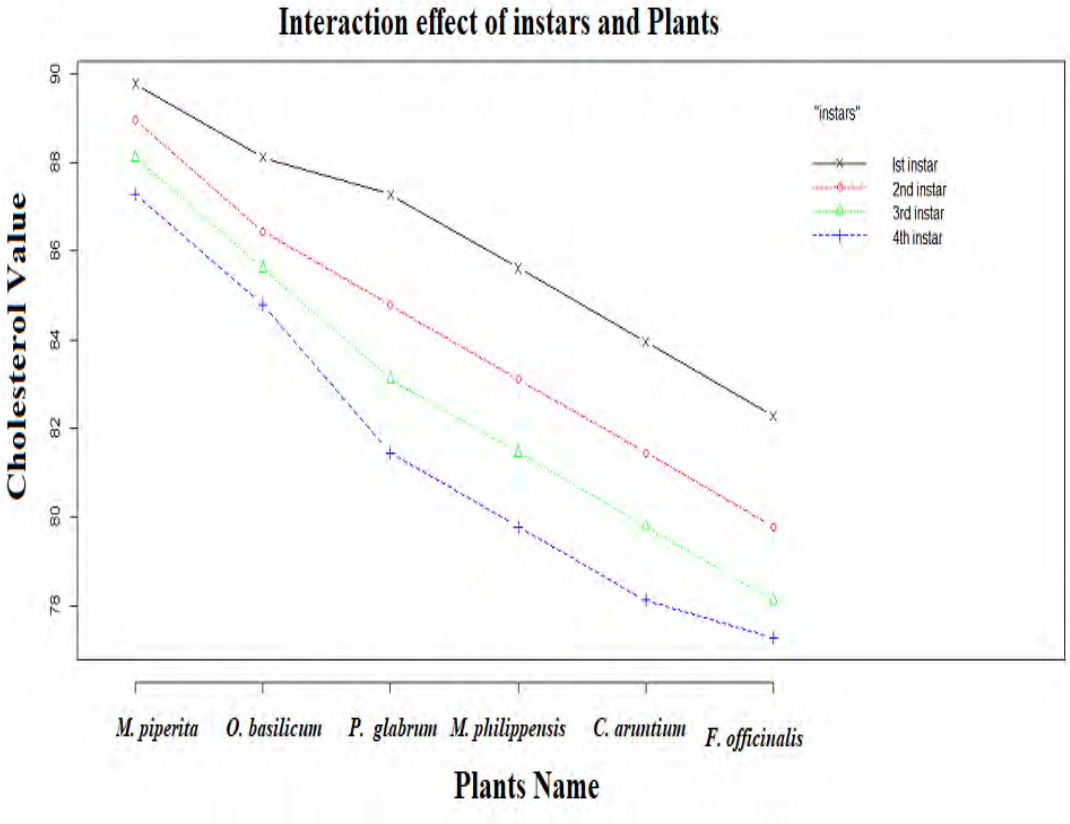


Figure 3.23: Interaction effects of instars and plants on cholesterol value.

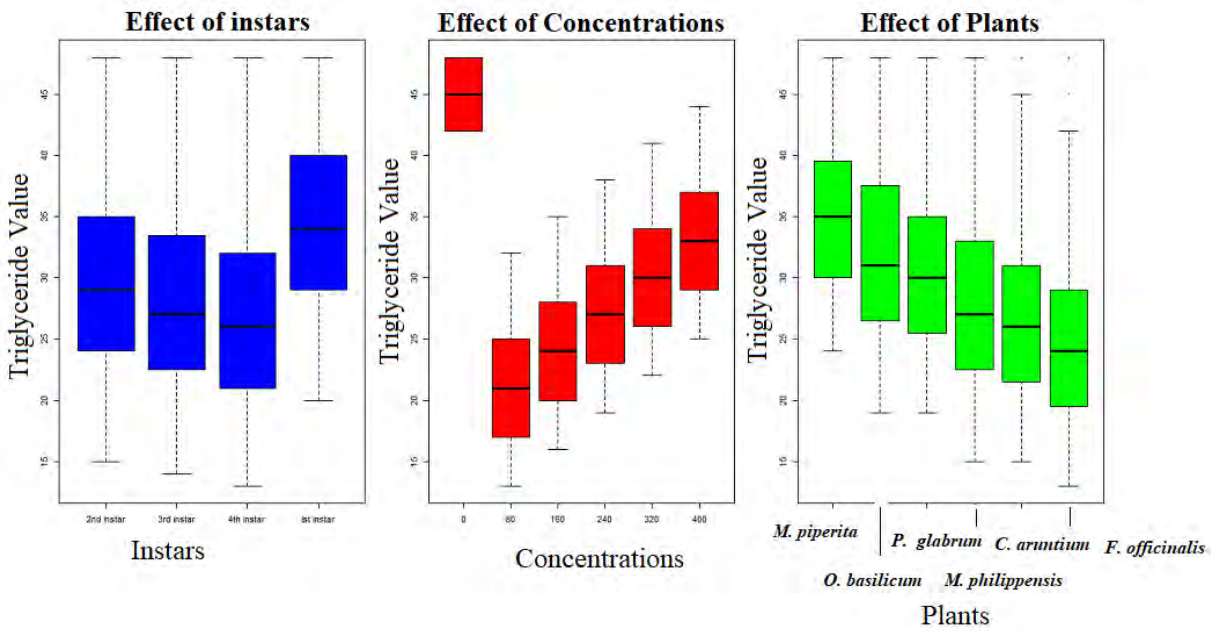


Figure 3.24: A box plot showing effect of instars, concentrations and plants on triglyceride value in ethanol solvent.

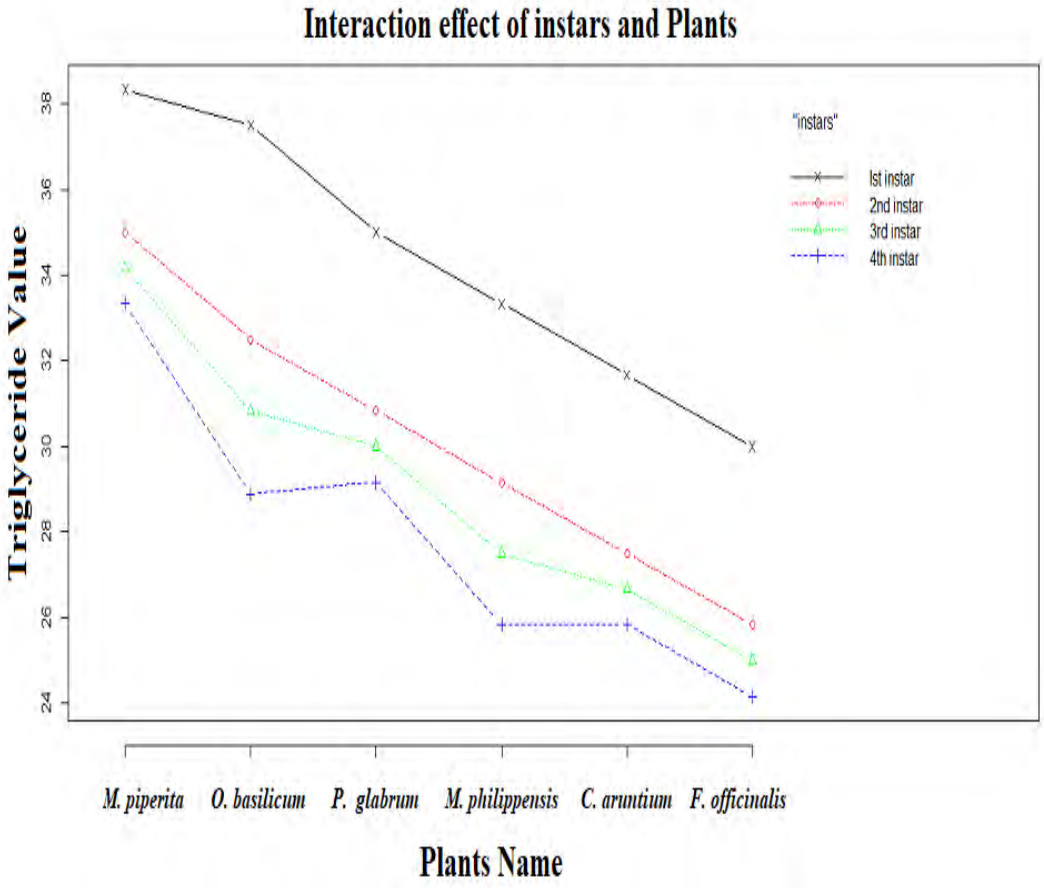


Figure 3.25: Interaction effects of instars and plants on triglyceride value.

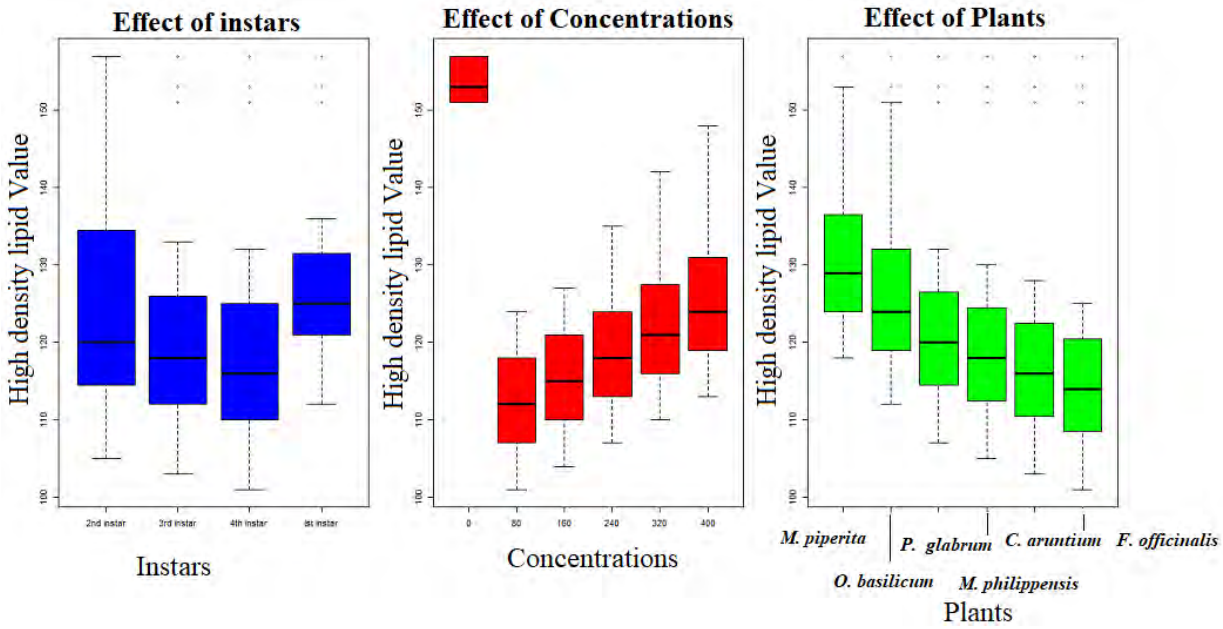


Figure 3.26:A box plot showing effect of instars, concentrations and plants on high density lipid value in ethanol solvent.

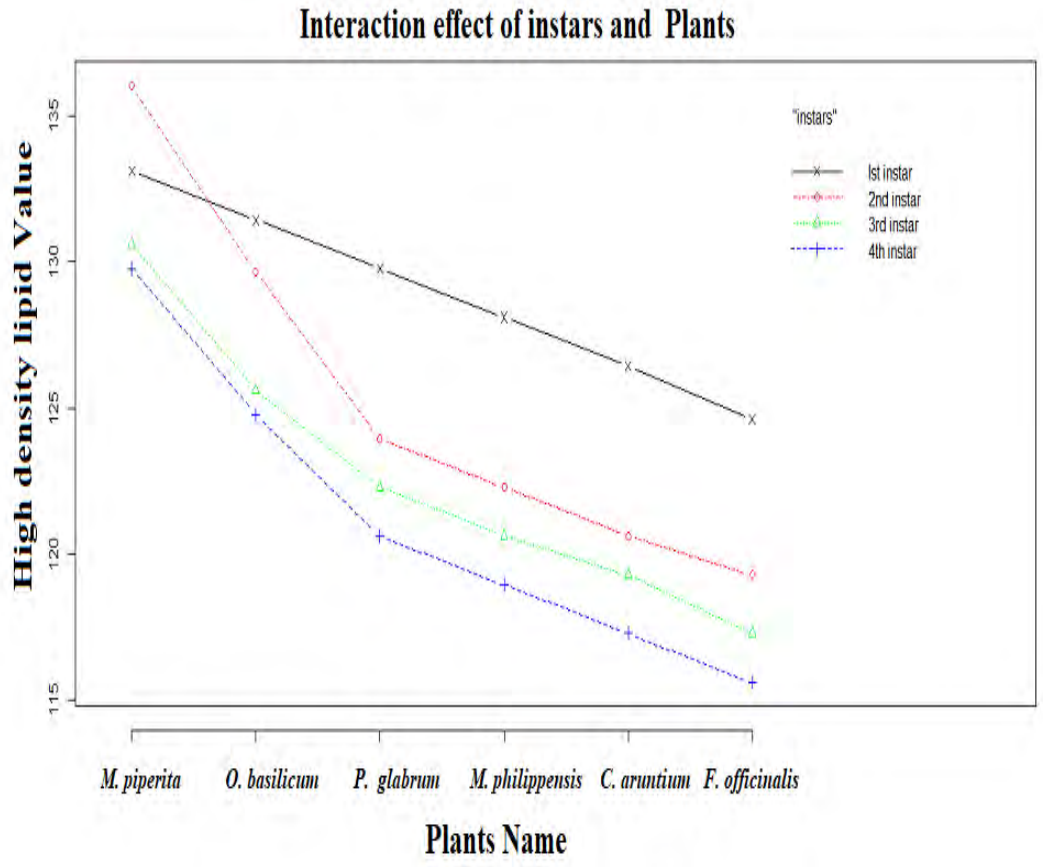


Figure 3.27: Interaction effects of instars and plants on high density lipid value.

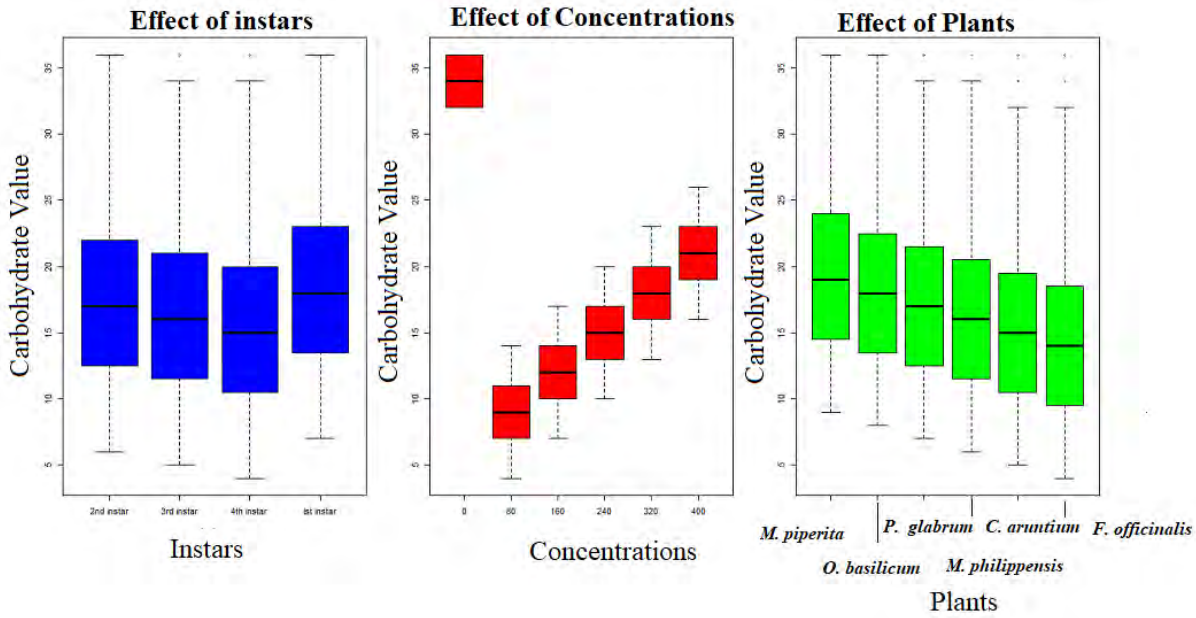


Figure 3.28: A box plot showing effect of instars, concentrations and plants on carbohydrate value in water solvent.

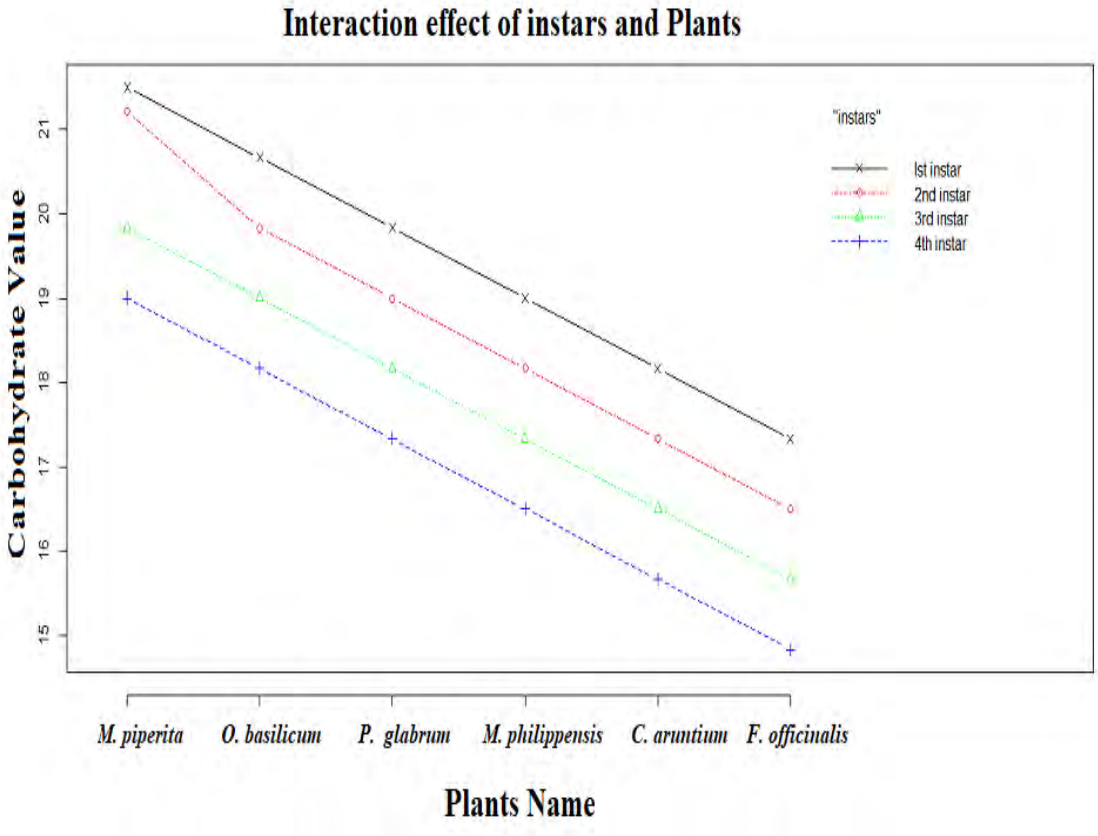


Figure 3.29: Interaction effects of instars and plants on carbohydrate value.

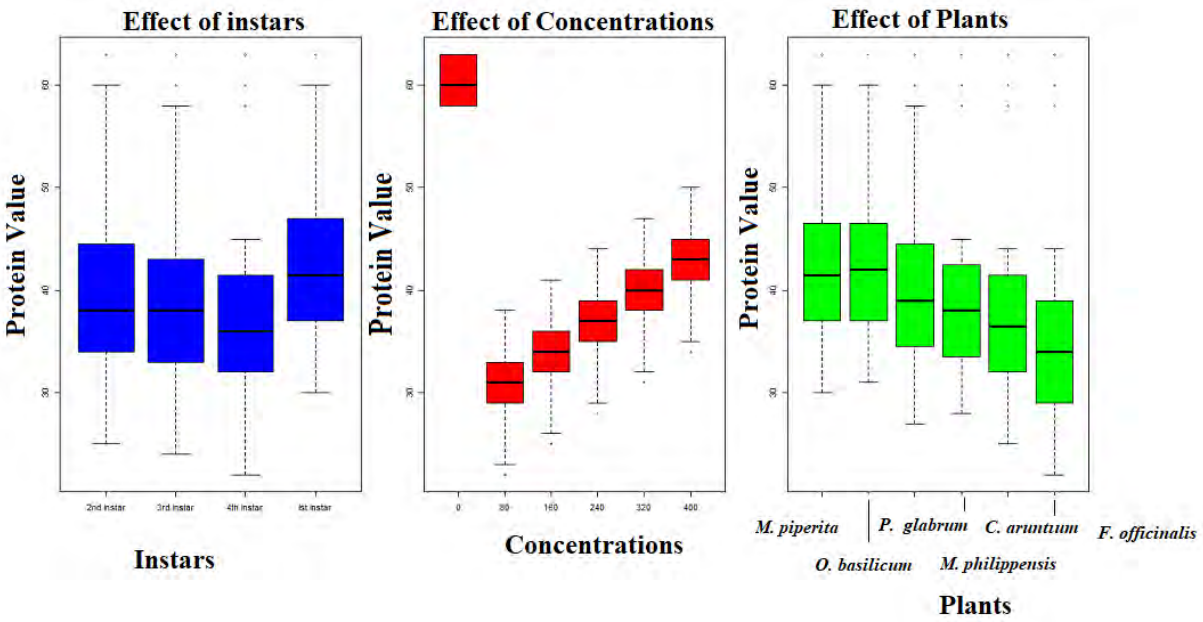


Figure 3.30: A box plot showing effect of instars, concentrations and plants on protein value in water solvent**.**

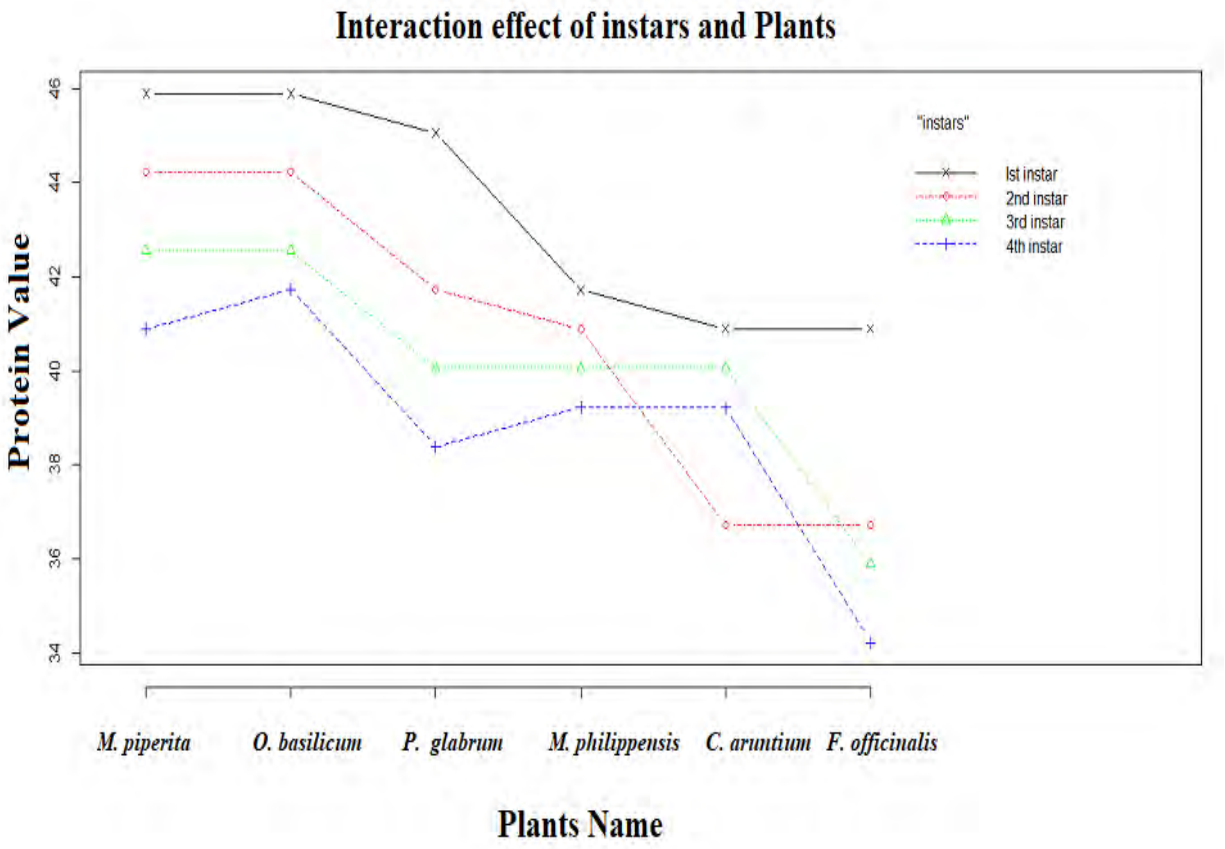


Figure 3.31: Interaction effects of instars and plants on protein values.

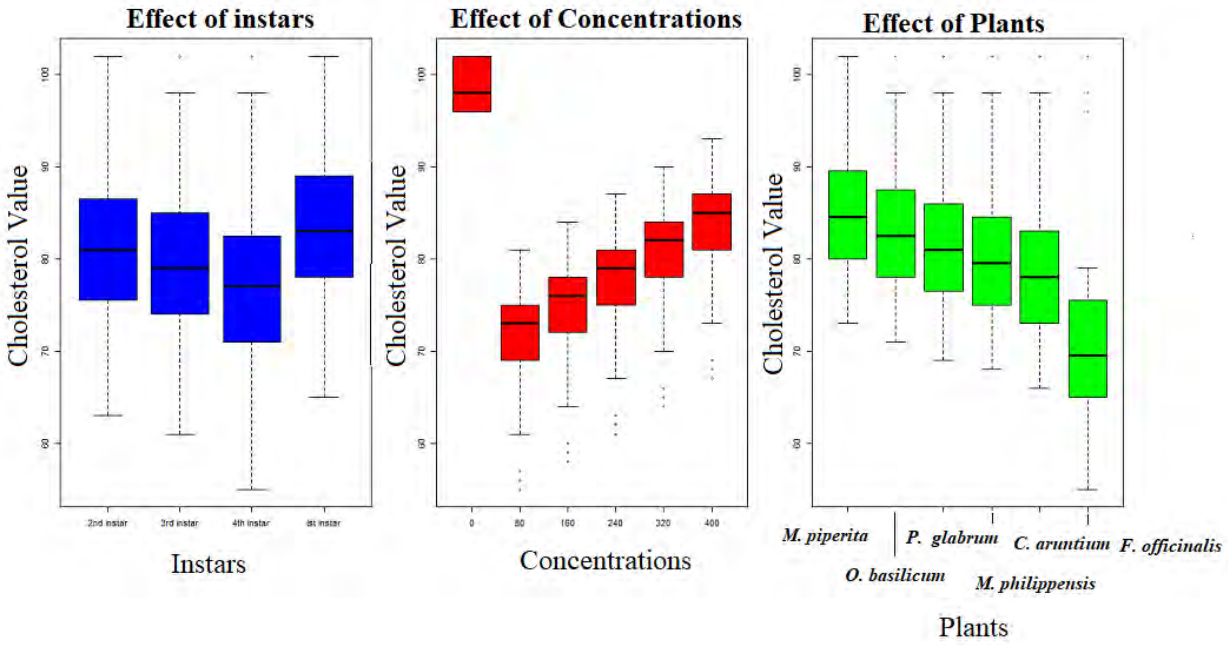


Figure 3.32: A box plot showing effect of instars, concentrations and plants on cholesterol value in water solvent.

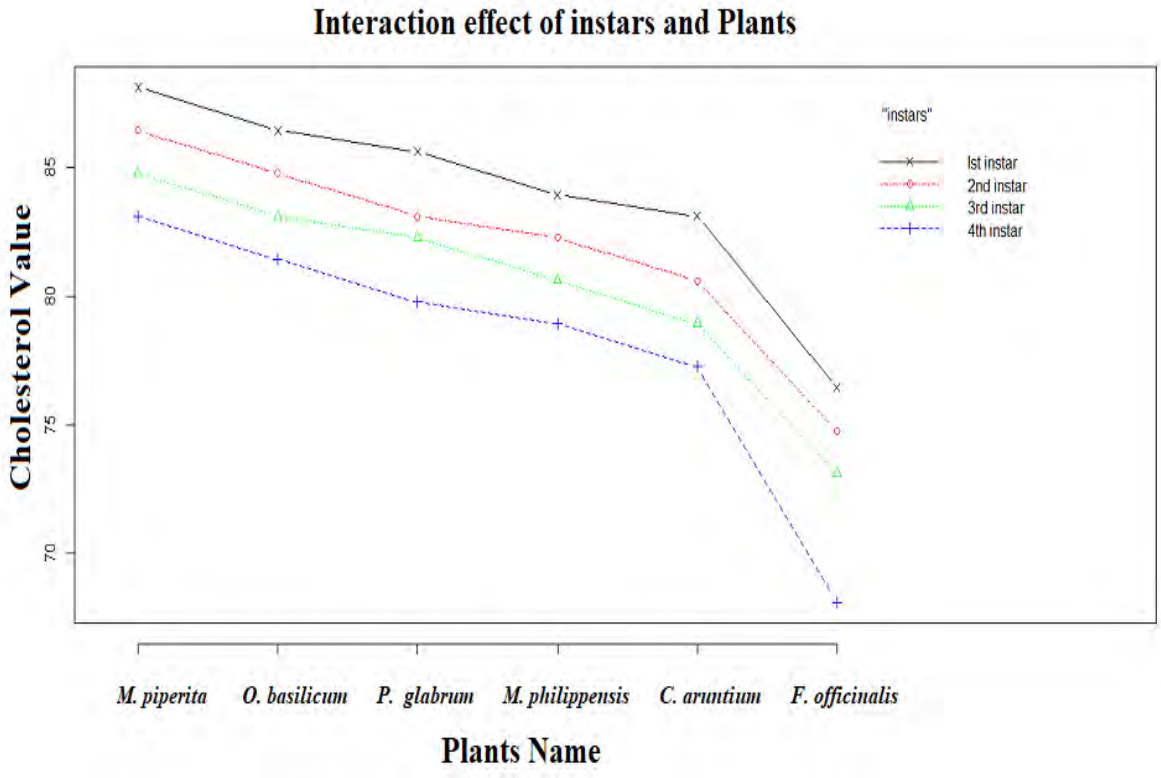


Figure 3.33: Interaction effects of instars and plants on cholesterol value.

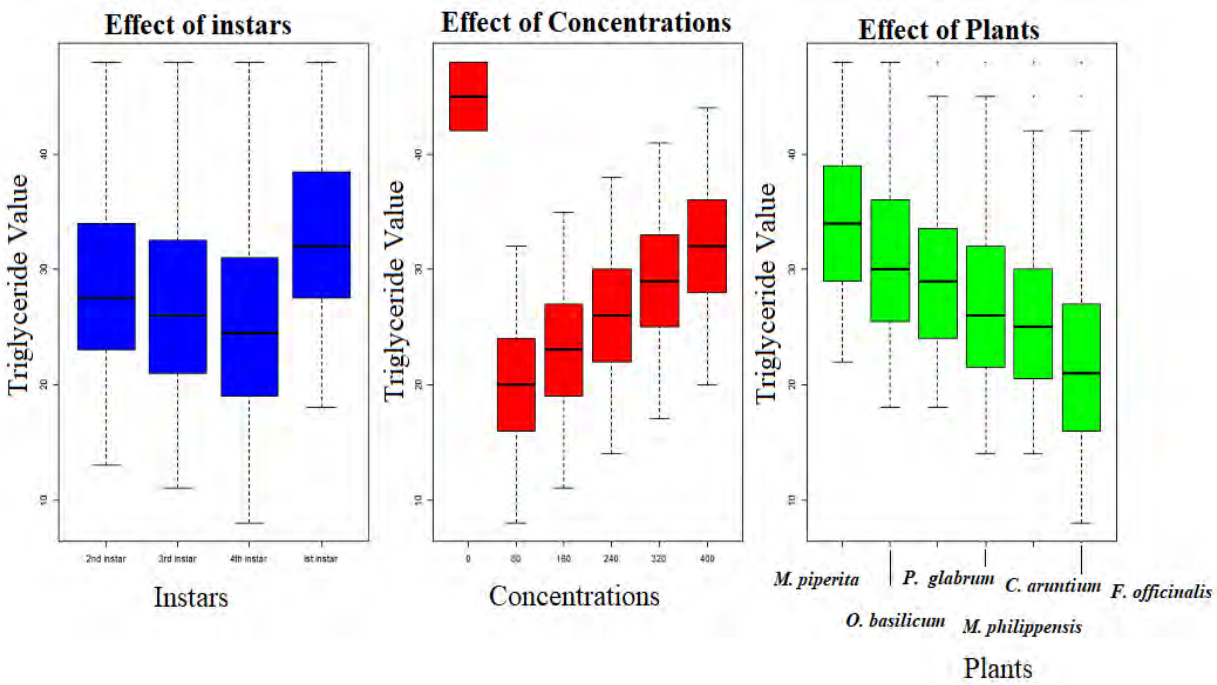


Figure 3.34: A box plot showing effect of instars, concentrations and plants on triglyceride value in water solvent.

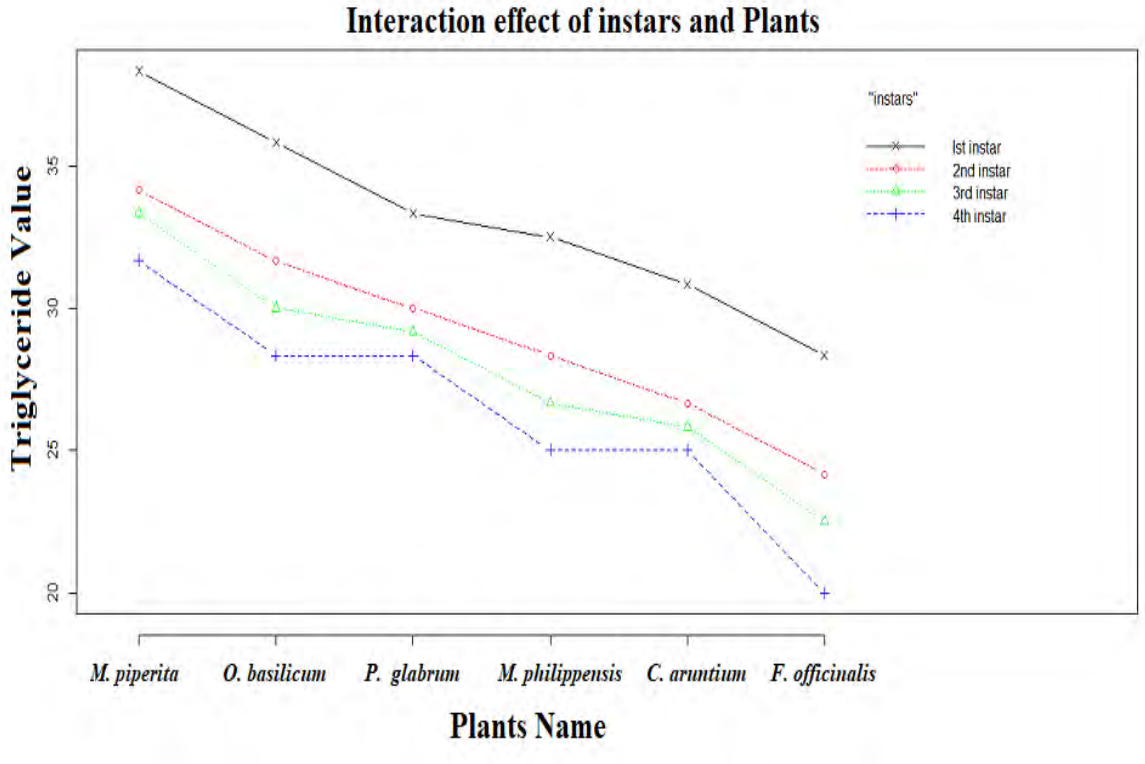


Figure 3.35: Interaction effects of instars and plants on triglyceride value**.**

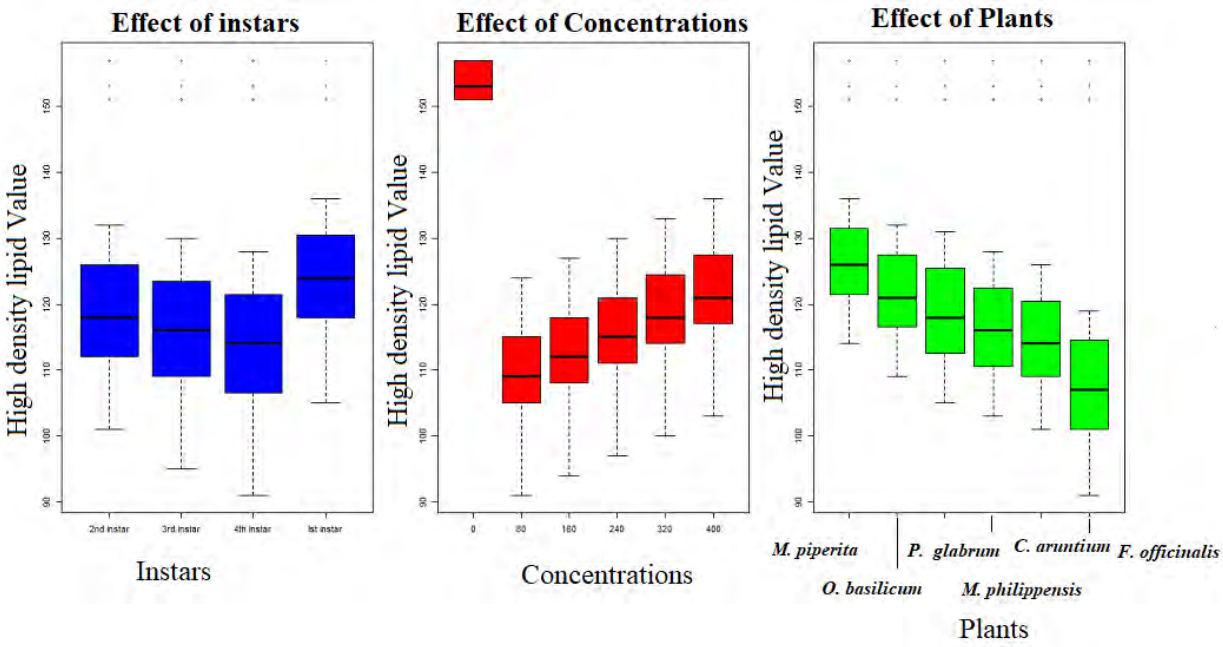


Figure 3.36: A box plot showing effect of instars, concentrations and plants on high density lipid value in water solvent.

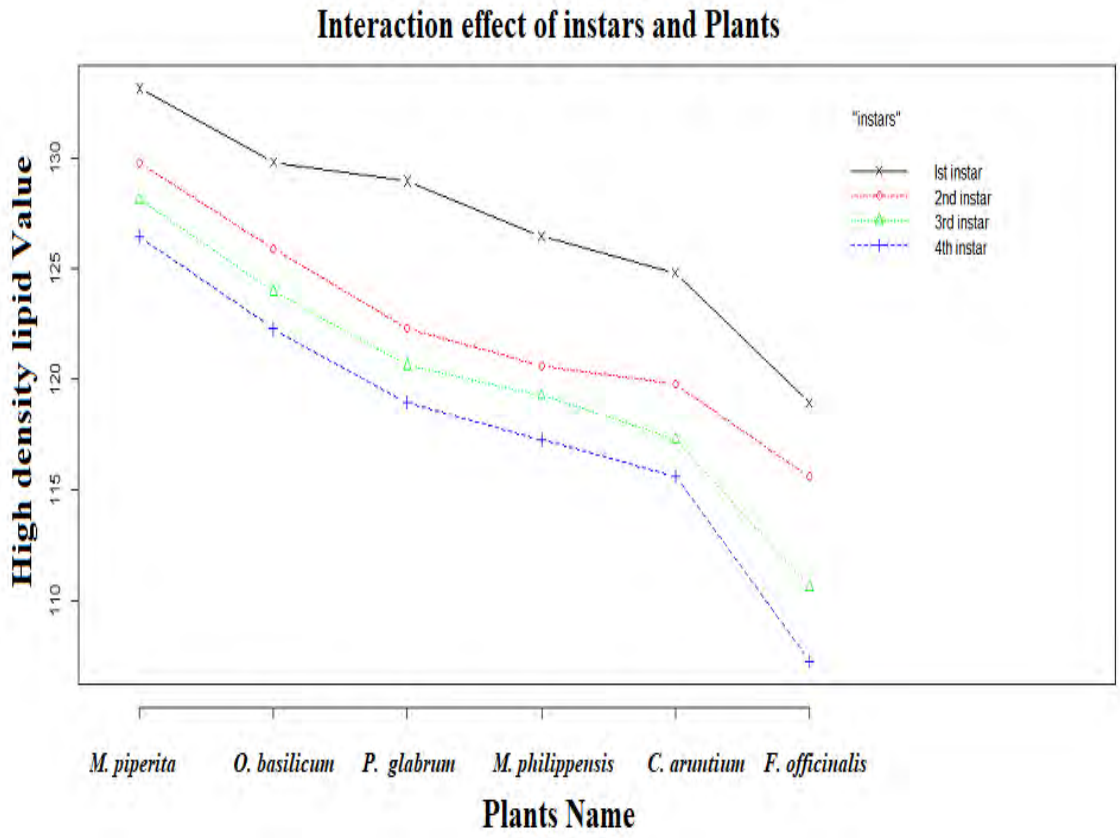


Figure 3.37: Interaction effects of instars and plants on high density lipid values.

Table. 3.4. LC_{50}, LC_{90}, P and χ2-values of plants used for antilarval activity.

Plants Name	Larval instars	LC_{50} (UCL-LCL)		LC_{90} (UCL-LCL)		P- value		(χ^2-value) df=4	
		Ethanol	Water	Ethanol	Water	Ethanol	Water	Ethanol	Water
O. basilicum	Ist instar	381.6 (426.3-347.8)	426.3 (478.9-387.5)	932.1 (1090.1-821.2)	968.8 (1135.6-852.3)	0.17	0.07	6.41	8.65
	2nd instar	286.8 (315.9-262.4)	325.9 (359.8-299.2)	820.5 (947.5-729.5)	848.5 (979.1-754.7)	0.000	0.000	35.96	38.77
	3rd instar	304.3 (335.1-278.9)	340.9 (376.1-313.2)	831.4 (959.2-739.7)	856.8 (987.9-762.6)	0.000	0.000	25.95	29.93
	4th instar	244.5 (264.2-226.2)	272.7 (294.1-253.8)	668.2 (743.5-610.3)	692.9 (771.4-632.6)	0.000	0.000	27.86	32.48
P. glabrum	Ist instar	408.3 (459.5-370.5)	418.5 (466.3-382.7)	971.9 (1144.4-852.3)	928.6 (1076.2-823.4)	0.48	0.29	3.48	4.96
	2nd instar	323.1 (358.6-294.9)	357.3 (396.6-326.9)	881.9 (1029.5-778.1)	893.7 (1038.6-790.9)	0.000	0.000	26.04	28.87
	3rd instar	352.9 (394.1-321.4)	390.3 (436.8-355.5)	920.8 (1080.6-809.4)	941.1 (1102.1-828.4)	0.002	0.001	16.95	19.98
	4th instar	269.26 (291.7 -249.3)	301.6 (326.5-280.4)	715.7 (802.9-649.7)	739.8 (829.7-671.7)	0.000	0.000	20.53	24.25
M. Piperita	Ist instar	290.7 (314.6-270.1)	325.6 (353.4-302.6)	730.4 (818.8-663.5)	768.5 (864.6-696.2)	0.030	0.004	10.72	15.45
	2nd instar	251.4 (274.9-230.2)	284.8 (311.3-262.1)	746.1 (849.1-670.4)	779.4 (888.7-699.2)	0.000	0.000	46.30	52.20
	3rd instar	273.2 (298.9-250.9)	305.8 (334.8-281.7)	774.3 (883.9-693.9)	803.9 (919.1-719.8)	0.000	0.000	35.64	41.24
	4th instar	208.9 (225.9-192.2)	246.9 (265.7-229.5)	599.6 (659.6-552.4)	649.37 (718.1-595.9)	0.000	0.000	36.45	41.03

M. philippensis	Ist instar	458.4 (524.4-411.9)	475.9 (539.9-429.9)	1054.5 (1261.4-914.8)	1022.8 (1207.2-895.4)	0.015	0.079	4.96	1.65
	2nd instar	363.9 (408.6-330.7)	4120.3 (530.5-486.5)	950.1 (1122.9-831.2)	962.8 (1133.1-844.6)	0.012	0.034	6.54	3.58
	3rd instar	401.5 (455.2-362.6)	437.4 (496.8-394.7)	1002.4 (1194.4-872.1)	1017.8 (1209.4-887.1)	0.049	0.013	9.54	12.69
	4th instar	300.1 (326.6-277.56)	334.9 (365.1-310.4)	768.7 (870.1-693.3)	795.2 (900.2-717.1)	0.008	0.002	13.72	16.69
C. Aruntium	Ist instar	510.2 (593.2-453.7)	532.3 (613.5-476.1)	1132.5 (1374.9-972.9)	1100.2 (1317.1-953.9)	0.983	0.97	0.38	0.52
	2nd instar	412.1 (468.9-371.3)	443.5 (503.7-400.4)	1020.6 (1220.6-885.7)	1018.7 (1209.3-888.5)	0.055	0.025	9.25	11.18
	3rd instar	462.7 (536.5-412.1)	500.9 (581.4-445.9)	1111.04 (1353.69 - 952.37)	1122.3 (1361.8-964.5)	0.35	0.177	4.42	6.31
	4th instar	334.9 (367.2-309.1)	366.7 (402.1-338.7)	823.47 (940.21-737.95)	837.9 (954.2-752.2)	0.114	0.051	7.44	9.44
F. officinalis	Ist instar	569.3 (674.9-500.1)	594.4 (704.2-522.3)	1222.6 (1511.4-1038.1)	1221.5 (1504.6-1039.7)	0.68	0.971	2.29	0.52
	2nd instar	524.7 (624.9-459.8)	567.2 (676.2-496.6)	1228.8 (1537.5-1035.7)	1240.1 (1545.2-1048.1)	0.610	0.443	2.69	3.73
	3rd instar	517.8 (608.1-457.7)	559.7 (664.3-491.5)	1172.5 (1440.9-999.4)	1221.4 (1513.9-1035.4)	0.87	0.56	1.23	2.99
	4th instar	449.6 (510.8-405.7)	490.9 (562.4-440.2)	1023.5 (1214.1 -892.9)	1066.5 (1272.9-93.7)	0.976	0.64	0.47	2.51

3.3: ANTIOXIDANT ASSAYS

3.3.1. DPPH radical scavenging method

Free radical scavenging activity of medicinal plants extracts was assessed by DPPH radical assay. A change from purple colour to yellow was detected. The results showed that for the comparison with other plants extract, maximum radical

scavenging activity was present in the ethanolic extract of *M. piperita* (85.43±1.22) at 400ppm. The lowest IC_{50} (8.57) values of *M. piperita* revealed the highest radical scavenging activity. Although water extracts of particular plants exhibited minimum inhibition at the highest IC_{50} values **(Table 4).**

3.3.2. Ferric reducing power

The experimented plants extract exhibited that concentration of extract increases, ferric reducing power also increases. Ethanolic and aqueous leaves extract of *M. piperita* showed the highest reducing power (378.1± 2.5, 275.3±2.2) as compared with other plants extracts **(Table 3).**

3.3.3. Total anti-oxidant capacity

In total antioxidant capacity estimation, Mo (VI) is converted into Mo (V) by the antioxidants of extracts. By estimating total antioxidant capacity, it was observed that ethanolic leaves extracts of *M. piperita* (125.4 ± 3.5µg/mg) and *O. basilicum* (115.6±2.8µg/mg) possess maximum antioxidant activity **(Table 3).**

Table 3.5: Total antioxidant capacity (TAC) and ferric reducing power (FRP) of different solvents extracts of selected plant.

Plant name	TAC (µg AAE/mg DW)		FRP (µg AAE/mg DW)	
	ethanol	water	Ethanol	Water
P. glabrum	105.81±0.95^{b}	94.21±1.52^{b}	354.76±2.34^{b}	221.87±1.64^{c}
O. basilicum	115.63±1.76^{ab}	104.65±0.45^{a}	365.12±0.31^{ab}	254.71±1.54^{ab}
M. philippensis	98.51±0.98^{d}	90.41±1.23^{ab}	344.76±1.85^{c}	164.51±0.54^{d}
C. aruntium	83.21±2.65^{c}	76.21±0.98^{c}	321.87±0.45^{d}	146.31±1.45^{e}
F. officinalis	65.76±1.43^{b}	54.65±2.32^{d}	276.54±0.57^{e}	103.61±1.34^{f}
M. piperita	125.46 ± 3.55^{a}	105.32±4.65^{a}	378.17± 1.04^{a}	275.38±2.23^{a}

Table 3.6: DPPH free radical scavenging activity (%) and IC_{50} values of different extracts of selected plants**.**

Concentrations (µg/ml) % inhibition by *M. piperita* and Vitamin C											IC_{50} µg/	
Plant name	80ppm		160ppm		240ppm		320ppm		400ppm			
	ethanol	Water	ethanol	water	ethanol	Water	ethanol	water	ethanol	Water	Ethanol	Water
M. piperita	63.84± 0.34^{a}	53.48± 0.44^{a}	65.75± 0.14^{a}	55.57± 0.24^{bc}	68.63± 0.42^{a}	62.26± 0.54^{ab}	75.74± 0.75^{ab}	73.48± 0.77^{fg}	85.43±0. 22^{cd}	78.33±0.42^{a}	9.57	11.73
O. basilicum	58.12± 0.21^{ab}	51.24± 0.65^{b}	61.54± 0.11^{ab}	44.24± 0.57^{c}	63.43± 0.71^{ab}	45.56± 0.38^{c}	65.77± 0.26^{cd}	46.11± 0.17^{ab}	68.64±0. 41^{fg}	48.11±0.71^{b}	10.67	12.87
M. philippensis	47.23± 0.45^{b}	41.45± 0.56^{c}	49.66± 0.31^{d}	38.53± 0.98^{d}	51.76± 0.38^{d}	39.45± 0.63^{d}	54.76± 0.63^{d}	41.44± 0.72^{c}	58.48±0. 53^{ab}	44.94±0.94^{b}	15.23	17.43
C. aruntium	36.43± 0.65^{c}	30.11± 0.31^{d}	39.31± 0.51^{e}	25.86± 0.17^{e}	43.65± 0.65^{e}	28.65± 0.72^{e}	45.21± 0.37^{gf}	31.45± 0.13^{d}	49.41±0. 62^{c}	35.28±0.38^{c}	18.34	20.56
F. officinalis	22.89± 0.54^{d}	15.67± 0.66^{e}	25.97± 0.76^{f}	11.32± 0.19^{f}	28.43± 0.38^{f}	14.21± 0.83^{f}	33.98± 0.72^{fg}	16.56± 0.49^{e}	37.66±0. 74^{d}	19.85±0.59^{d}	22.34	25.76
P. glabrum	54.67± 0.43^{ab}	49.87± 0.87^{ab}	57.22± 0.73^{db}	41.97± 0.28^{g}	58.32± 0.59^{c}	42.33± 0.91^{cd}	61.32± 0.84^{bc}	44.78± 0.38^{abc}	64.72±0. 59^{b}	48.93±0.65^{b}	12.45	14.67
Vitamin C	66.23± 1.05^{a}	56.23± 1.25^{ab}	69.26± 1.21^{a}	58.62± 1.29^{abc}	73.65± 1.00^{d}	71.54± 1.11^{d}	87.54± 1.28^{e}	79.23± 1.30^{f}	103±1.0 9^{g}	85.12±1.19^{f}	6.51	8.52

3.4. Cytotoxicity Assay

Evaluation of pharmacological plants provides an attractive and good basis for safe and innovative medical plant development. It is vital to check the cytotoxicity of particular plants to progress protection. For the assessment of toxicity purpose, brine shrimp toxicity bioassay is considered as fast, reliable, and low cost. The ethanolic and water extracts of six plants indicated increased proportionally with the increasing concentrations of the extract and diverse mortality rate of brine shrimp. The inhibitory result of the extract might be due to the toxic compounds present in the crude extracts. The ethanolic extract of *M. piperita* at 4^{th} instar larvae was originated to be the most active at which 50% mortality ($LC_{50=}$ 208.976ppm) and 90% mortality (LC_{90}= 599.576) of brine shrimp nauplii that happened were found to be 208.976 and 599.576ppm, respectively (Table 1). Aqueous extracts of all plants specified less cytotoxicity.

3.5. CHARACTERIZATION:

3.5.1. QUALITATIVE PHYTOCHEMICAL ANALYSIS

The qualitative phytochemical analysis of *M. piperita* showed the occurrence of several bioactive secondary components, which might be responsible for their medicinal characteristics. The leaves extract of *M. piperita* in ethanol were used for the evaluation of phytochemical qualitative analysis being more toxic as compared to *O. basilicum,* showed the presence of medicinally active components like alkaloids, carbohydrates, flavonoid, cardiac glycosides, tannins, reducing sugar, quinones, terpenoids, phenol, amino acid, starch, cellulose, anthocyanin, betacyanin, anthraquinones, and steroid. The leaves extract of *M. piperita* in water was also assessed for phytochemical qualitative analysis indicated the presence of pharmaceutically active constituents like tannins, quinones, phenol, cellulose, alkaloids, carbohydrates, flavonoid, reducing sugar, cardiac glycosides, starch and steroid **(Table and Figure: 3.38)**

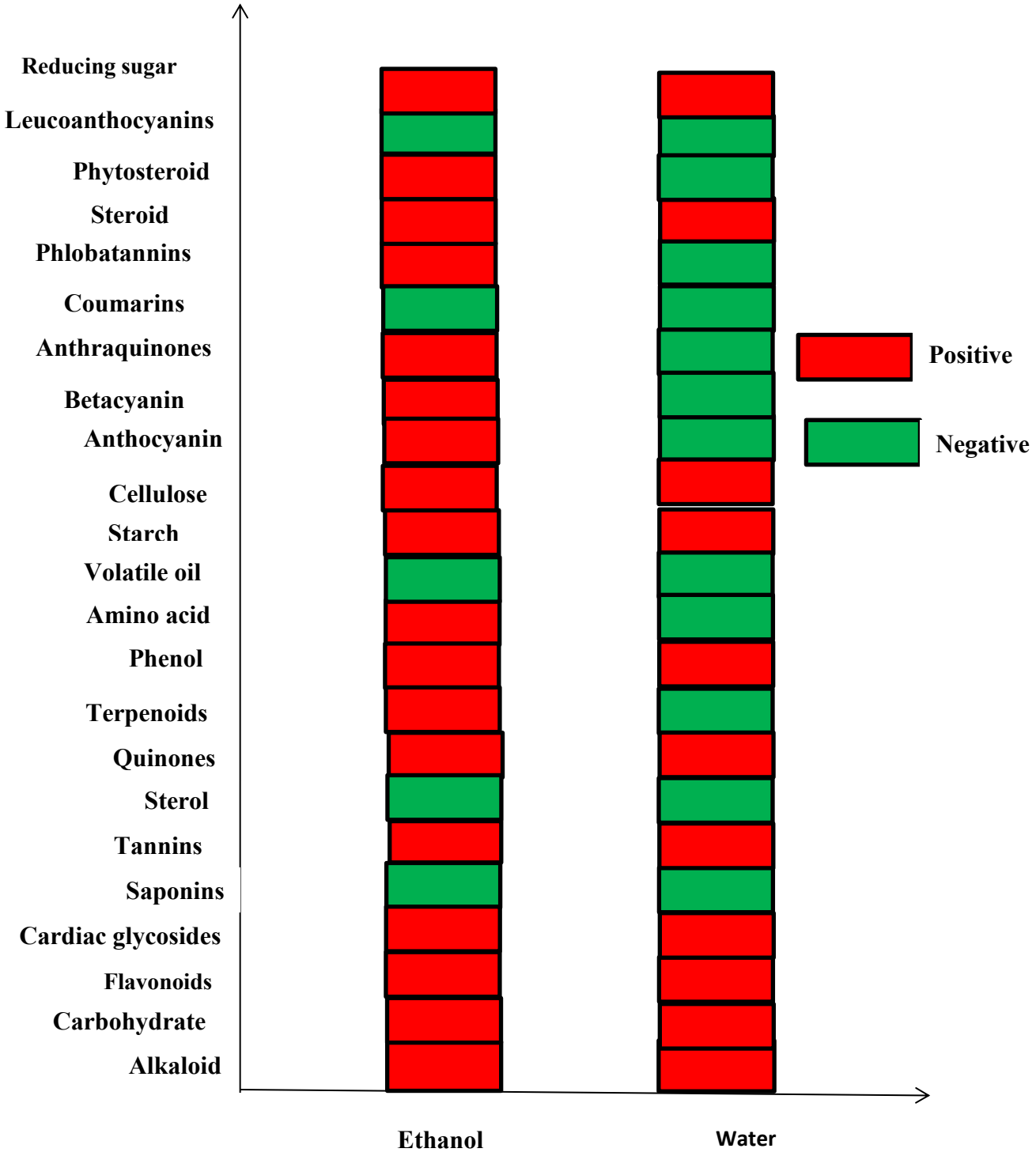


Figure 3.38: Phytochemical components in the ethanolic and aqueous leaves extract of *M. piperita.*

Table 3.7: Qualitative phytochemical analysis of *M. piperita* in ethanolic and waterleaves extracts.

Phytochemical Compounds	**Ethanol**	**Water**
Alkaloids	+	+
Carbohydrates	+	+
Flavonoid	+	+
Cardiac glycosides	+	+
Saponins	-	-
Tannin	+	+
Reducing Sugar	+	+
Sterol	-	-
Quinones	+	+
Terpenoids	+	-
Phenol	+	+
Amino Acid	+	-
Volatile Oil	-	-
Starch	+	+
Cellulose	+	+
Anthocyanin	+	-
BetaCyanin	+	-
Anthraquinones	+	-
Coumarins	-	-
Phlobatannins	+	-
Steroid	+	+
Phytosteroid	+	-
Leucoanthocyanins	-	-

Table 3.8: Quantitative estimation of the secondary metabolites (%) in ethanolic and aqueous extract of *M. piperita.*

Sr. No	Phytochemical constituents	Mean ± STD (ethanol) (µgGAE/mgDW)	Mean ± STD (water) µgGAE/mgDW)
1	Alkaloids	6.23 ± 0.0025^{ab}	5.43 ± 0.0023^{ab}
2	Tannins	5.75 ± 0.0021^{c}	4.70 ± 0.0031^{c}
3	Flavonoids	10.33 ± 0.0011^{a}	8.47 ± 0.0017^{a}
4	Terpenoids	3.95 ± 0.0033^{d}	1.97 ± 0.0029^{g}
5	Proteins	3.35 ± 0.0027^{e}	2.37 ± 0.0025^{e}
6	Steroids	2.66 ± 0.0026^{g}	0.66±0.0021
7	Cardiac Glycosides	5.55 ± 0.0017^{c}	3.55 ± 0.0015^{d}
8	Reducing sugar	4.90 ± 0.0029^{f}	4.21 ± 0.0027^{f}
9	Carbohydrates	3.23 ± 0.0033^{e}	2.68 ± 0.0028^{e}
10	Saponins	3.85 ± 0.0031^{d}	3.67 ± 0.0030^{cd}
11	Phenols	7.45 ± 0.0020^{b}	$6.\ 44\pm0.0018^{b}$

It showed the degree of abundance of these phytochemicals in the percentage of the extract is as follows; 3.23±0.0033 of carbohydrates, 5.75±0.0031 of tannins, 3.35 ± 0.0027of Proteins, 4.90 ± 0.0029 of reducing sugar, 3.85 ± 0.0031of saponins, 2.66 ± 0.0026 of steroids, 10.33 ± 0.0011of flavonoids, 6.23 ± 0.0025 of alkaloids, 5.55 ± 0.0017 of cardiac glycosides, 3.95 ± 0.0033 of terpenoids and 7.45 ± 0.0020 (µgGAE/mgDW) of phenol. The phytochemical, with the maximum quantity, like phenol, followed by alkaloids, tannins, reducing sugar, saponins, cardiac glycosides, flavonoids, carbohydrates, proteins, terpenoids, and steroid respectively.

It showed the degree of abundance of these phytochemicals in the percentage of the extract is as follows; 2.68±0.0028 of carbohydrates, 4.70±0.0031 of tannins, 2.37±0.0025 of Proteins, 4.21±0.0027 of reducing sugar, 3.67±0.0030 of saponins, 0.66±0.0021 of steroid, 8.47±0.0017 of flavonoids, 5.43± 0.0023 of alkaloid, 3.55±0.0015 of cardiac glycoside, 1.97±0.0029 of terpenoids and 6.44±0.0018 (µgGAE/mgDW) phenol. The phytochemical with the maximum quantity was phenol, followed by alkaloids, tannins, reducing sugar, saponins, cardiac glycosides, flavonoids, carbohydrates, proteins, terpenoids, and steroids respectively (Table 3.8).

3.6. UV-VIS SPECTROSCOPY

Table 3.9: UV-VIS peak values of extract of *M. piperita*.

Sr. No	Wavelength (nm)	Absorbance
1	209.509	2.338
2	282.814	0.796

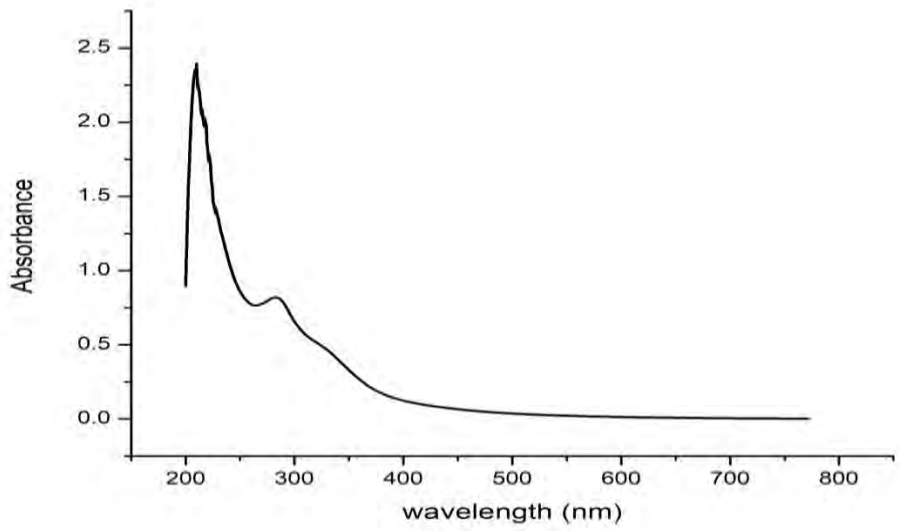


Figure 3.38: UV-Visible spectrum of ethanol extract of *M. piperita.*

The UV-VIS analysis was performed for the identification of phytoconstituents present in ethanolic extract of *M. piperita.* The qualitative UV-VIS spectrum profile was selected at the wavelength of 200 to 800nm due to the sharpness of the peaks and proper baseline. The UV-VIS spectrum showed two peaks at 209.509 and 282.814nm with the absorption of 2.338 and 0.796 respectively (Figure and table:1). The spectrum of *M. piperita* shows two peaks at 209.509 and 282.814nm. This confirms the presence of unsaturated groups and heteroatoms such as N, S, and O.

3.7: Fourier Transform infrared spectroscopy (FT-IR)

Table 3.10: FT-IR spectral peak values and functional groups obtained from ethanolic leaves extract of *M. piperita.*

Peak No	Characteristic Absorption (cm-1)	Types of vibration	Functional group	Intensity
1	3314.47	O-H stretch	Alcohol	strong
2	2943.50	C-H stretch	Alkanes	Weak
3	2831.44	C-H stretch	Aldehyde	variable
4	1448.74	C=C stretch	Aromatic ring	Weak
5	1088.41	C-F stretch	Fluoro compound	strong
6	1023.34	C-O-C stretch	Ether linkage	strong
7	880.84	C-H bend/ S-OR	1,2,4trisubstituted/Ester	strong
8	607.36	C-Br stretch	Halo compound	strong
9	584.06	C-I stretch	Halo compound	strong
10	568.79	C-Cl stretch	Halo compound	strong
11	554.94	C-X stretch	Halo compound	strong
12	542.87	C-X stretch	Halo compound	strong
13	535.31	C-X stretch	Halo compound	strong
14	526.95	C-X stretch	Halo compound	strong

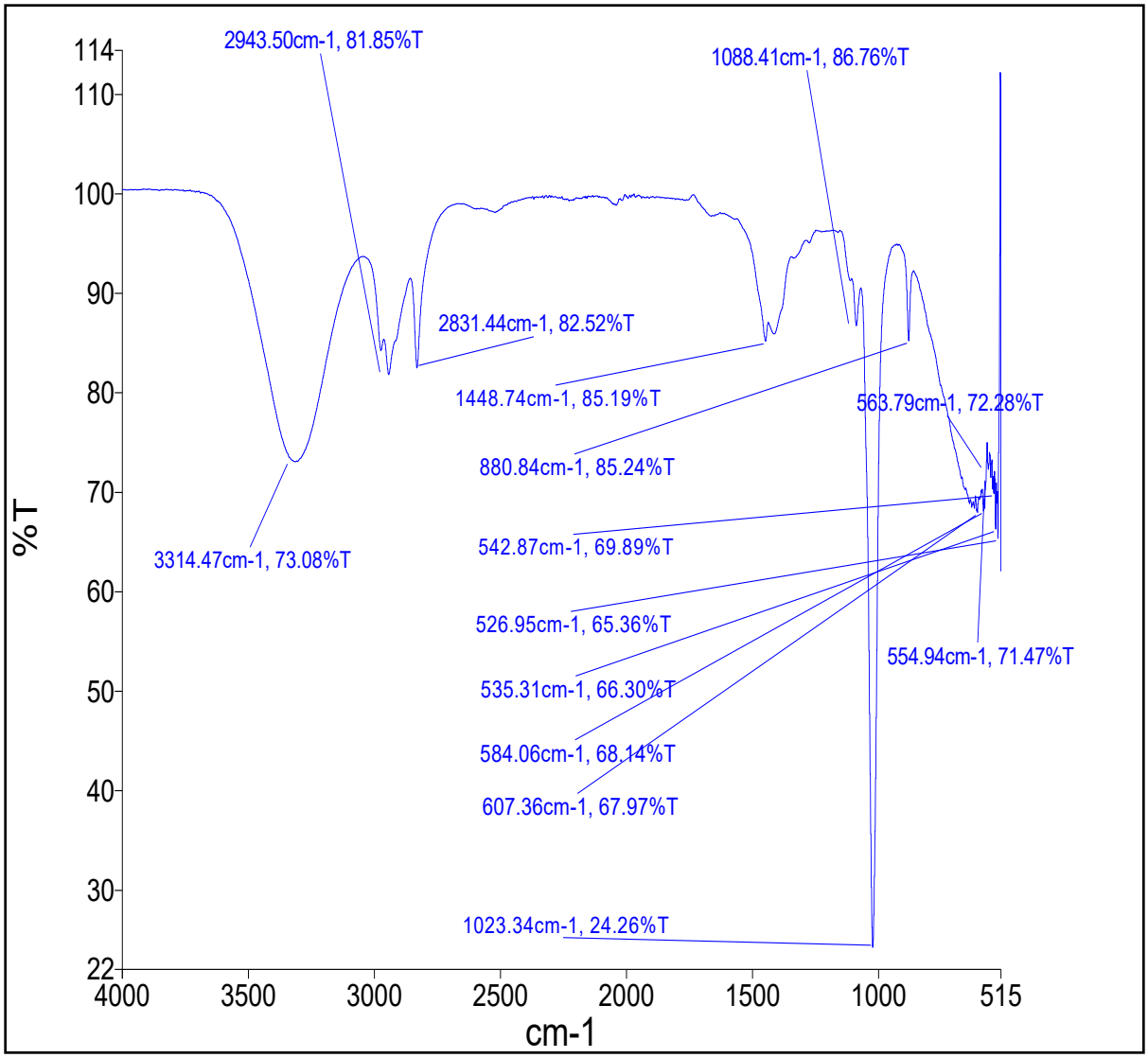
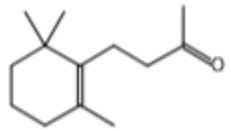
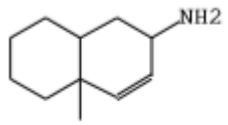
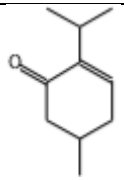


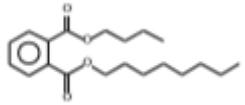
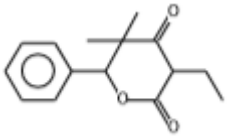
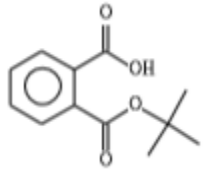
Figure 3.39: FT-IR spectra of ethanol extract of *M. piperita*.

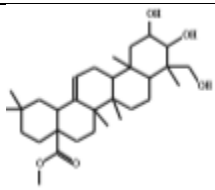
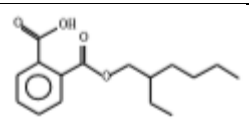
The FT-IR spectrum was used to detect the functional group of the active constituents centered on the peak value in the area of infrared radiation. The results of FT-IR analysis confirmed the presence of alcohols, alkanes, aldehyde, aromatic ring, ether linkage, ester, and halo- compounds (Figure and Table 2). These were confirmed by FT-IR spectra study that revealed the presence of the functional groups: - O-H, C-H, C==C, C-F, C-O-C, C-Br, and C-I.

3.8. GC-MS Analysis:

Table 3.11: GC-MS spectral analysis of ethanolic extract of *M. piperita.*

Peak No	Retention time	Name of the compound IUPAC/Common name	Molecular formula	Molecular weight	Peak area %	Compound nature and uses	Biological activity	Structure
1	5.755	2-Butanone, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-	$C_{13}H24O$	194	7.75	Terpenes Flavouring Agent	Antimicrobial, anticancer.	
2	6.013	2-Napthalenamine ,1,2,4a,5,6,7,8,8a-octahydro-4a-methyl	$C_{11}H_{19}N$	165	7.29	insecticide and pest repellent	Antibacterial, Anti-inflammatory, Analgestic, antimicrobial.	
3	6.694	2-Cyclohexen-1-one,5-methyl-2-(1-methylethyl)	$C_{10}H_{16}O$	152	7.45	Monoterpene ketone, production of synthetic men thol	Antimicrobial, antioxidant.	

4	7.809	1,2-Benzene dicarboxylicacid,	$C_{20}H_{30}O_4$	334	10.36	Plasticizer compound, lubricant	Antibacterial	
5	8.051	8-Octadecen-1-ol acetate	$C_{20}H_{38}O_2$	310	9.64	Oleic alcohol, cosmetic agent	Antioxidant	
6	8.117	Pentadecanal	$C_{15}H_{30}O$	226	7.48	2,3-saturated fatty aldehyde, fragrance use	Antioxidant	
7	8.667	2H-Pyran-2,4(3H)-dione,3-ethyl-5,5-dimethyl-6-phenyl	$C_{18}H18O_3$	246	13.15	Heterocyclic, nonaromatic compound, treatment of dental infections	Antifungal, phytotoxic	
8	8.730	t-Butyl hydrogen phthalate	$C_{12}H_{14}O_4$	222	13.99	Ester, Plasticizer	Antioxidant	

9	8.904	Olean-12-en-28-oic acid, 2. beta., 3.beta.,23-trihydroxy-methyl ester	$C_{31}H_{50}O_5$	502	10.74	Ester compound, emulsifying or solubilizing agent	Anticancer, antioxidant	
10	9.705	Mono-(2-ethylhexyl) ester	$C_{16}H_{22}O_4$	278	12.45	Plasticizer compound/ Phthalate ester, stabilizers and lubricants	Antimicrobial, Mutagenic	

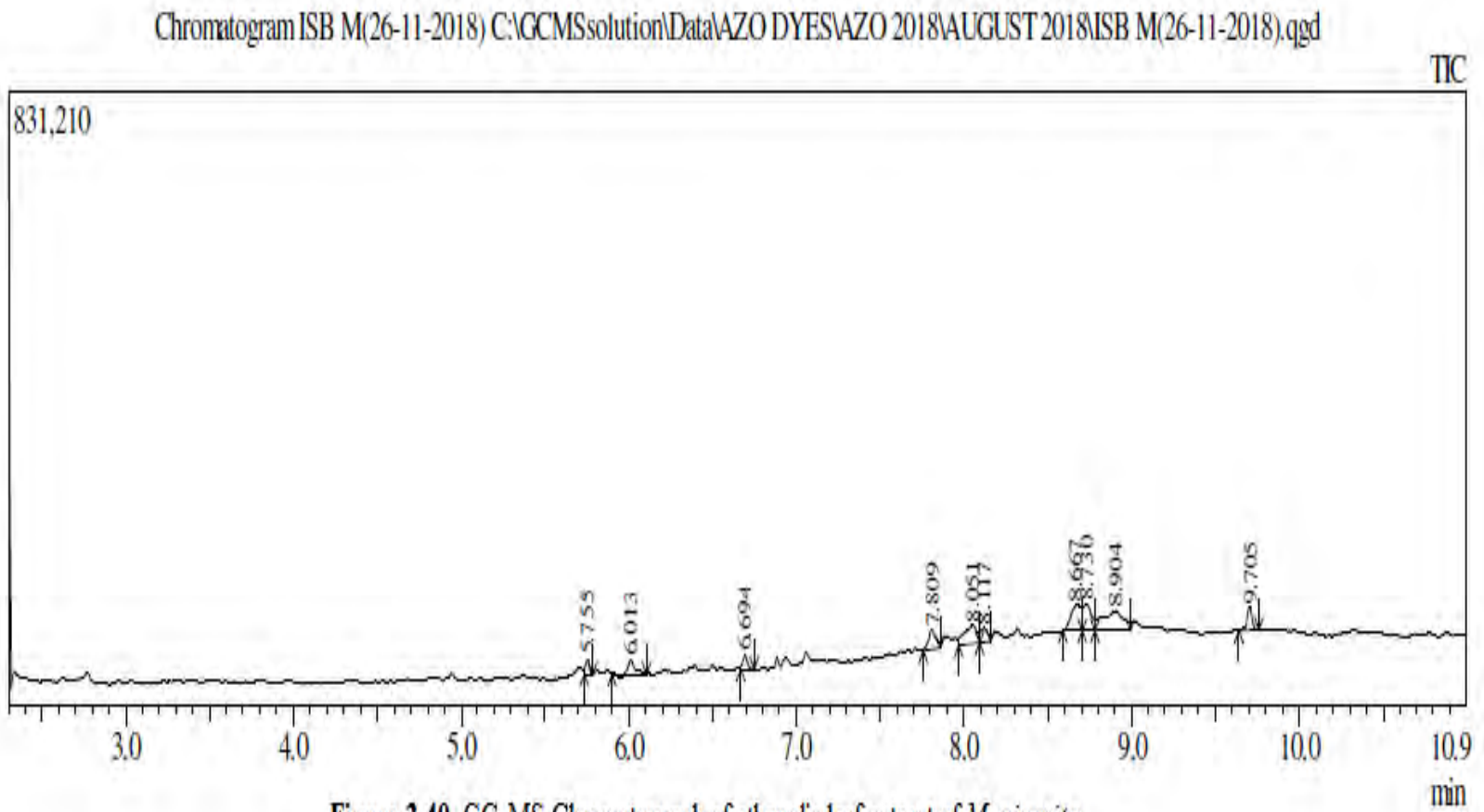


Figure 3.40. GC-MS Chromatograph of ethanolic leaf extract of *M. piperita*.

GC-MS is the convenient method to detect the components of volatile matter, long and branched-chain hydrocarbons, ester, ketones, carboxylic acid, alcohol, aldehyde, terpenes, substituted alkenes, phenolic compounds, heterocyclic nonaromatic compound, plasticizer, and monoterpenes Ketone. Retention time, peak area%, molecular formula, molecular weight, compound nature, uses, and biological activity were used for the identification of phytochemical components (Table 4). From the GC-MS study of *M. piperita* leaves the presence of twenty-one compounds (phytochemical constituents) was shown the medicinal value of the plant (Table 4). The mass spectra of these compounds were present.

The GC-MS chromatograms of ethanolic leaves extract of *M. piperita* revealed ten peaks at different retention times and confirm the presence of ten compounds **(Table 3.11).** T-Butyl hydrogen phthalate **(13.99%),** 2H-Pyran-2,4(3H)-dione,3-ethyl-5,5-dimethyl -6-phenyl **(13.15%),** Mono-(2-ethylhexyl) ester **(12.45%),** Olean-12-en-28-oic acid, 2.beta., 3.beta.,23-trihydroxy-methyl ester **(10.74%),** 1,2-Benzene dicarboxylic acid **(10.36%)** , and 8-Octadecen-1-ol acetate **(9.64%)** are the major components of fraction 4. Other bioactive compounds are present in minor quantity include 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)- **(7.75%),** 2-Butanone, and Pentadecanal **(7.48%),** 2-Cyclohexen-1-one,5-methyl-2-(1-methylethyl) **(7.45%),** 2-Napthalenamine ,1,2,4a,5,6,7,8,8a-octahydro-4a-methyl **(7.29%).** The GC-MS chromatogram of fraction 7 is represented in **figure 3.40**

3.9: Molecular docking

The three-dimensional crystal structure of NS3 protease is designated for docking. The ten compounds from *M. piperita* were evaluated based on binding affinity with NS3 to justify the antilarval potency. The best docked of ligand-protein complex determined by best binding affinity values, number of hydrogen bonds, and also residues involved in hydrogen bonding. Out of all compounds, 1, 2-Benzenedicarboxylic acid and 3-ethyl-5, 5-dimethyl -6-phenyl showed significant results with the highest E value (-6.9107 and -6.8340) kcal/mol respectively.

The 2D and 3D interaction diagram showing hydrogen bonds of compound 1,2-Benzenedicarboxylic acid and 3-ethyl-5,5-dimethyl -6-phenyl with NS3 protease. The important contact residues for the docked legends were (Leu, Ala, Gly, Val, lle, trp, Asn, lys, Thr) and (Lys, Val, Thr, lle, Asp, Asn, Ala, Met, Trp, Leu, Gly,) for 1,2-

Benzenedicarboxylic acid and 3-ethyl-5,5-dimethyl -6-phenyl respectively. Both compounds with the NS3 protease specify their interaction with the protein through diverse chemical forces, i.e., bonds. Four hydrogen bonds are seemed to be elaborate; one is between the acidic hydrogen of the compound and electronegative part of the protein residual, two hydrogen bonds between the electronegative oxygen and positive part of the residual protein, and a fourth hydrogen bond between the electronegative nitrogen of the compound and residual protein. Other than hydrogen bonding, many other important electrochemical forces are also involved, i.e., Van der Waals, carbon-hydrogen interaction, covalent bond, etc. Covalent inhibition is an irreversible inhibition process. The irreversible inhibitors act together with their specific targets in a time-dependent manner and the reaction proceeds to end before proceeding into equilibrium. This study indicates that a strong interaction based on electrochemical interaction forces is possible between the 1, 2-Benzenedicarboxylic acid, 3-ethyl-5,5-dimethyl -6-phenyl, and the protein. This showed that compounds 1, 2-Benzenedicarboxylic acid, and 3-ethyl-5,5-dimethyl -6-phenyl bound well to the NS3 receptor. Molecular docking has contributed a lot in the identification of novel small drug-like scaffolds showing the highest binding selectivity and affinity for the target. Out of ten compounds, only two compounds 1, 2-Benzenedicarboxylic acid and 3-ethyl-5, 5-dimethyl -6-phenyl i.e. showed significant results **(Figure 8 and 9).**

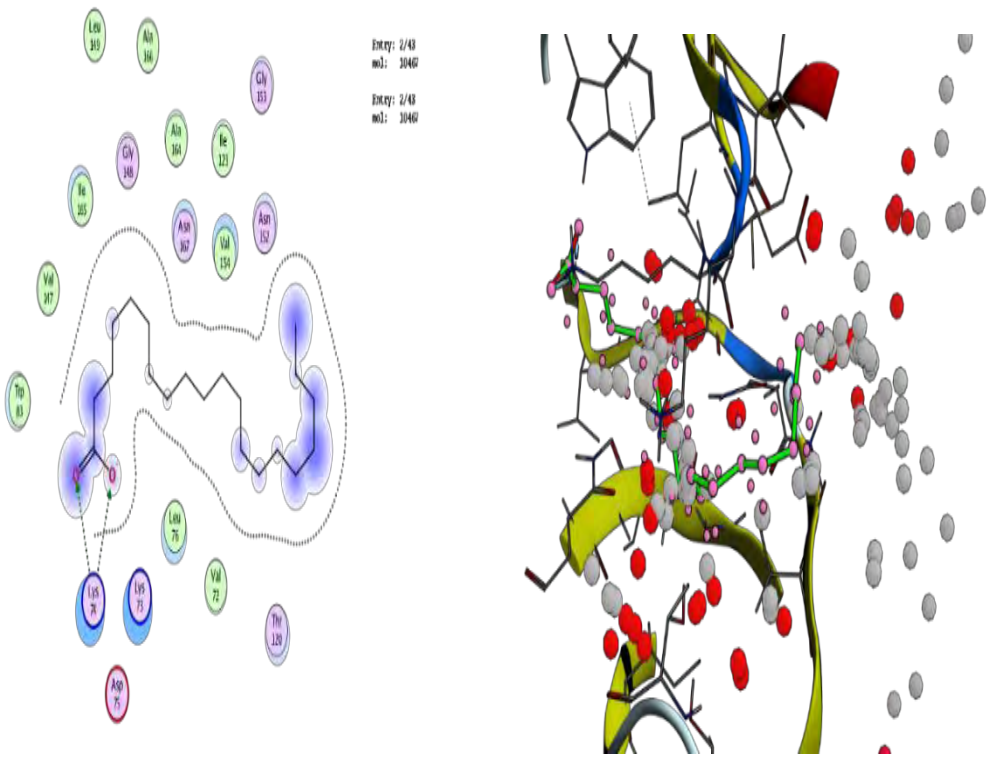


Figure 3.41: 2D and 3D interaction of compound (1, 2-Benzenedicarboxylic acid) with the target protein (PDB ID: 2FOM).

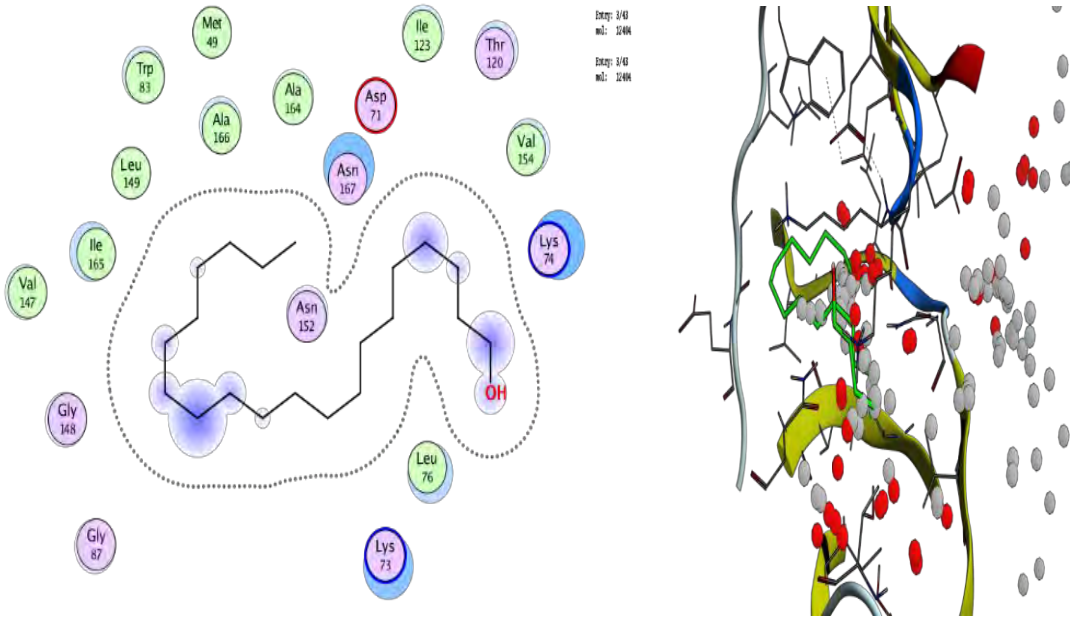


Figure 3.42: 2D and 3D interaction of compound (3-ethyl-5,5-dimethyl -6-phenyl) with the target protein (PDB ID: 2FOM).

DISCUSSION

Mosquitoes are effective vectors for the spread of variability of diseases in humans and other animals like malaria, filariasis, dengue fever, yellow fever, Japanese encephalitis and virus infections, etc. universally (Bagavan *et al*., 2009).

Currently, vector control programs are concentrating on the eradication of mosquitoes at larval stages using natural products (plant derivatives) as an alternative to synthetic insecticides. Since environmental concerns and the least resistance by mosquito larvae are paramount targets along with viable habitat conditions. The desirable point in targeting mosquito larvae is that they are unable to outflow from target/breeding sites till the adult stage. Also, it decreases the quantity of pesticides needs as required for the adult stage more ever the aerial application is non-specific (Tennyson *et al*., 2012).

The therapeutic model of crude plant extracts is often recognized for the multifaceted invention of toxic and other dynamic constituents. For this purpose, primary screening is a good suggestion to investigate the potential and active compounds in crude plant extracts against mosquito larvae. In the current study ethanolic extract of *M. piperita* was significantly ($P<0.05$) active to induce the highest average mortality in each larval stage of *Cx. quinquefasciatus* as compared to other experimental plants. Although the water extract also showed significant ($P<0.05$) potential against mosquito larvae, but it is lesser than ethanolic extracts This larvicidal activity may be attributed due to the higher solubility of mint into ethanol and the presence of flavonoids and anthraquinones. It has been reported earlier that flavonoids induced significant mortality against III and IV larval instars of *Ae. aegypti* and *An. stephensi* isolated from the crude extract of *Vitex negundo* and *Andrographis paniculata* (Gautam *et al*., 2013).

Furthermore, aqueous crude extracts of *Hemidesmus indicus, Gymnema Sylvestre,* and *Eclipta prostrata* were induced 100 % mortality in *Cx. quinquefasciatus* mosquito larvae at 5% concentration due to the presence of flavonoids and phenols (Khanna and Kannabiran, 2007). In a study, 31 plant extracts of Europe and Asiatic origin were studied to determine larvicidal potential against larvae of *Cx. quinquefasciatus* (Pavela, 2008). In another study, petroleum ether extracts of sixty-three plants were screened for larvicidal potential besides larvae

of *Cx. quinquefasciatus, An. stephensi, and Ae. aegypti among* which six plants were well-known as strong larvicides due to the presence of flavonoids (Sakthivadivel and Daniel, 2008).

The treatment of artificial substances for extinction and control of insects mainly mosquitoes grows conflict against chemicals. Then, the progress of novel approaches is significant. Bio-pesticides are less toxic, destructive and cause fewer environmental contaminations than chemical pesticides (Knio *et al*., 2008). Plants are rich in bioactive compounds because they have herbal medicine and pesticide properties. The mixtures ready from diverse fragments of plants are used against mosquitoes are operative. Moreover, the natural environment disturbs by synthetic chemicals. These compounds decrease the resistance of living beings act as an immunosuppressant (De Omena *et al*., 2007).

Botanical pesticides are also environment friendly and are exposed to be effective against mosquitoes. For the control of mosquito larvae, the sap of *C. procera* was originated effective as identified by the rate of mortalities (Kaushik and Saini, 2008). The causes of high mortalities in larvae of *An. stephensi,* extract of *Citrullus vulgaris* have been used. In terms of mortalities and larvicidal potential of botanical extracts, the present-day conclusions are equivalent to the mentioned study. More than 2000 plant species with insecticidal activity are already identified (Fan *et al*., 2011).

The larvicidal action of some legumes was experienced against *A. aegypti* and *Cx. pipiens.* As well as the toxicity of *Mentha pulegium* (lipped) was confirmed on mosquito larvae. The larvicidal activity of the extracts from aromatic plants was also reported (Bhagat and Kulkarni, 2012). Several plant species have been tested as aqueous extracts against *Cx. pipiens* such as *Myrtus communis*, *Eucalyptus camaldulensis*, and the *Neriumo leander*, *Azadirachta indica*, *T. vulgaris* used as an aqueous extract or in the form of essential oils was found to present larvicidal activity. Essential oils obtained from *T. vulgaris* were also tested against larvae of *C. quinquefasciatus* (Govindarajan and Karuppannan, 2011).

In this research, it was revealed that the concentrations of ethanolic and aqueous leaves extracts at 400ppm were found to be the most effective against larvae and indicated 93% death of larvae after 24hrs. The results exhibited that leaves of

medicinal plants can be used for the prevention and control of mosquitoes due to their larvicidal effect.

The current conclusion is that *M. piperita, O. basilicum,* and *p. glabrum* leaf extracts have the ability to be used efficiently against mosquito's larvae. The present consequences can also be associated with a previous study that 5% aqueous extract of *C. procera* leaves showed 98% mortality of *Henosepilachna elaterii* larvae (Haldar *et al*., 2011). Extract of *M. piperita* can be effectively used for the control of various insect species, exhibited similarity with our study. In the present finding, it was detected that the death rate of larvae was directly related to the dose and hours of exposure. The larvae were treated after 24, 48, and 72hrs with concentrations of 80, 160, 240, 320, 400ppm.

In a previous report, *the Calotropis procera* plant is considered an effective and natural larvicide against *Anopheles arabiensis* and *Cx. quinquefasciatus* with low LC_{50} values. *C. sinensis* peels crude extract provided LC_{50} (58.25ppm) and LC_{90} (298.31ppm) values against *An. subpictus* larvae (Dasgupta *et al.,* 2013). In another study, lemon oil (*Citrus limon*) and orange oil (*C. sinensis*) exhibited at 250ppm maximum larvicidal activity of 99.20% against *An. stephensi* with LC_{50} and LC_{90} values of 35.95 and 138.86ppm (Jang *et al*., 2002). These results are almost similar to those found in our study.

In my study, the ethanolic leaves extract of the most effective plant with a low LC_{50} value of 208.98 ppm as compared to other extracts indicated concentration-dependent larvicidal activity. The results recommended that the crude extract of *M. piperita* can be used as natural biocides for mosquito control and has significant larvicidal, adult emergence inhibitor, and repellent effect against *Cx. quinquefasciatus*.

Biochemical analysis of experimented larvae revealed that carbohydrates, proteins, and lipids profile were significantly reduced in the extract-treated larvae as related to the control group (Huang *et al*., 2004). This suggests that at 400ppm concentration of plant extracts possess some toxic constituents which affected the biochemistry of larvae and induced high mortality. It might be owing to modifications in energy absorption, peroxidation, and insecticidal concern (Qureshi *et al*., 2015).

These consequences are following the described reduction in oocyte hemolymph and fat substances of *Tenebrio molitor* once revealing to Malathion (Sharma *et al*., 2011).

Microscopic explanations of treated larvae showed the lethal outcome of plant extracts in the form of sluggish spinning, loss of steadiness, and rupturing of the gut. This may be due to deterioration in the level of glucose as related to control. It is also proposed that it might be due to insecticidal pressure stimulated by these extracts lead to the reduction in scavenging presentation, limited depletion of food material, and deprivation of body wall guts (Lohar and Wright, 1993).

High radical scavenging activities of ethanolic extract of *M. piperita* in the present study are in favor with the previous study reported by Deeksha and Arunachalam, (2019). During the purpose of the reducing power of extracts, $Fe^{3}+$ is converted into $Fe^{2}+$, $Fe^{2}+$ then reacts $FeCl_2$ and results from the formation of complex ferrous. Like the present study, Kifayatullah *et al*., (2015) reported that ethanolic extracts of *Pericampylus glaucus* (Lamk) exhibit an increase in reducing power as the concentration of the extract increased. According to the Eshwarappa *et al*., (2014), *S. cumini* methanol extract had better reducing power and possessed equal potential with the standard ascorbic acid used. In the Phospho molybdenum method of TAC estimation, through antioxidant mediators present in extract green colour phosphate/Mo (V) complex formation occurred. Ethanolic leaves extracts of *M. piperita* and *O. basilicum* possess maximum antioxidant power. Munira *et al*. (2018) reported that the total antioxidant activity of *M. piperita* ethanolic leaf extracts is in the range of standard catechin.

The *F. glomerata* extract showed moderate toxicity having LC_{50} values of 454.34µg/ml having a significance value of P=0.147 (Nguta *et al.,* 2012). Diverse collection location, the tissue of plants, time of harvest, and solvent extraction noticed that toxicity showed variations significantly (Bussmann *et al.* (2011). Due to this natural variability, the leaves identified LC_{50} between 100 and 500µg/ml cytotoxicity have served us for more research on biologically active extracts against fleas (*Xenopsylla cheopis*), mosquitoes (*Aedes aegypti*), ticks (*Ixodes scapularis*), microbes affecting forest and health of living thing (Johnston *et al*., 2001; Dietrich *et al*., 2006). In the current study, *M. piperita, O. basilicum,* and *p. glabrum* plants extract exhibited moderate toxicity against brine shrimp.

Various bioactive compounds like phenols, flavonoids, alkaloids, saponins, terpenoids, etc. commonly used as medicinal attributes were confirmed in all selected plants. In *M. piperita* extracts preliminary qualitative phytochemical screening confirmed tannins, alkaloids, flavonoids, saponins, etc. (Bagyalakshmi *et al*., 2019). Pavai *et al*. (2019) confirmed the presence of different phytochemicals like saponins, carbohydrates, alkaloids, phenols, etc. in *M. piperita and O. basilicum* extracts. *P. glabrum* leaves extracts contained different medicinally important phytochemicals like phenols, tannins, saponins, proteins, etc. (Ramos and Bandiola, 2019) which is in accordance with the present study. Among all the tested extracts, maximum total phenolic and flavonoids contents were found in ethanolic extract of *M. piperita and O. basilicum*. Deeksha and Arunachalam, (2019) also reported maximum TP and TF contents in *M. piperita* extract. In contrast, the work of Sumi *et al*. (2016) reported that *Mallotus philippensis, Citrus aruntium* ethanolic leave extract also contained a significant amount of phenolic (20.2mgQE/g) and flavonoid (22.81mgQE/g) contents. According to Kaneria *et al*., (2013), antioxidant activities of extracts are highly related to their total phenols and flavonoids contents.Due to the presence of these phytochemicals (Phenols, flavonoids, Saponins, alkaloids, and flavonoids), medicinal plants play a key role in the treatment of numerous diseases. Plant bioactive components are designated to keep strong antioxidant, antibacterial, and several biological activities (Cedric *et al*., 2018).

So, in the existing study characterization of *M. piperita* was carried out by using UV-VIS Spectroscopy, FT-IR, and GC-MS analysis. The UV-VIS spectrum profile was selected at the wavelength of 200 to 800nm. The UV- VIS spectrum showed the maximum absorption at (209.509) 2.338 and (2825.814) 0.796 respectively. A similar peals spectrum was reported in leaf extract of *Meizotropis pellita* (Rani *et al*., 2016). The outcomes of FT-IR analyzed the presence of functional groups of carboxylic acids, amines, amides, Sulphur derivatives, polysaccharides, organic hydrocarbons, halogens. Similar active components based on peak values in the region of infrared radiation were observed by (Starlin *et al*., 2012) that are responsible for various medicinal properties of *Aerva lanata* (Nithyadevi and Sivakumar, 2015) also worked in the methanol leaf extracts of *Ichnocarpus frutescens.*

The GC-MS analysis of *M. piperita* leaves extracts characterized ten phytocomponents by comparing their mass spectrum with the standard library of Nist. Of the five compounds identified, the most dominant compounds were t-Butyl hydrogen phthalate (13.99%), 2H-Pyran-2,4(3H)-dione,3-ethyl-5,5-dimethyl -6-phenyl (13.15%), 1,2-Benzenedicarboxylic acid, mono-(2-ethylhexyl) ester (12.45%), Olean-12-en-28-oic acid, 2.beta., 3.beta.,23-trihydroxy-methyl ester (10.74%), 1,2-Benzene dicarboxylic acid, butyl octyl ester (10.36%), 8-Octadecen-1-ol acetate (9.64%). Among the compounds, three compounds were reported to have antimicrobial activity. In addition to antimicrobial activity, Pentadecanal, 8-Octadecen-1-ol acetate, 2-Cyclohexen-1-one,5-methyl-2-(1-methylethyl), %), Olean-12-en-28-oic acid, 2.beta., 3.beta.,23-trihydroxy-methyl ester was also reported to have antioxidant and anti-cancer properties. The enzyme NS3 protease that plays an important role in larvicidal efficacy was designated as a target for docking study. A total of ten compounds were used as ligands in docking and evaluated based on binding affinities to justify their larvicidal potential. In current docking, different forces present between the best-docked compounds i.e., 1,2-Benzenedicarboxylic acid and 3-ethyl-5, 5-dimethyl -6-phenyl, and the target protein. The two hydrogen bonds are present between each compound and target protein. Hydrogen bonds are very important in docking as they act as the facilitator of protein-ligand binding and are important in protein folding (Salentin *et al*., 2014)**.** Besides these, some other forces like Vander wall forces, alkyl, and Pi-alkyl interactions are present in the ligand-protein interacted complex. Vander wall forces stabilize the formed complex by the creation of a strong cohesive environment. The alkyl and Pi-alkyl interactions are crucial for charge transfer help in inserting the drug in the binding site of the receptor (Arthur and Uzairu, 2019).

The current conclusion revealed that due to the presence of medicinally vital phytochemicals, medicinal plants have noteworthy larvicidal and antioxidant activities. These biochemicals can be considered as best substitutes to chemical larvicides. Extract of *M. piperita* can be used efficiently for the control of mosquito species and acts as a powerful botanical pesticide. Furthermore, these plants are existing in native zones of Pakistan and are easily affordable. This study leads that further explorations are essential for the identification, isolation, and characterization

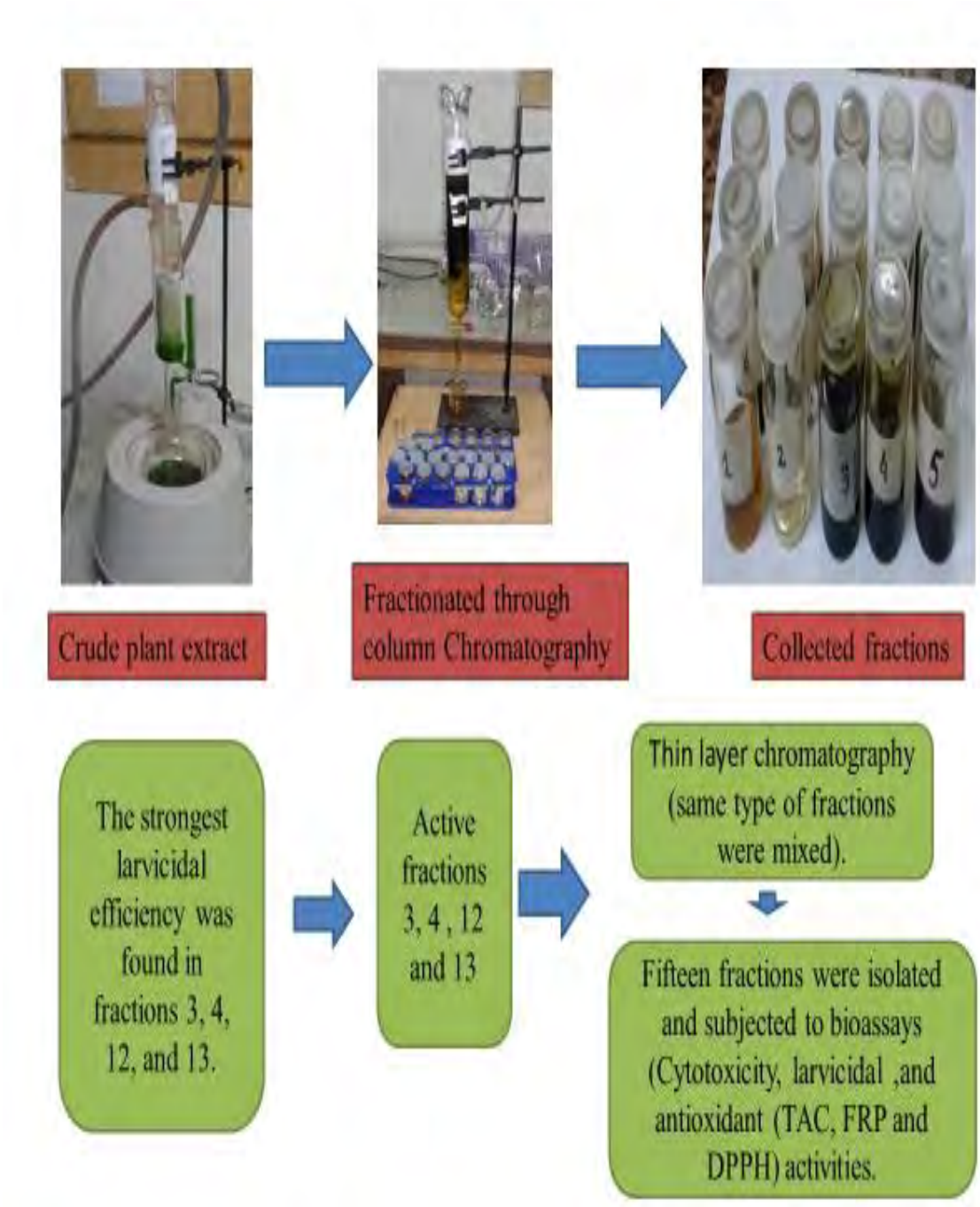
of bioactive constituents from *M. piperita* exhibiting larvicidal potential. The ethanolic extract of *M. piperita* may be directly used at the breeding sites of mosquitoes in stagnant water and localized conditions.

It has antilarval potential and can provide a renewable source of natural bioactive compounds. Insecticides of plant sources may offer applicable substitute bio-control methods in the future. Plants extracts are environment friendly and can be used effectively to change the chemical control processes because they are hazardous for the complete biodiversity is the utmost vital feature of the current discoveries. Among all identified compounds, 1, 2-Benzenedicarboxylic acid, and 3-ethyl-5, 5-dimethyl -6-phenyl are ideal for the production of the novel drug against larvicidal potential due to their improved interaction with the target during docking. Further exploration is extremely suggested on the separation of the antioxidant compounds from these plants.

CHAPTER 4 (Part-1)

ISOLATION AND IDENTIFICATION OF BIOACTIVE FRACTIONS FROM CRUDE LEAVES EXTRACT OF *MENTHA PIPERITA* USING CHROMATOGRAPHIC TECHNIQUES

GRAPHICAL ABSTRACT



ABSTRACT

Chromatographic techniques have significantly contributed to the discovery of novel compounds of biomedical and pharmaceutical importance. It opened a new gateway for isolation of pure secondary metabolites from crude plant extracts. Leaves of *Mentha piperita* (Lamiaceae) contain certain allelochemicals that inhibit the growth of surrounding plants. In the present study, the crude leaves extract of *M. piperita* was fractionated by column chromatography and further characterized by thin-layer chromatography using various solvents with different ratios such as n-hexane, ethyl acetate, chloroform and methanol for isolation of bioactive secondary metabolite. Fifteen fractions were isolated and applied for cytotoxicity, larvicidal and antioxidant activities (total antioxidant capacity, ferric reducing power, and DPPH) against *Cx. quinquefasciatus.* Different concentrations (80, 160, 240, 320 and 400ppm) were used in dose-response bioassay. Among all the fractions used 3, 4, 12 and 13 exhibited the strongest antilarval activity against having an LC_{50} values of all four instars larvae (127.9, 121.7, 105.1, 112.5), (131.5, 128.1, 118.6, 95.1), (136.6, 112.5, 102.4, 52.6), (104.1, 110.9, 130.7, 46.9) respectively. F3, F4, F12, and F13 showed the maximum total antioxidant (70±0.54, 72±0.34, 77±0.21, 85±0.25), ferric reducing (250±0.85, 255±0.65, 265±0.69, 275±0.94), and DPPH radical scavenging activities (90±0.13, 96±0.11, 97±0.87, 98±0.34).All derived fractions exhibited toxicity in the safety range IC_{50}>100µg/ml. The fraction (F13) (7.65±0.19ug/ml) showed significantly the highest antilarval activity with IC_{50} compared with control i.e. 7.23±0.55 µg/ml. The present study, it is concluded that out of fifteen isolated fractions 3, 4, 12 and 13 was found to most effective against I^{st}, $2^{nd,}$ 3^{rd} and 4^{th} instars larvae of *Cx. quinquefasciatus*.

INTRODUCTION

Plants are a natural and healthy source of life (Russell and Duthie, 2011). Natural products such as plant extracts open up a fresh perspective for the development of new medicinal agents, with the added benefit of being natural. In most poor nations, the use of traditional medicine and medicinal plants as a normative foundation for the preservation of worthy health has been commonly identified. About 80% of the world's population relies on herbal treatments (Cos *et al.,* 2006).

Plants have a wide range of chemical components that can be used to treat both chronic and infectious disorders (El-Shemy *et al*, 2007). The microbial fight against chemically produced medicines necessitates a shift toward ethanopharmacology. They are the source of hundreds of phytochemicals with biological properties such as antibacterial, antidiarrheal, analgesic, anticancer, and wound healing, as well as antioxidant activity (Hazra *et al*., 2007).

Plant phytoconstituents can be generated from any part including bark, leaves, flowers, roots, fruits, seeds, etc. in combination with secondary metabolites which are taxonomically different and extremely prospective for any specific plant (Wink, 1999; Pausas and Austin, 2001) helpful in the field of medicine (Gordon and Dravid, 2001).

Plant metabolites often occur as complex mixtures of many substances of a wide range of polarity and hydrophobicity. The most important groups of substances in plant material are: low polar (waxes, terpenoids), semi-polar (lipids, phenolic compounds, low-polar alkaloids), and high-polar (polar glycosides, polar alkaloids, saccharides, peptides, proteins) (Romanik *et al*., 2007). Secondary metabolites are those chemical substances that are not directly involved in the growth and development of plants. They lead to the biosynthesis of simple to complex metabolites, consuming the intermediates from the primary metabolites through precise pathways (Devika and Koilpillai, 2015). Mostly are poisonous and play defensive roles against biotic factors like protection from attack of pathogens and herbivores, allelopathy, etc. (Achakzai *et al*., 2009). The concentrations of secondary metabolites are fluctuating from plant to plant species stated numerous investigators and even in the different parts of the same species (Burda and Oleszek, 2001).

In present years, the screening of medicinal plants for innovation and separation of new secondary metabolites has increased. The ethanopharmacologist, botanists, microbiologists, and natural product chemists are improving diverse methodologies to enhance phytochemicals that might provide operative treatment for infectious ailments. Herbal drugs have been frequently and broadly used for numerous centuries, owing to less harmfulness and well-known pharmacological activity. Numerous crude preparations of herbal drugs are in experimental use in the administration of human and plant infections (Rates, 2001).

Not until the 1990s that researchers fully understand that these secondary metabolites mostly remain from an organism's metabolic processes. These substances can function as communications tools, defense mechanisms, or sensory devices. The biological activity of these chemicals is valuable to the organism that produces them, but it is often harmful to other species, including humans (Swerdlow, 2000). This toxicity can adversely affect the functions of the entire human body or only a specific biological process, such as the growth of cancer cells. In this way, certain foreign, naturally produced chemicals can act as powerful drugs when directed at the proper concentration (Newman and Cragg, 2007).

The use of secondary metabolites from plant sources could be an improvement and best resolution to compact the use of unnatural products with probable pharmacological properties. In the earliest, the plant or microbial extracts in crude or partially-purified forms were the simple causes of medicine available for the treatment of human and animal diseases. This indicated that the significance of medicine in the human body is due to the contact of the drug with biological molecules. This opened novel doors in pharmacology, as clean, isolated compounds, instead of extracts, as the standard for the cure of diseases. At current, there is an uncountable number of such bioactive compounds isolated from crude extracts (Bajpai *et al.,* 2016).

Almost 20,000 therapeutic plants are present in 91 countries comprising 12 mega biodiversity countries according to the World Health Organization (WHO). The main phases to make use of the biologically active constituents from plant resources are extraction, pharmacological screening, separation and characterization of bioactive compounds, toxicological assessment, and experimental evaluation

(Sasidharan *et al*., 2011). Since time ancient, man utilized the strong biochemical from plants and converted them into wonderful collections of industrial use in many subdivisions like pharmacy, chemical, dye, pesticides, etc. (Mahesh and Vidya, 2008).

For separation of bioactive constituents of medicinal plant extracts into diverse solvent fractions depending on the polarity, activity showed fractionation has been used. The fractions achieved by liquid-liquid extraction still contain a combination of several kinds of bioactive compounds. Several research studies proved that natural antioxidants from plant sources can effectively inhibit the oxidation of food and reduce the risk of age-dependent diseases. Recently, the separation of natural antioxidants from plant sources has considerably improved since synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tertiary butyl hydroquinone (TBHQ) are supposed to be responsible for liver harm and carcinogenesis (Belhaddad *et al*., 2018).

Mentha piperita belonging to the family Lamiaceae is a hybrid mint commonly used as a folk medicine, having antioxidant, antimicrobial, larvicidal, antiproliferative, antimicrobial, anti-inflammatory, and cancer-preventive activities (Duraipandiyan *et al.,* 2006). The plant extracts have to be exposed to numerous biochemical screening methods to determine novel bioactive compounds from plant sources, which can be used as innovative main molecules, such as high-performance thin-layer chromatography (TLC), gas chromatography-mass spectrometry (GC-MS), which provides structural evidence, resulting in partial or complete structure determination of natural products (Wolfender *et al*., 2003).

The purity of bioactive compounds can be determined by column chromatography, thin-layer chromatography (TLC), and high-performance liquid chromatography (HPLC). Column chromatography is one of the analytical techniques used for the isolation and quantification of individual bioactive compounds from a mixture of compounds. It is commonly used for the preparation of synthetic chemicals from a very small amount to kilograms on a large scale. Column chromatography is advantageous over other analytical techniques because it is comparatively less expensive and the stationary phase used during the process is easy to dispose of which avoids cross-contamination and its degradation during the recycling process.

Purification of these compounds can be achieved with the help of column chromatographic techniques. Silica gel column chromatography is usually used for the separation of phytochemicals with variable biochemical nature. Multiple mobile phases with increasing polarity are consequently convenient for good separation (Zygmunt and Namieśnik, 2003).

Thin-layer chromatography and HPLC is also an analytical technique used for the identification of compounds and to determine their purity (Shahverdi *et al*., 2007). Recent analytical spectroscopic techniques like Nuclear Magnetic Resonance Spectroscopy (NMR), Liquid Chromatography Electrospray Ionization Mass Spectroscopy (LC-ESI-MS), and Fourier Transform Infrared Spectroscopy (FT-IR) play a key role in the identification and characterization of bioactive principles having antimicrobial, antioxidant, and antiproliferative activities (Mahesh *et al*., 2015).

So, the main purpose of this research was isolation and identification of bioactive fractions from crude plant extract by using column chromatography and TLC and the application of isolated fractions in the laboratory for assessment of larvicidal, antioxidant activity, and cytotoxicity.

MATERIALS AND METHODS

4.1: *M. piperita* leaves collection and identification

Fresh *M. piperita* leaves were taken near Quaid-i-Azam University, Islamabad, Pakistan. The plant was identified with the help of taxonomist (Abdullah *et al.,* 2018). After collection, the leaves of plants were washed with tap water and shadow dried for three weeks at room temperature (27-37ºC)

4.2: Preparation of Crude plant Extract

M. piperita powdered leaves (65gm) were extracted in 400ml ethanol solvent using the Soxhlet extraction device (Shanghai Heqi, China) at 60°C for 8-10hrs (6 cycles per hour). The methodology is described in detail in Chapter 3. The resultant extracts were filtered by Whatman No. 1 filter and the solvent was removed using a rotatory vacuum evaporator at 50◦C (R-300, Rotavapor, Germany). The resultant extract was stored at 4°C for further column chromatography.

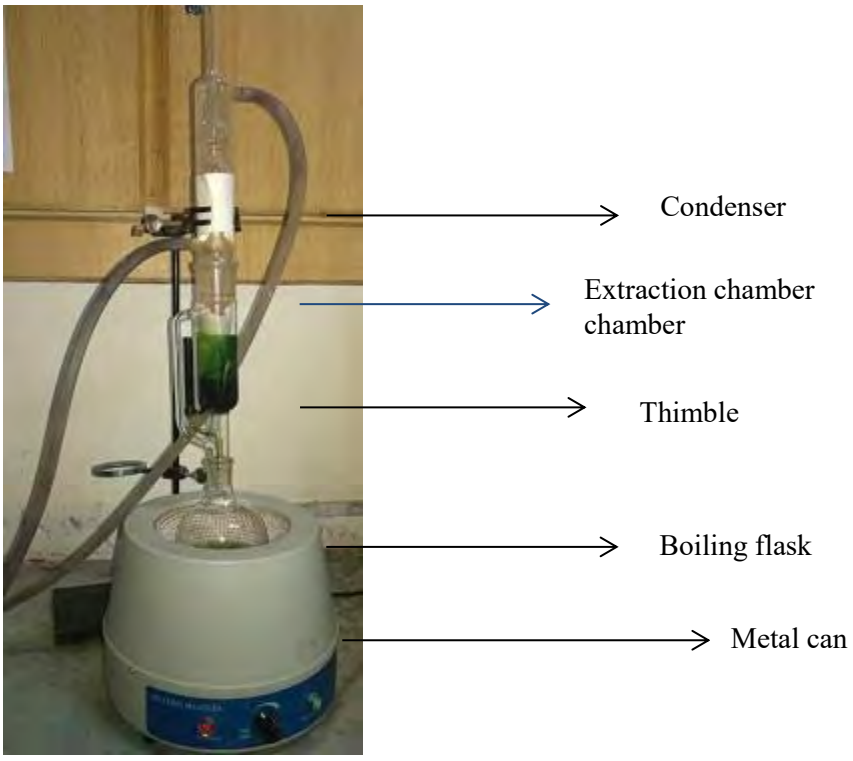


Figure 4.1: Soxhlet extraction apparatus.

4.3: Fraction isolation through column chromatography

4.3.1: Column Packing

A column (28.3 inches) was made with silica gel (0.063-0.200mm) in dry powder form and stored overnight for the isolation of various fractions from plant extract. For settling, the column was run with 300ml of n-hexane (Figure 4.2).

Figure 4.2: Isolation of fractions from *M. piperita* crude extracts using a column

4.3.2: Loading of Sample and Column treatment

The crude ethanolic extract of *M. piperita* (15gm) was placed into a silica gel open chromatographic column, and the column was run in three distinct solvents, n-hexane, ethyl acetate, chloroform, and methanol in increasing order of their polarity, using the step gradient technique. Different ratios of n-hexane and ethyl acetate (pure n-hexane, 20:80, 40:60, 80:20, and pure ethyl acetate) were used to isolate fractions at varied rates, followed by ethyl acetate and chloroform (pure ethyl acetate, 20:80, 40:60, 80:20, and pure chloroform)

4.4: Thin Layer Chromatography

Thin-layer chromatography (TLC) was used to further characterize the fraction recovered from column chromatography in various eluents. On fractions eluted using column chromatography, thin layer chromatography (TLC) was performed using silica covered TLC plates (15.8cm) (Merck, Mumbai). On the TLC plate, a start line of 1.5cm was drawn using a pencil. Each fraction (0.1µl) was carefully highlighted on the line. After that, each fraction TLC plate was immersed in its corresponding solvent until colour spots appeared. UV light was used to examine the plates after they had dried.

The retention factor (Rf) value of the spots was calculated using the following formula;

$$Rf = \frac{Distance\ traveled\ by\ solute}{Distance\ traveld\ by\ solvent}$$

Different fractions with similar Rf values were mixed (Table 1). The stock solution was made by evaporating the solvents and using the crude fractions.

4.5: Preparation of stock solution and Dilutions

The separated fractions stock solution was made by dissolving 1g of crude extract in 100ml distilled water, and several dilutions (80, 160, 240, 320, and 400ppm) were made using the formula $C_1V_1=C_2V_2$.

$$V1 = \frac{Required\ ppm \times Required\ Volume}{Stock\ Solution}$$

4.6: Bioassays or experimental design of isolated Fractions

4.6.1: Larvicidal Bioassay

The Larvicidal bioassay was designed to determine the toxicity of plant extracts against mosquito larvae. The methodology is described in detail in Chapter 3.

4.6.2: Antioxidant Bioassay

The overall antioxidant activity, DPPH radical scavenging, and total reducing power of all pooled fractions were assessed according to the method as described by Rashid (*et al.*) 2017. The methodology is described in detail in Chapter 3.

4.6.3: Brine shrimp cytotoxicity assay

Shrimp eggs were placed on one side of the partitioned little tank, which was then enclosed, in man-made seawater (38g NaCl/1000 ml tap water). The shrimps were allowed to develop nauplii for 48hrs after hatching and were tested for bioactivity. The ethanolic extract of *M. piperita* was taken in different quantities (5, 7.5, 10, 12.5, and 15mg/10mL) in sample tubes. Ethanolic extracts were dissolved in DMSO for the cytotoxic assay. A disinfected pipette was used to release 20 live shrimps into each test tube, along with a control group. The tubes were examined with a magnifying glass after 24hrs for the counting of survived nauplii in each vial and interpretations were recorded for each container. Each test consisted of five recurrences that were repeated three times. We calculated the LC_{50}, values. As a positive control, tricaine methanesulfonate was utilized (El Badry *et al.,* 2015).

4.7: Statistical Analysis

The mean mortality of mosquito larvae was compared for each plant extract and concentration, and statistically significant differences were found using one-way ANOVA with Tukey's multiple comparison tests in the "R language." Results with a P value of less than 0.05 were significant, while those with a P value of less than 0.005 were considered highly significant. At $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, statistically significant differences are indicated by stars (*, **, ***). The LC_{50} value of each plant extract against termites was obtained using SPSS software version 19's "Probit analysis."

RESULTS

4.1: Fractions collected through column chromatography

Initially, 143 fractions (20ml) were collected in fraction collecting tubes through column chromatography having silica gel column, which were further characterized by thin-layer chromatography (TLC) in different eluents (hexane, ethyl acetate, methanol) and the same type of fractions was mixed. Fifteen samples were collected after TLC **(Figure.4.1).** Fraction 1 was a simple hexane because it didn't reveal the presence of any brand on the TLC plate.

Figure 4.3: Fractions collected through column chromatography

4.2. Fractionation of crude extract and Rf values

The ethanolic extract of *M. piperita* crude extract was fractionated by column chromatography with the help of silica gel (0.063-0.200mm Merck). About 143 fractions with different polarity, each 50ml in volume were collected. Based on the TLC profile, fractions with similar Rf values were combined and crystallized. After mixing of same Rf value fractions, total of 15 fractions with different Rf values were finally collected (Table 3). During column chromatography increasing order of polarity from non-polar to polar mobile phase was followed for elution of fractions.

Table 4.1: Observations on TLC and Rf values of the fractions of ethanolic leaves extract of *M. piperita* using a gradient solvent system in column chromatograph.

Sr. No	Solvent system	Ratio	Fractions	Volume (ml)	Observations on TLC	Final fractions	Rf Values
1	n- Hexane	250-300ml	1-4	50,48,50,44,50,50,	F1-F4: No spot	-	-
							-
2	n-Hexane: Ethyl acetate	80:20	5-12	47,50,50	F9-F12: 3 similar spots	2	0.59
3	n-Hexane: Ethyl acetate	60:40	13-30	48,48,45	F21: Single spot	3	0.37
					F19 and 23: No spots	-	-
4	n- Hexane: Ethyl acetate	40:60	31-44	44,45,45	F26_28: 3 similar spots	4	0.44
5	n- Hexane: Ethyl acetate	20:80	45-50	45,50,50,44	F31 and 34: 4 similar spots	5	0.39
6	Ethyl acetate	250-300ml	51-59	48,45,50,43,50	F40: Single spot	6	0.37
					F43-55: 3 similar spots	7	0.25
7	Ethyl acetate: Chloroform	80:20	60-65	50,44,45	F60: No spot	-	-
					F62-54: 2 similar spots	8	0.38
8	Ethyl acetate: Chloroform	60:40	66-74	46,48,45,50,47,44	F67: Single spot	-	0.41
					F68-74: 3 similar spots	9	0.17
9	Ethyl acetate: Chloroform	40:60	75-80	48, 45,44,50	F77-79: 3 similar spots	10	0.27
10	Ethyl acetate: Chloroform	20:80	81-106	50,48	F90-97: 2 spots	11	0.26
11	Chloroform	250-300ml	107-112	48,49,46,50,43,44,48,50	F107-: No spot	-	-
					F108-112: 4 similar spots	12	0.28
12	Chloroform: Methanol	80:20	113-118	50,48,44,48,50,48	F113: single spot	-	0.25
					F116 and 117 :4 similar spots	13	0.54
13	Chloroform: Methanol	60:40	119-126	48,44,46,50	F119: No spot	-	-
					F120-126: 5 similar spots	14	0.47
14	Chloroform: Methanol	40:60	127-132	50,48,44,50,47	F127-128: No spot	-	-
					F129-:132 similar spots	-	0.29
15	Chloroform: Methanol	20:80	133-139	44,50,48	F133-139: 5 similar spots	-	0.42
16	Methanol	250-300ml	140-143	50, 50	F140-143: 8 Similar spots	15	0.57

4.3: Antilarval activity of Fractions Isolated from *M. piperita*

4.3.1: Antilarval activity against *M. piperita*

Antilarval activity of different fractions was investigated by using dose-response bioassay against *Culex quinquefasciatus*. Fifteen (2-15) fractions were used in 72hrs experiment and the percentage mortality of mosquito larvae was calculated by using different concentrations (80, 160, 240, 320 and 400ppm). All concentrations were found to have significant effect on larval mortality as compared to control ($F\ (df) = 511.5\ (5)$; $P < 0.05$) in time-dependent manner, that also differ significantly from each other ($F\ (df) = 412.6\ (13)$; $P < 0.05$). Similarly, all fractions showed differential antilarval activity in dose and time-dependent manner ($F\ (df) = 255.7\ (15)$; $P < 0.05$). The effect of hours, concentrations and fractions on mean mortality of larvae is represented in figure 4.1. Fraction 3, 4, 12 and 13 were found to be most effective ($p<0.05$) having a 50% decline in larvae population after 72hrs with 94%, 95%, 96% and 97% decrease respectively at the end of the experiment. Fraction 11 caused 28% decline after 24 hours of the experiment. Fraction 9, 10 and 11 also significantly ($p<0.05$) reduce larvae mortality up to 63%, 67% and 75% respectively. Similarly, observed larvae mortality due to the toxic effect of fractions 5, 6, 7, 8 was 42%, 40%, 52% and 55% respectively. While fraction 1, 2, 14 and 15 were found to be least effective with non- significant ($p>0.05$) decrease in larval population. The level of significance of all fractions and their LC_{50} values are represented in **table 4.2 and 4.3.**

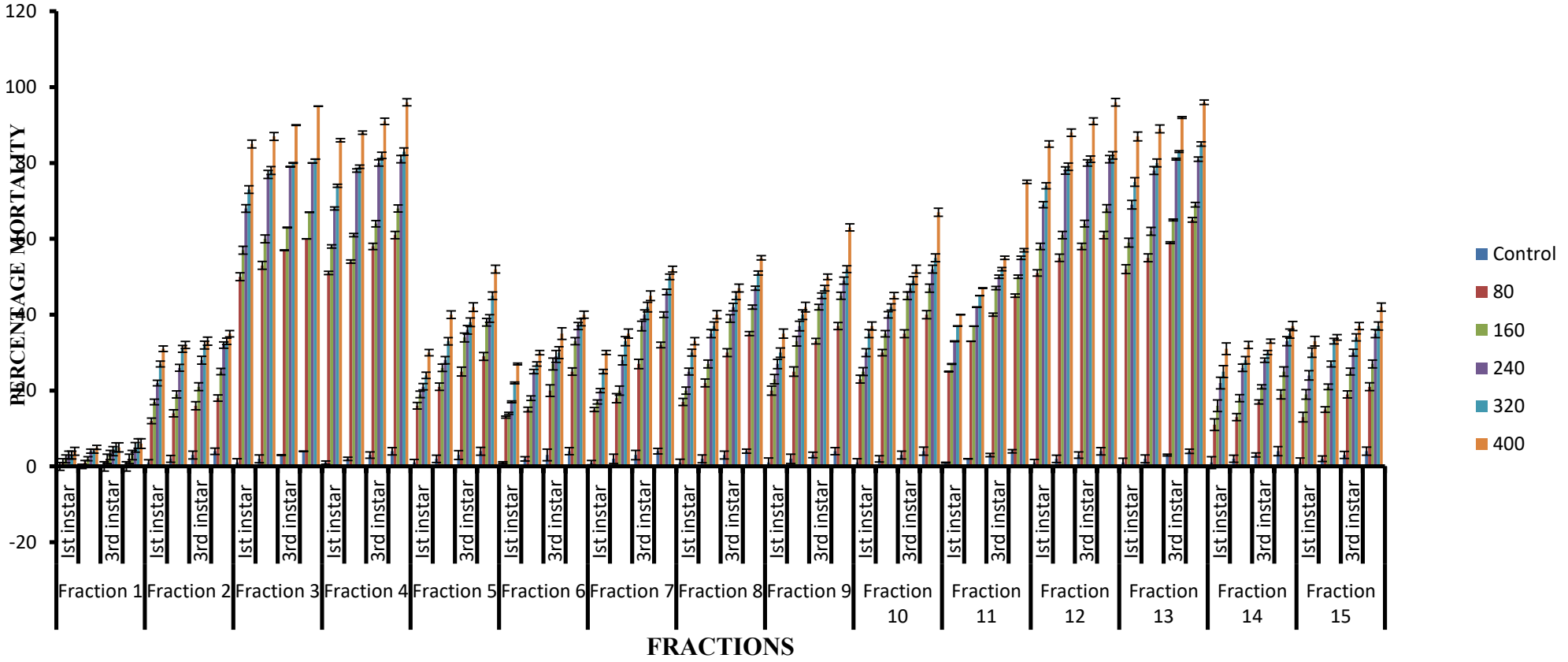


Figure 4.4: Percentage mortality of all instars larvae of *Cx. quinquefasciatus* of different concentrations of *M. piperita* fractions in ethanol solvent.

Table 4.2: LC_{50} values of different fractions of *M. piperita* leaves extract used for larvicidal potential.

Number of Fractions	LC_{50} (ppm) Upper confidence limit - Lower confidence limit			
	Ist instar	**2nd instar**	**3rd instar**	**4th instar**
2	365.8 (363.6-334.2)	384.5 (364.9-320.2)	319.9 (383.3-354.9)	387.3 (391.9-306.3)
3	127.9 (312.5-281.1)	131.5 (116.7-109.3)	136.6 (143.9-134.2)	104.1 (143.8-128.3)
4	121.7 (287.3-254.2)	128.1 (126.2-104.2)	112.5 (139.4-121.5)	110.9 (114.9-106.9)
5	272.8 (285.4-234.7)	332.1 (348.3-331.5)	228.7 (274.5-241.7)	318.3 (282.5-174.2)
6	253.1 (345.9-321.2)	324.1 (335.5-330.4)	217.2 (217.1-201.6)	298.9 (273.7-164.3)
7	209.5 (328.5-321.3)	304.3 (303.8-295.9)	197.7 (198.6-133.2)	265.8 (229.3-222.2)
8	193.6 (369.9-376.5)	282.1 (278.1-298.9)	177.2 (173.6-121.1)	229.3 (224.9-213.7)
9	181.6 (393.1-386.7)	262.3 (263.1-248.1)	167.7 (169.6-141.4)	218.8 (216.2-207.6)
10	172.8 (236.5-211.9)	220.1 (251.4-207.1)	148.6 (163.6-131.2)	196.9 (200.5-133.2)
11	152.2 (268.6-226.6)	212.1(142.4-124.1)	138.7 (156.6-101.3)	166.3 (145.3-139.4)
12	112.5 (175.4-166.6)	118.6 (123.1-126.8)	102.4 (123.6-139.7)	130.7 (148.8-103.6)
13	105.1 (142.4-124.1)	95.2 (101.1-96.6)	52.6 (93.6-101.8)	46.9 (121.5-112.4)
14	338.1 (253.1-2342.3)	354.8 (359.7-305.8)	292.1 (313.6-301.5)	353.1 (367.4-318.6)
15	304.3 (334.2-6534.1)	342.8 (361.3-345.5)	242.8 (283.6-211.3)	321.5 (324.6-258.5)

Table 4.3: Significance level of fractions used for larvicidal potential against all instars larvae of *Cx. quinquefasciatus.*

Number of Fractions	Level of Significance			
	Ist instar	**2nd *instar***	**3rd instar**	**4th instar**
2	Non-significant*	Non-significant*	Non-significant*	Non-significant*
3	Significant***	Significant t***	Significant***	Significant*
4	Significant***	Significant***	Significant***	Significant***
5	Significant*	Significant***	Non-significant*	Significant***
6	Non-significant*	Non-significant*	Non-significant*	Significant*
7	Significant**	Significant*	Significant**	Significant***
8	Significant**	Non-significant*	Non-significant*	Significant**
9	Significant **	Non-significant*	Significant**	Non-significant*
10	Significant**	Significant**	Significant**	Significant**
11	Significant**	Non-significant*	Non-significant*	Significant*
12	Significant***	Significant***	Significant***	Significant***
13	Significant***	Significant***	Significant***	Significant***
14	Non-significant*	Non-significant*	Non-significant*	Non-significant*
15	Non-significant*	Non-significant*	Non-significant*	Non-significant*

***= Highly significant, **= low significant *= Non-significant

4.4. BIOCHEMICAL TESTS:

The result exhibited that carbohydrate, proteins, and lipids (cholesterol, triglycerides and high-density lipid) level of all instars larvae of *Cx. quinquefasciatus* was lower as compared to control in all fractions after treatment with *M. piperita* extract respectively.

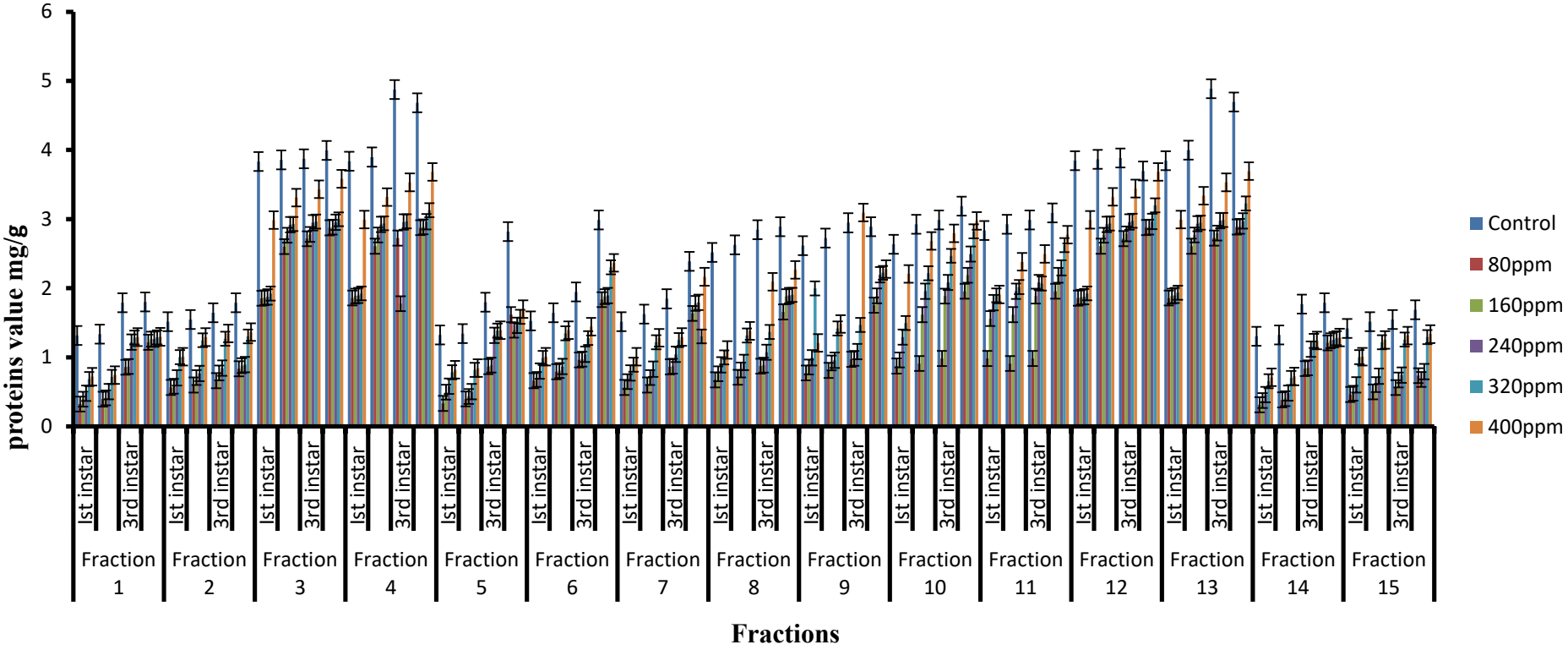


Figure 4.5: Protein values of Ist, 2nd, 3rd and 4th instars larvae of *Cx. quinquefasciatus* in different concentrations of *M. piperita* fractions in ethanol Solvent.

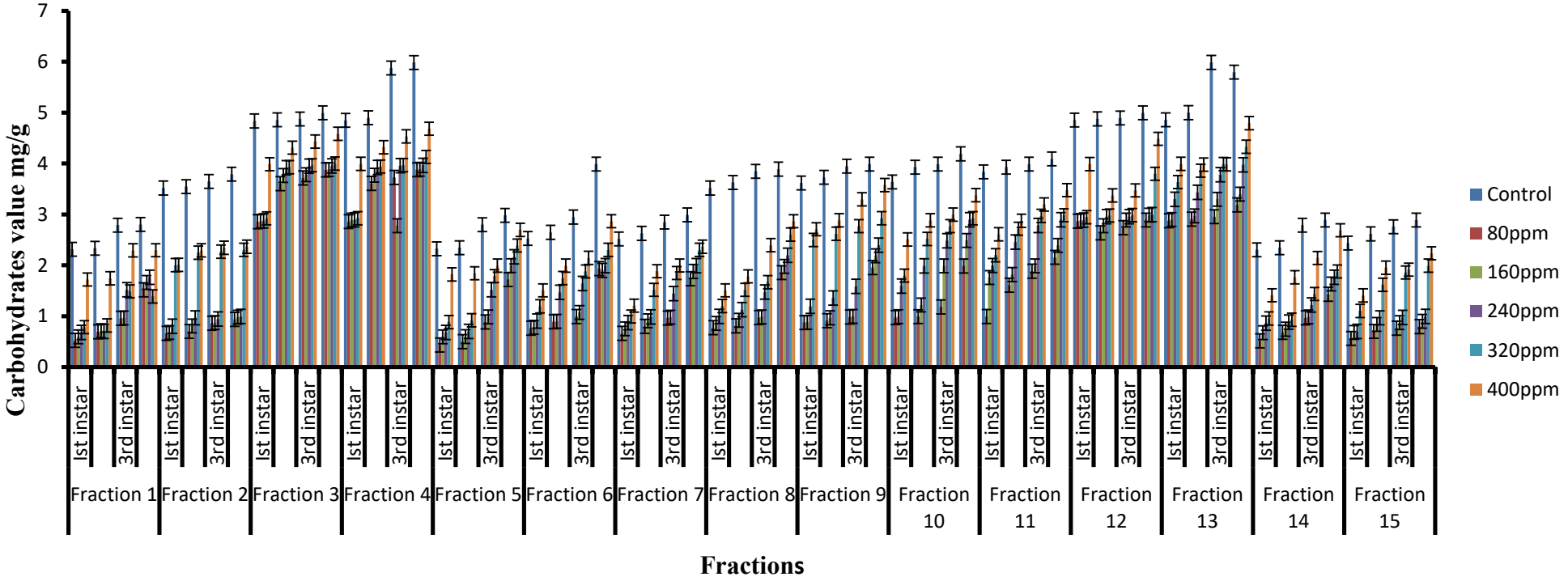


Figure 4.6: Carbohydrates values of Ist, 2nd, 3rd and 4th instars larvae of *Cx. quinquefasciatus* in different concentrations of *M. piperita* fractions in ethanol solvent.

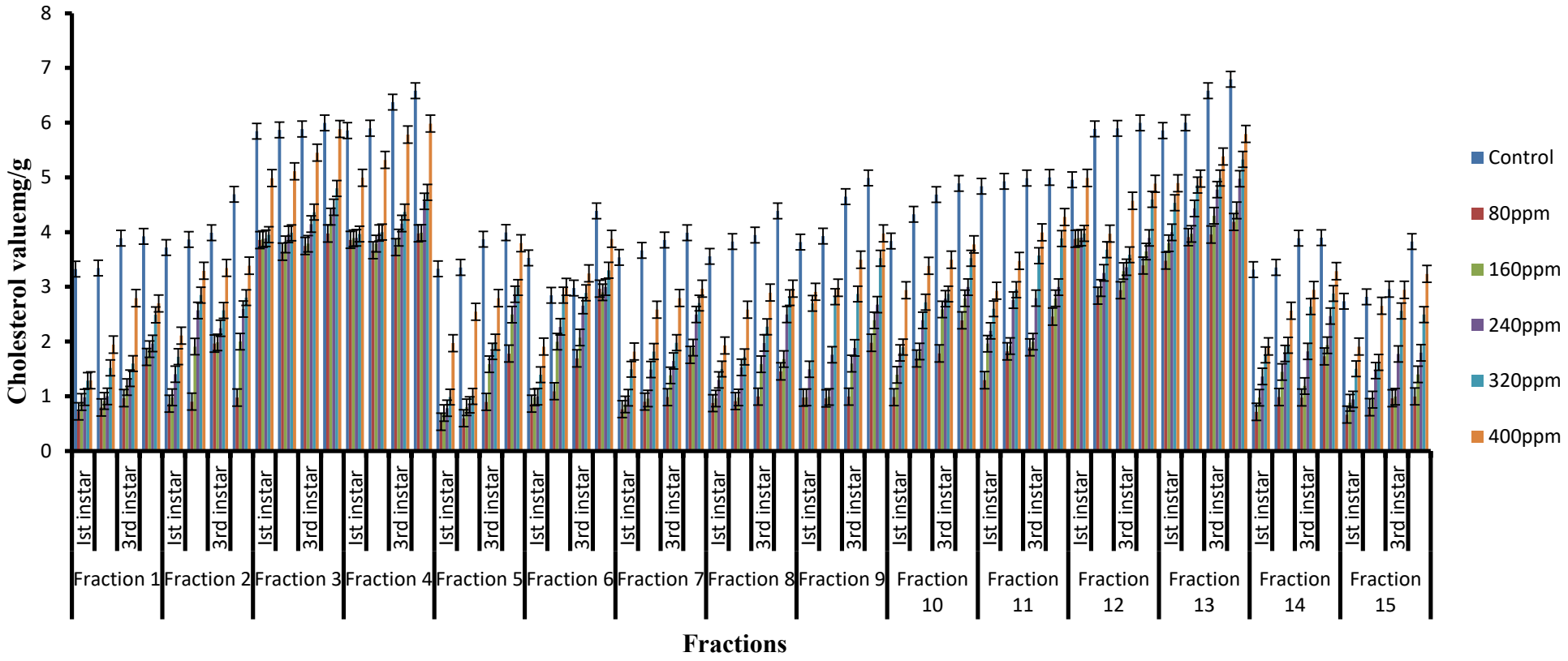


Figure 4.7: Cholesterol values of Ist, 2nd, 3rd and 4th instars larvae of *Cx. quinquefasciatus* in different concentrations of *M. piperita* fractions in ethanol solvent.

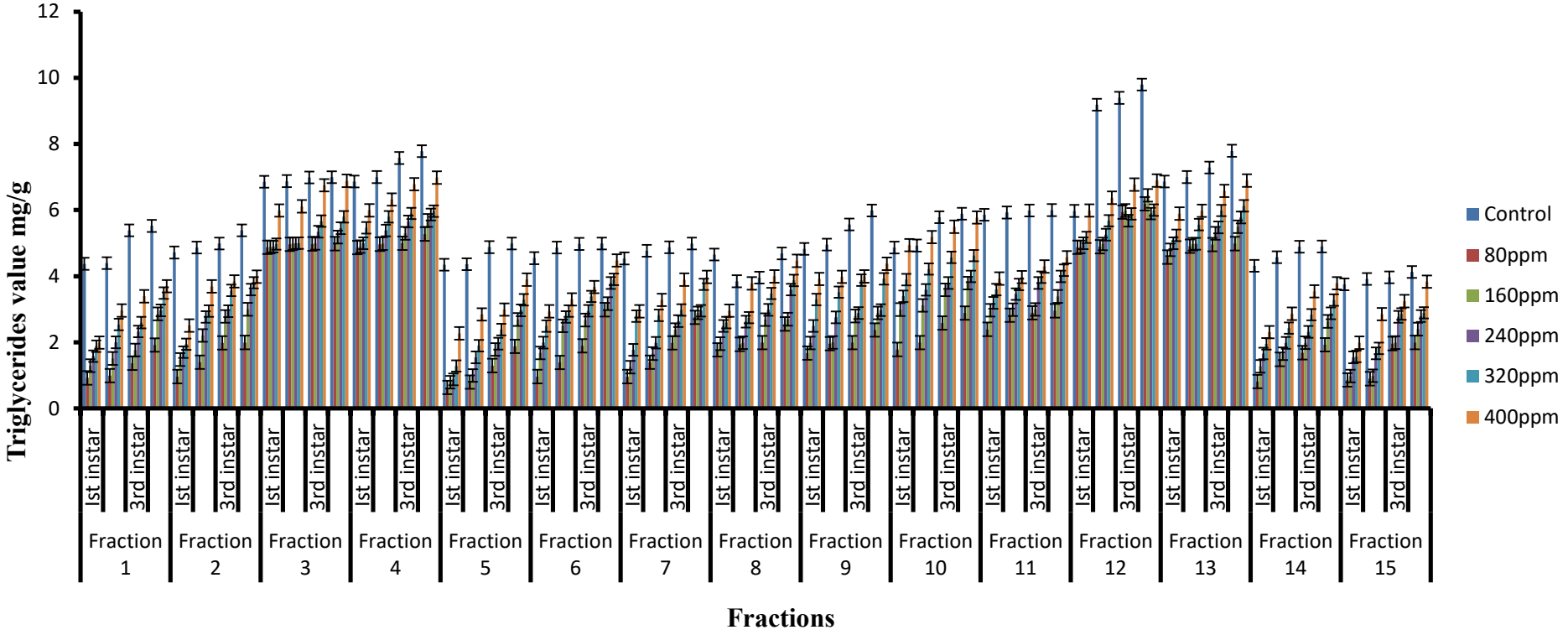


Figure 4 8: Triglycerides values of I[st], 2[nd], 3[rd] and 4[th] instars larvae of *Cx. quinquefasciatus* in different concentrations of *M. piperita* fractions in ethanol solvent.

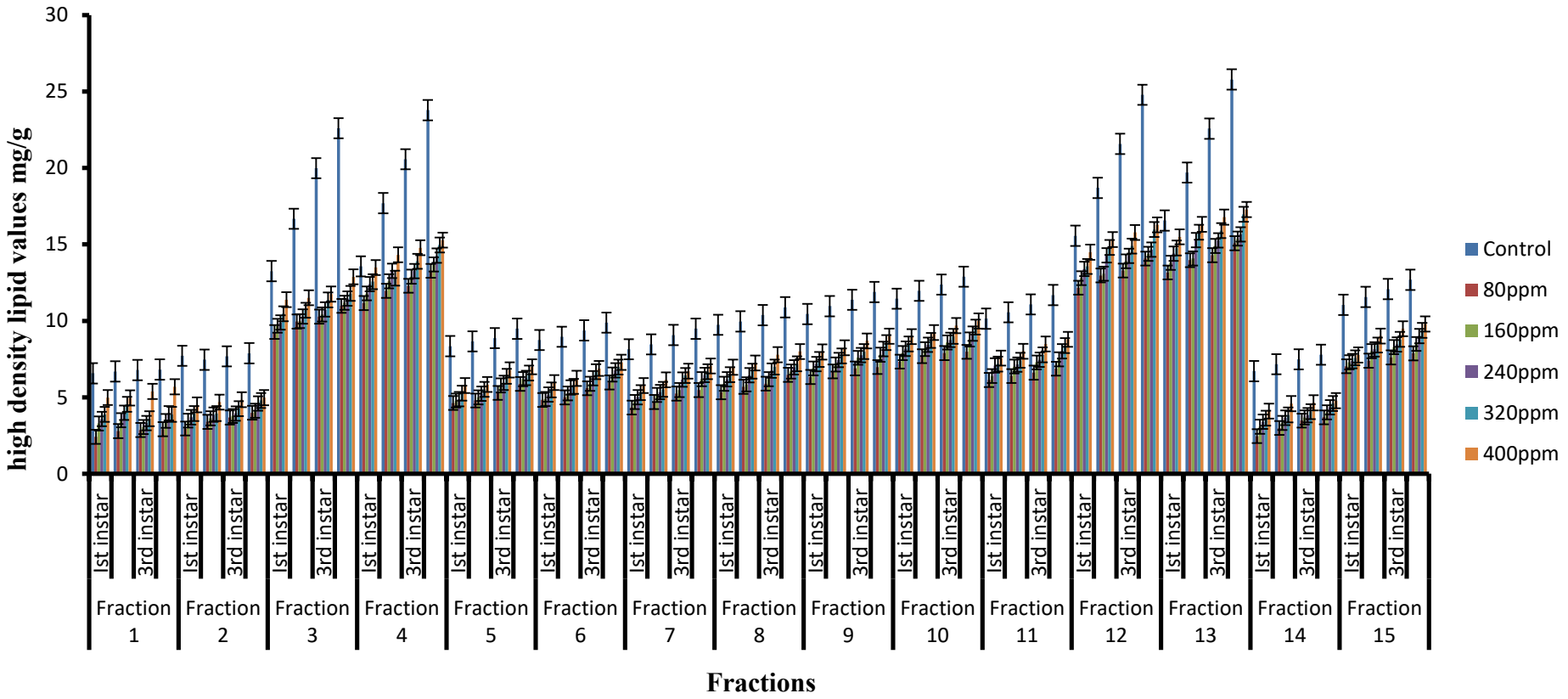


Figure 4.9: High density lipid values of Ist, 2nd, 3rd and 4th instars larvae of *Cx. quinquefasciatus* in different concentrations of *M. piperita* fractions in ethanol solvent.

4.4. Antioxidant assays

Antioxidant activities of isolated fractions were determined using three assays including DPPH radical scavenging activity, ferric reducing power and total antioxidant capacity. Ascorbic acid was used as standard for all assays.

4.4.1. 2,2-diphenyl-1-picrylhydrazyl (DPPH)

Highest antioxidant activities based on the DPPH assay were detected in fraction 3, 4, 12 and 13 with lowest IC_{50} values (7.56, 8.23, 8.27 and 8.06) µg/ml respectively (Table 4). These four active fractions eluted with ethanol solvent indicating that compounds eluted were relatively polar.

4.4.2. Ferric reducing power (FRP)

The reducing power assay results indicated an increasing trend of reducing antioxidant power with increasing extract concentration in each fraction. Among the isolated fractions, fractions 3, 4, 12 and 13 possess the maximum reducing power (250±0.85), (255±0.65), (265±0.69) and (275±0.94) at 600µg/ml respectively. That is slightly lower but statistically similar to the reducing power of standard Ascorbic acid (3.9±1.04) on same concentrations. The antioxidant activity of the tested samples evaluated by FRAP method could be ranked in following order; F13>F12>F4>F3>F11>F10>F9>F8>F7>F6>F5>F15>F14>F2>F1 (Table 3). Fractions having maximum reducing power were eluted with polar solvent.

4.4.3. Total antioxidant capacity (TAC)

Among the tested fractions, F13 exhibited maximum total antioxidant capacity (85±0.25) µg/ml followed by F12, F4 and F3 (77±0.21, 72±0.34 and 70±0.54) µg/ml, lower than standard ascorbic acid total antioxidant capacity (59.32±1.47) (Table 3).

Table 4.4: Different fractions of *M. piperita* were tested for total antioxidant capacity (TAC) and ferric reduction power (FRP)**.**

Fractions	TAC (µg AAE/ml DW)	FRP (µg AAE/ml DW)
1	12 ± 1.02^{h}	95 ± 2.21^{i}
2	25 ± 1.11^{g}	110 ± 2.11^{h}
3	70 ± 0.54^{abc}	250 ± 0.85^{b}
4	72 ± 0.34^{ab}	255 ± 0.65^{b}
5	50 ± 0.78^{de}	151 ± 0.96
6	53 ± 0.65^{de}	165 ± 1.02^{f}
7	55 ± 0.43^{de}	173 ± 1.31^{f}
8	58 ± 0.23^{e}	189 ± 1.67^{de}
9	60 ± 0.87^{d}	193 ± 0.68^{e}
10	62 ± 1.06^{d}	210 ± 0.41^{d}
11	65 ± 0.94^{d}	220 ± 0.35^{c}
12	77 ± 0.21^{b}	265 ± 0.69^{a}
13	85 ± 0.25^{a}	275 ± 0.94^{a}
14	39 ± 0.68^{f}	135 ± 1.35^{g}
15	41 ± 0.96^{f}	142 ± 1.69^{g}

In the table, each value is presented as a ± SD (n=3) mean; AAE=ascorbic acid; DW=dry weight. TAC = total antioxidant assay, FRP= ferric reducing power.

Table 4.5: DPPH free radical scavenging activity (%) and IC_{50} values of different fractions isolated from *M. piperita*.

Concentrations (ug/ml) % inhibition by fractions and Vitamin C						IC_{50} ug/ml
Fractions	**Concentrations (ppm)**					
	80	**160**	**240**	**320**	**400**	
2	30±2.33^{gh}	33±2.87^{ij}	35±2.97^{f}	40±2.68^{g}	42±2.13	40.87±2.41
3	80±1.02^{ab}	82±0.12^{ab}	86±0.34^{ab}	94±0.41^{a}	90±0.13^{ab}	8.36±0.11
4	81±0.85^{ab}	83±0.65^{ab}	89±0.64^{ab}	91±0.32^{ab}	96±0.11^{b}	8.27±0.41
5	40±1.33^{f}	50±0.78^{gh}	55±0.78^{e}	57±0.87^{f}	58±0.18^{e}	29.21±0.31
6	45±0.95^{f}	55±0.88^{gh}	60±0.85^{cd}	67±0.94^{de}	71±0.21^{bd}	27.41±0.21
7	53±0.98^{e}	59±0.98^{h}	62±0.97^{ad}	63±0.48^{de}	65±0.29^{cd}	23.11±0.14
8	59±0.97^{e}	61±0.87^{g}	65±0.87^{bd}	66±0.51^{e}	69±0.64^{cd}	21.89±0.51
9	62±1.41^{d}	65±0.97^{f}	69±1.54^{d}	73±0.97^{d}	75±0.25^{d}	18.45±0.54
10	65±1.56^{d}	68±0.82^{f}	70±1.64^{d}	75±1.54^{d}	78±0.35^{d}	17.35±0.87
11	75±1.65^{c}	77±1.56^{e}	80±0.41^{c}	82±1.64^{c}	89±0.54^{c}	15.41±0.97
12	83±0.41^{ab}	84±0.13^{d}	90±0.34^{a}	94±0.87^{a}	97±0.87^{b}	8.23±0.13
13	85±0.21^{b}	86±0.51^{c}	92±0.24^{a}	95±0.34^{a}	98±0.34^{a}	7.65±0.19
14	33±1.87^{h}	34±1.96ij	36±1.84^{f}	39±2.65^{h}	41±1.96^{g}	39.21±1.59
15	37±1.97^{g}	39±2.34^{i}	42±1.67^{fg}	45±2.42^{g}	47±2.17^{f}	35.87±2.18
Vitamin C	95±0.11^{a}	105±0.21^{b}	110±0.25^{c}	112±0.31^{c}	120±0.45^{d}	7.23±0.55

DPPH = 2, 2-diphenyl-1-picryl hydrazyl, IC= inhibitory concentration

4.5. Cytotoxicity assay of derived fractions:

In the derived fractions of the current study, LC_{50} of all fractions ranged from 5.1±0.1 to 85.2±1.4µg/ml (Table 4.6). The fraction (F13) exhibited the lowest (5.1±0.1) while fraction (F2) presents the highest (85.2±1.4) LC_{50} values. The LC_{50} values for fractions (F3-F9) were 13.4±0.2, 10.5±0.1, 45.7±0.9, 43.1±0.9, 41.5±0.7, 40.6±0.4, and 39.1±0.4 µg/ml respectively. The fractions (F10 and F11-F15) presented 38.6±0.3, 35.9±0.2, 7.5±0.2, 5.1±0.1, 55.1±1.1, and 47.9±0.9µg/ml respectively. Interestingly all fractions showed LC50 <100µg/ml which indicates the safety range of all fractions for antilarval assays. The p-value of all fractions was significant (0.001). The toxicological effect of the plant extract is comparable to the effect of the standard drug (tricaine methanesulfonate). The results hence indicate that the plant extract has highly potent cytotoxic constituents.

Table 4.6: Cytotoxic potential of crude ethanolic extract fractions of *M. piperita* leaves.

Fractions	Total number of shrimps tested	Total number of shrimps killed	% mortality	LC_{50} (ppm)
2	20	3	15	85.2±1.4
3	20	15	75	13.4±0.2
4	20	16	80	10.5±0.1
5	20	6	30	45.7±0.9
6	20	7	35	43.1±0.9
7	20	9	45	41.5±0.7
8	20	10	50	40.6±0.4
9	20	11	55	39.1±0.4
10	20	13	65	38.6±0.3
11	20	14	70	35.9±0.2
12	20	17	85	7.5±0.2
13	20	18	90	5.1±0.1
`14	20	4	20	55.1±1.1
15	20	5	25	47.9±0.8

DISCUSSION

Because of their disease prevention and therapy actions, medicinal plants are becoming increasingly important in the pharmaceutical sector. Some physiologically active compounds were detected in all of the plant crude extracts at very high quantities. The high concentration of essential compounds could be a part of the plants defence system. The leaves of *M. piperita* were used to make acceptable crude extracts for bioactive chemical components, which were chosen based on the GC-MS analysis mentioned earlier. They are part of a large group of defensive substances known as 'phytoanticipins' or 'phytoprotectants' that have been discovered in this plant (Hossain and Nagooru, 2011). As a result, various crude extracts from *M. piperita* leaves were separated and identified using GC-MS. The chemical compounds found could have far-reaching repercussions for the environment (Al Hashmi *et al*., 2013).

The majority of bioactive chemicals with larvicidal activities have yet to be discovered. The active component Azadirachtin extracted from neem oil has been shown to have larvicidal potential against *Culex* mosquitoes, whereas limonoids, another active component of neem oil, has shown considerable antilarval activity against *Ae. aegypti* mosquitoes (Maistrello *et al*., 2003). The effect of bioactive fractions (clerodin, 15-methoxy14, 15-dihydroclerodin, and 15-hydroxy-14, 15-dihyroclerodin) isolated from methanolic leaves extract of *Clerodendron infortunatum* L. against the highly polyphagous pest *Helicocoverpa armigera*, the cotton bollworm, was reported by Abbaszadeh *et al*., (2014). Clerodin and 15-methoxy14, 15-dihydroclerodin has much stronger antilarval action in a dose-response bioassay than the major constituents in commercial pesticides.

Ahmed *et al*. (2016) discovered that extracts and their derived fractions from *Nepeta leavigata*, *Nepeta kurramensis*, and *Rhynchosia reniformis* have moderate to strong insecticidal properties. The insecticidal activity of the N-butanol fraction extracted from *Nepeta kurramensis* was the strongest, with 89% death against *Tribolium castaneum*, followed by 88% mortality in the case of *Nepeta leanigata*. Almeida *et al*., for example, conducted similar research (2015). They used a dose-response bioassay to assess the toxicity of phytochemical compounds derived from six medicinal plants, including *Schinus teribinthifolius*, *Pittosporum undulatum*, *Lippia sidoides*, *Lippia adracilis*, *Mentha arvensis*, and *Croton eajucara*, against *Heteotermes sulcatus*,

and found that the bioactive compounds limonene.

Mentha piperita was shown to be the most effective among the eight plants tested, with substantial antilarval, antioxidant, and cytotoxicity properties. Using a dose-response bioassay against *Cx. quinquefasciatus*, the antilarval activity of different fractions was studied. In a 72hr experiment, fifteen (2-15) fractions were employed, and the percentage mortality of mosquito larvae was measured using varied concentrations (80, 160, 240, 320 and 400ppm). In a time-dependent manner, all concentrations were found to have a significant influence on larvae mortality when compared to control ($F (df) = 511.5 (5); P<0.05$) and differ significantly from each other ($F (df) = 412.6 (13); P 0.05$).

In a dose and time-dependent manner, all fractions demonstrated differential antilarval activity ($F (df) = 255.7 (15; P <0.05$). Figure 4.1 depicts the influence of hours, concentrations, and fractions on the mean mortality of larvae. Fractions 3, 4, 12, and 13 were shown to be the most successful ($P<0.05$), with a 50% decrease in larval population after 72hrs and 94%, 95%,, 96% and 97% decreases at the end of the trial, respectively. After 24hrs of testing, fraction 11 caused 28% decrease. Fractions 9, 10 and 11 also lower larvae mortality by up to 63%, 67%, and 75%, respectively ($P<0.05$). Similarly, observed larvae mortality due to the toxic effect of fractions 5, 6, 7, 8 was 42%, 40%, 52%, and 55%, respectively. With a non-significant ($P>0.05$) drop in larval population, fractions 1, 2, 14, and 15 were determined to be the least effective.

They looked at the effects of vetiver oil, nootkatone, and disodium octaborate tetrahydride on the larvae population of *Cx. quinquefasciatus* and discovered that nootkatone and vetiver oil reduced larvae population significantly ($P<0.05$), which could be related to their antilarval actions. These findings are likewise equivalent to those of Hassan *et al*. study (2017). They studied the mortality rate of two mosquito species using heartwood extracts from *Cedrus deodara*, *Pinus roxburghii*, *Tectona grandis*, and *Dalbergia sissoo* (*Ae. aegypti* and *An. stephensi*). All plant extracts rapidly reduce the larvae population, eventually resulting in larvae mortality.

Biochemical analysis of the experimental larvae found that the extract-treated larvae had a much lower carbohydrate, protein, and lipid profile than the control group (Huang *et al*., 2004).

These effects are the result of Tenebrio molitor's oocyte hemolymph and fat components being reduced after being shown to Malathion (Sharma *et al*., 2011).

Microscopic examinations of treated larvae revealed that plant extracts caused sluggish spinning, loss of stability, and gut rupturing, all of which were fatal. This could be attributable to a decline in glucose levels compared to control. It is also possible that the insecticidal pressure induced by these extracts causes a decrease in scavenging presentation, restricted food depletion, and gut deprivation in the body wall (Lohar and Wright, 1993). The results of the current investigation demonstrated that after treatment with *M. piperita* extract, carbohydrate, proteins, and lipids (cholesterol, triglycerides, and high-density lipid level of all instars larvae of *Cx. quinquefasciatus*) were all lower than control in all fractions.

The present study high radical scavenging capabilities of fractions of *M. piperita* ethanolic extract are consistent with Deeksha and Arunachalam's prior investigation (2019). Fe3+ is transformed to Fe2+ for the purpose of obtaining reducing power, and Fe2+ combines with FeCl2 to generate complex ferrous. Kifayatullah *et al*., (2015) found that ethanolic extracts of *Pericampylus glaucus* (Lamk) increase in reducing power as the concentration of the extract increases, similar to the current investigation.

According to Eshwarappa *et al*., (2014), the methanol extract of *S. cumini* had better reducing power and potential than the usual ascorbic acid utilized. Through antioxidant mediators present in extract green colour phosphate/Mo (V) complex formation occurs in the Phospho molybdenum technique of TAC measurement. *M. piperita* and *O. basilicum* ethanolic leaf extracts have the highest antioxidant activity. Munira *et al*. (2018) found that *M. piperita* ethanolic leaves extract had total antioxidant activity comparable to standard catechin.

In the current research highest antioxidant activities based on the DPPH assay were detected in fraction 3, 4, 12 and 13 with lowest IC_{50} values (7.56, 8.23, 8.27 and 8.06) µg/ml respectively. These four active fractions eluted with ethanol solvent indicating that compounds eluted were relatively polar. The reducing power assay results indicated an increasing trend of reducing antioxidant power with increasing extract concentration in each fraction. Among the isolated fractions, fractions 3, 4, 12 and 13 possess the maximum reducing power (250±0.85), (255±0.65), (265±0.69) and (275±0.94) at 600 µg/ml respectively. That is slightly lower but

statistically similar to the reducing power of standard Ascorbic acid (3.9±1.04) on same concentrations. The Antioxidant activity of the tested samples evaluated by FRAP method could be ranked in following order; F13>F12>F4>F3>F11>F10>F9>F8>F7>F6>F5>F15>F14>F2>F1. Fractions having maximum reducing power were eluted with polar solvent. Among the tested fractions, F13 exhibited maximum total antioxidant capacity (85±0.25) µg/ml followed by F12, F4 and F3 (77±0.21, 72±0.34 and 70±0.54) µg/ml, lower than standard ascorbic acid total antioxidant capacity (59.32±1.47).

The *F. glomerata* extract showed moderate toxicity having LC_{50} values of 454.34µg/ml having a significance value of P=0.147 (Nguta *et al.,* 2012). Diverse collection location, the tissue of plants, time of harvest, and solvent extraction noticed that toxicity showed variations significantly (Bussmann *et al.* (2011). The leaves identified LC_{50} between 100 and 500g/ml cytotoxicity, which has served us for more research on biologically active extracts against fleas (*Xenopsylla cheopis*), mosquitoes (*Ae. aegypti*), ticks (*Ixodes scapularis*), microbes affecting forest and living thing health, and microbes affecting forest and living thing health (Johnston *et al*., 2001; Dietrich *et al*., 2006).

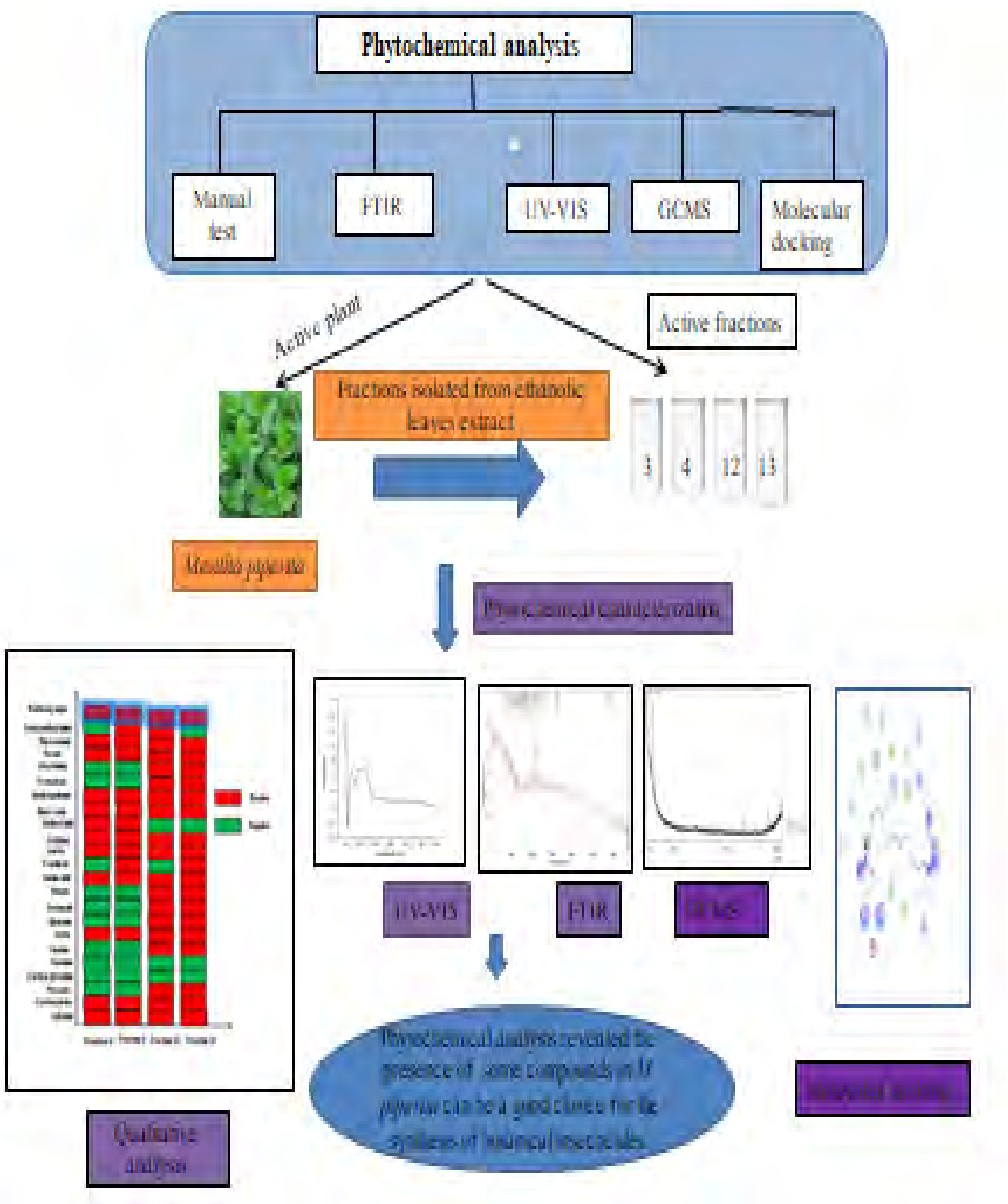
The LC_{50} of all fractions in the current investigation ranged from 5.12 to 85.24 g/ml in the derived fractions (Table 4.6). The fraction (F13) has the lowest LC_{50} value (5.12), while the fraction (F2) has the highest (85.21). Fractions (F3-F9) had LC_{50} values of 13.37, 10.45, 45.74, 43.11, 41.54, 40.56, and 39.12 g/ml, respectively. F10 and F11-15 showed 38.64, 35.87, 7.54, 5.12, 55.12, and 47.87 g/ml, respectively. Interestingly, all fractions had an LC_{50} of 100g/ml, indicating that all fractions are safe for antilarval assays. All fractions had a significant p-value (0.001). The plant extracts toxicological effect is comparable to that of a typical medication (tricaine methanesulfonate). As a result, the findings suggest that the plant extract contains cytotoxic constituents in high concentrations.

The current study findings show that bioactive fractions from *M. piperita* crude leaves extract contain a wide range of phytochemical compounds and have promising larvicidal properties as evidenced by a significant decrease in the population of mosquito larvae.

CHAPTER 4 (Part-II)

PHYTOCHEMICAL ANALYSIS OF BIOACTIVE FRACTIONS ISOLATED FROM *MENTHA PIPERITA* LEAVES EXTRACT USING UV-VIS, FT-IR, GC-MS AND MOLECULAR DOCKING

GRAPHICAL ABSTRACT



ABSTRACT

Phytochemical screening is a feasible method for identifying novel chemical compounds in plant extracts, and it has ethnopharmacological uses. Many secondary metabolites isolated from crude plant extract had very different chemical properties depending on the plant species. The composition of these chemical compounds can be determined through phytochemical analysis utilizing various spectroscopic techniques. The current work used preliminary phytochemical assays, UV-VIS, FT-IR, and GC-MS analysis to assess the bioactive components present in ethanolic leaves extract of *Mentha piperita*. In all fractions, qualitative phytochemical analysis revealed the presence of alkaloids, carbohydrates, sterol, amino acid, starch, cellulose, flavonoids, phenol, Betacyanin, anthraquinones, anthocyanin, steroid, and Phytosteroid. The UV-VIS profile revealed typical peaks spanning from 300-800nm with varied absorptions using the Perkin Elmer Spectrophotometer. The well-accepted FT-IR analysis was performed to identify different functional groups based on typical peak values, and the spectrum was recorded between 400 and 4000cm-1.The FT-IR spectrum of active fractions 3, 4, 12, and 13 confirmed the existence of alcohol, primary amine, aliphatic primary amine, secondary amine, alkane, carboxylic acid, alkene, anhydride, nitro-compound, conjugated anhydride, aromatic ester/aromatic amine, alkyl aryl ether, ester/tertiary alcohol, aliphatic ether, sulfoxide, aliphatic phosphate, aromatic phosphate, halo-compound, aliphatic-primary amine, alkyne, aldehyde, acid-anhydrides, amide, isothiocyanate, secondary alcohol, and nitrile. The results of the GC-MS analysis provide different peaks determining the presence of some phytochemical compounds e:g ethanol, ammonium oxalate, carbazic acid, propanamide, isobornylthiocyanoacetate, 1, 2-Benzenedicarboxylic acid, N-[5-(5-phenyl-3,4-dihydro-2H-pyrrol-4-yl)nonan-5-yl]benzamide, eicosanoic acid, phytol, n-hexadecenoic acid with different therapeutic activities. The 1, 2-Benzenedicarboxylic acid of F3, eicosanoic acid of F4, Phytol of F12, and N-[5-phenyl-3, 4-dihydro-2H-pyrrol-4-yl) nonan-5-yl] benzamide of F13 have the highest binding affinity for the target NS3 protease, according to an in silico molecular docking research. F3, F4, F12, and F13 revealed larvicidal activity among all fractions, according to the findings. The presence of these phytochemicals in

bioactive fractions suggest that *M. piperita* could be a good substitute for botanical pesticide production. The phytochemical profile of *M. piperita* leaf extract revealed that it could be a best source of larvicides.

INTRODUCTION

Medicinal plants are a gift from nature that plays an important role in disease prevention and treatment. They can be a source of biologically and pharmacologically important chemical substances. Photochemistry is a discipline of chemistry that deals with phytochemicals that come from plants. Plants make primary metabolites like proteins, lipids, nucleic acids, and carbohydrates from modest amounts of water, carbon dioxide, nitrogen, and inorganic salts. Secondary metabolites (alkaloids, steroids, terpenoids, saponins, flavonoids) are produced from these main metabolites used as medications (Yakubu *et al*., 2018). Generally, they are a vital source with different structural arrangements (Koduru *et al*., 2006) and biological properties for example antioxidant activity, antimicrobial effect, detoxification enzyme modulation, immune system stimulation, platelet aggregation reduction, hormone metabolism modulation, and anticancer property (Padma *et al*., 2016). They are causes of effective pharmaceuticals and will continue to be relevant from the early times for screening of new compounds. According to the World Health Organization (WHO) in 2008, more than 80% of the world's population depends on traditional medicine for their basic healthcare necessities (Priyanka *et al*., 2018). Vegetations, fruits, and vegetables, as well as microbes contain bioactive chemicals (Dharmaaj, 2010**;** Liu, 2013).

Plants bioactive chemicals are classified according to a number of factors. Bioactive chemicals are one of the most common ingredients found in botanicals and herbal preparations for therapeutic use. Bioactive chemicals found in fruits include polyphenols, flavonoids, and tannins (Park *et al*., 2012). Some examples of bioactive chemicals found in plants are terpenoids and alkaloids (Bernhoft, 2010). As one of the important ingredients present in natural sources, bioactive chemicals have an impact on living organisms, tissues, and cells. The physician, pharmacist, or toxicologist is interested in the pharmacological and toxicological consequences of their clinical role. Almost 20,000 medicinal plants occur in 91 countries, according to the World Health Organization (WHO) (Pervez *et al*., 2015).

Its therapeutic potential is shown by the presence of phytochemical substances in the plants. Tannins indicate that plants have antiparasitic, antiviral, and

antibacterial properties. Flavonoids are phenolic chemicals that have antioxidant, anti-inflammatory, anti-allergic, and cancer-preventive properties. Saponins are antifeedants that are utilized in vaccines as an adjuvant. Alkaloids possess antibacterial, anticancer, antiarrhythmic, and analgesic properties. Steroids are vital in the prevention of cardiotonic activity and function as signaling molecules (Chhetri *et al*., 2008). Carmex and Chloraseptic spray are oral analgesics contain phenols as an antiseptic and active component. Plant chemical components are necessary since they may disclose novel basis of cost-effective chemicals such as tannins, oils, and gums, as well as the precursor for the production of new chemical substances that can be used in medications (Sonam *et al*., 2017).

As a result of today's unhealthy lifestyles among humans is one of the dynamic forces behind the search for natural products that have been shown to have numerous health benefits and fewer side effects (Almeida *et al*., 2011). There have been numerous efforts to enhance the number of chemicals available for therapeutic prospects. In vitro screening has become the favoured method because of the need to minimize costs and speed up the drug development process (Yuliana *et al*., 2011). Further research into new bioactive compounds in plants may lead to drug formulation (Theng and Korpenwar, 2015), lead structure for medicine (Duarte *et al.*, 2012), development of new therapeutic agents (Lakshmi *et al*., 2014), and herbal alternatives for the treatment of various diseases (Karpagasundari and Kulothungan, 2014). Multiple drug resistance has hampered the development of new synthetic antimicrobial medications, forcing researchers to look for new antimicrobials from other sources (De Fátima *et al*., 2006).

Antibiotics have traditionally been derived from natural compounds and from microbial sources. However, as herbal therapy becomes more widely recognized as a viable alternative to conventional treatment, screening medicinal plants for active chemicals has become increasingly important. Sahaya *et al*. (2012) found that a substantial number of medicinal plants and their refined compounds had therapeutic potential. Bioactive molecules are gaining popularity in a variety of fields, including modern pharmacology, agrochemicals, cosmetics, food business, and nanobioscience.

One of the most important components for therapeutic reasons is bioactive

chemicals. The majority of scientists are interested in extracting and isolating the bioactive molecule. Identification of bioactive substances is also critical (Ibrahim *et al*., 2008; Guaadaoui *et al*., 2014).

Mentha piperita (peppermint) belongs to family Lamiaceae and genus *Mentha.* It contains fruit preserves, alcoholic beverages, chewing gum, toothpaste, soaps, and skincare products. *Mentha* has 25 species that can be found in many different parts of the world. Peppermint has a lot of menthol, macrocyclic phenols, tannin derivatives, flavonoids, and alkaloids found in earlier research (Khanna *et al*., 2014).

The chemical complexity of phytoconstituents is a major obstacle to determining plant product quality characteristics for the practice of in vitro screening methods to control potential phytocomponents (Deattu *et al*., 2013). It is difficult to fully characterize all of the chemical compounds in herbal medicines due to the large number of chemical compounds and pharmacological studies (Mathekaga and Meyer, 1998; Pierangeli *et al*., 2009). The technique of column chromatography is used to quantify and isolate active phytocompounds from plant crude extracts (Church *et al*., 2005). Thin-layer chromatography (TLC) is used for compound identification and purity determination (Abdulhamid *et al*., 2017). The discovery of novel sources of significant therapeutic chemicals is aided by phytochemical research. Gas chromatography-mass spectrometry (GC-MS) is an instrumental method that separates, identifies, and quantifies complex mixtures of substances using a gas chromatograph (GC) and a mass spectrometer (MS). It is suitable for evaluating hundreds of low-molecular-weight compounds present in environmental materials. To be examined by GC/MS, a molecule must be volatile and thermally stable (Nivetha and Prasanna, 2016).

Phytoconstituents are mostly determined using relatively expensive and time-consuming chromatographic techniques like gas (GC) and liquid (LC) chromatography combined with specific detection schemes (Uzer *et al*., 2005), thin Layer Chromatography, high-Performance Liquid Chromatography (HPLC), (TLC), and bio-autographic methods, as well as non-chromatographic techniques like immunoassay (Dharmaraj, 2010; Csernatoni, *et al*., 2013). The FT-IR is a valuable technique for detecting and characterizing chemicals or functional groups (chemical bonds) in an unidentified mixture of plant extracts (Aysal *et al*., 2007). It is a quick

and efficient method that needs very little sample preparation (Singh *et al*., 2011). At a given frequency, the development of bands in the infrared spectrum is further influenced by the functional groups allows for the qualitative assessment of organic molecules (Schulz *et al*., 2003). Phytocomponents are detected using UV-VIS spectroscopy (Gunasekaran *et al*., 2003).

The application of a UV-VIS spectrophotometer in complicated analysis is forced by its inability to assign absorption peaks to any specific system component. Other analytical techniques, such as GC/MS, etc., must be used in conjunction with UV-VIS findings to allow correct extract characterization and content identification. (Karpagasundari and Kulothungan, 2014). The most advanced method of GC-MS (gas chromatography-mass spectrometry), which also enables the mass spectrum of each component to be recorded, is used to analyze the bioactive components (Krishnakumari and Nagaraj, 2012). The development of hyphenated chromatographic methods like GC or LC-MS has simplified and reduced the cost of evaluating small amounts of chemicals. GC-MS can identify pure compounds present at less than 1ng (Eisenhauer *et al*., 2009).

The in-silico method now offers a supply of potential drug options, and all parasite therapies are known (Fu *et al*., 2015). In the case of neglected illnesses, where an exact drug improvement method is not possible and there is no commercial return on a significant investment, repurposing drug aspirants is an elegant in-silico policy difference (Feng *et al*., 2020). Target-based drug design is a second strategy that has gained adhesion in the post-genomic era, in which possible pharmacological goals are reformatted using in silico methods that include genome and protein databases (Azam *et al*., 2013). The molecular in silico docking technique is a computer technique used in structure-based rational drug design to identify optimal ligand conformation and protein-ligand interactions, which usually include one ligand and one receptor (Tantar *et al*., 2008; Yuriev *et al*., 2009; Mura and McAnany, 2014). Autodock Vina (Trott and Olson, 2010), Flex X (Rarey *et al*., 1996), GOLD (Jones *et al*., 1997), and Autodock Vina (Trott and Olson, 2010) are the most commonly used applications and programmes (Morris *et al*., 2009). In the contemporary drug discovery process, molecular docking techniques are routinely utilized to recognize protein-ligand interactions. The protein-ligand composite's three-dimensional

structure may be a useful tool for understanding how proteins interact with one another and perform biological functions.

The exact atomic structure of protein-ligand and their complexes is one of the most crucial subjects in biological sciences as a result. The majority of the docking experiments are then conducted in the protein databank, where conformational changes are brought about by ligand binding. To improve interactions with the ligand, small side-chain rotations can be necessary. Molecular docking and virtual screening methods have been applied in numerous recent structure-based drug discovery endeavors. Therefore, information on the interactions of proteins and ligands with particular drugs may offer important understanding of the binding interactions and relativeness of the drug (Li *et al*., 2004). The growing demand for new pharmaceuticals has led to drug docking, a computer approach that predicts prospective drugs. The procedure needs predicting the structure and orientation of the ligand within the targeted binding site. The key proteins involved in the delivery of odorants and pheromones to the olfactory receptors in insects are assumed to be odorant binding proteins. Members of these protein families have been found in a variety of insect species, including *Cx. quinquefasciatus*, which aids in the identification of its host (Seyoum *et al*., 2000).

Molecular recognitions, including drug-protein interactions, are essential for a variety of biological activities, such as signal transmission, cell regulation, and other macromolecular assemblies. Determining the binding method and affinity of the constituent molecules in molecular recognition is crucial for comprehending interaction mechanisms and developing treatment approaches. For anticipating likely binding modes and affinities, computational methods like molecular docking are required because experimental methods for determining the structures of complexes are difficult and expensive. The current study was carried out to better understand the mechanism of interaction between the mosquito species *Cx. quinquefasciatus*, odorant binding protein and many important ligands from the plant *Calotropis gigantean* (Weigel *et al*., 2002).

Until date, these and other comparable methods have mostly focused on docking between two molecules through non-covalent interactions like as hydrogen bonding, electro statistic contact, and Van der Waals interaction, or by using scoring

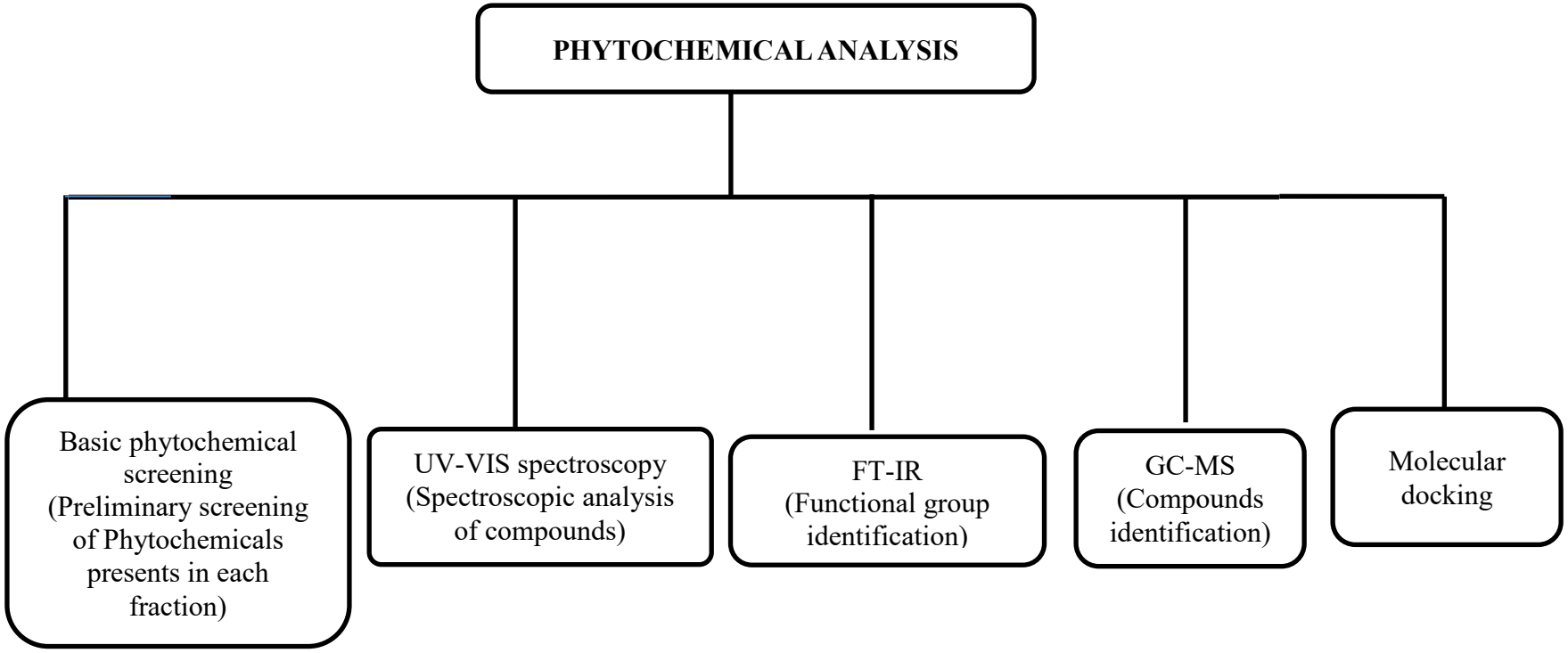
systems to identify these non-covalent interactions (Rarey *et al*., 1996). Though not all medications have a non-covalent interaction with the active site, there are a variety of drugs classified as covalent (Singh *et al*., 2011). Nowadays, non-covalent ligand-receptor interactions are more common. A crucial tool for the development of antilarval medicines is the successful prediction of the binding mechanism of non-covalent inhibitors (Yuriev and Ramsland, 2013) using the major computational technique known as molecular docking (Kellenberger *et al*., 2004; Yuriev and Ramsland, 2013).

However, in this research, we discuss active fractions isolated from *M. piperita* and larvicidal effect against *Cx. quinquefasciatus* and then characterized for phytocomponents identification, as well as molecular docking studies to determine the NS3 Protease gene's active binding site.

MATERIALS AND METHODS

5.1. Phytochemical Characterization

Different phytochemical tests were used to undertake a qualitative phytochemical analysis of effective fractions (3, 4, 12, and 14) extracted from ethanolic leaves extract of *M. Piperita*.



4.1.1: Phytochemical Screening

The qualitative phytochemical analysis of effective fractions (3, 4, 12, 13) isolated from *M. piperita* was conducted by using different phytochemical tests. Further the bioactive fractions are examined by qualitative phytochemical screening, Ultraviolet visible spectroscopy (UV-VIS), Fourier transforms infrared spectroscopy (FT-IR) and Gas chromatograph coupled spectroscopy (GC-MS). Detailed methodology of each analytical technique is already described in chapter 3.

RESULTS

5.1: Qualitative phytochemical characterization

Preliminary phytochemical investigation of bioactive fractions 3, 4, 12, and 13 exhibited the occurrence of different secondary metabolites such as alkaloids, carbohydrates, sterol, amino acid, starch, cellulose, flavonoids, phenol, betacyanin, anthraquinones, anthocyanin, steroid, phytosteroid was present in all fractions. Cardiac glycosides and saponins were absent in all fractions. Tannin, quinones, terpenoids, coumarins, and phlobatannins were absent in fractions 3and 4 while present in fractions 12 and 13. The volatile oil was present only in fraction 4 and absent in the other three fractions. Anthocyanins were present in fractions 3 and 4 but absent in fractions 12 and 13. Leucoanthocyanin was present in fractions 4 and 12 while absent in fractions 3 and 13 (Table 5.1 and figure 5.1).

Table 5.1: Qualitative phytochemical analysis of bioactive fractions isolated from *M. piperita.*

Phytochemical Compounds	Fraction 3	Fraction 4	Fraction 12	Fraction 13
Alkaloids	+	+	+	+
Carbohydrates	+	+	+	+
Flavonoid	+	+	+	+
Cardiac glycosides	-	-	-	-
Saponins	-	-	-	-
Tannin	-	-	+	+
Sterol	+	+	+	+
Quinones	-	-	+	+
Terpenoids	-	-	+	+
Phenol	+	+	+	+
Amino Acid	+	+	+	+
Volatile Oil	-	+	-	-
Starch	+	+	+	+
Cellulose	+	+	+	+
Anthocyanin	+	+	-	-
Betacyanin	+	+	+	+
Anthraquinones	+	+	+	+
Coumarins	-	-	+	+
Phlobatannins	-	-	+	+
Steroid	+	+	+	+
Phytosteroid	+	+	+	+
Leucoanthocyanin	-	+	+	-

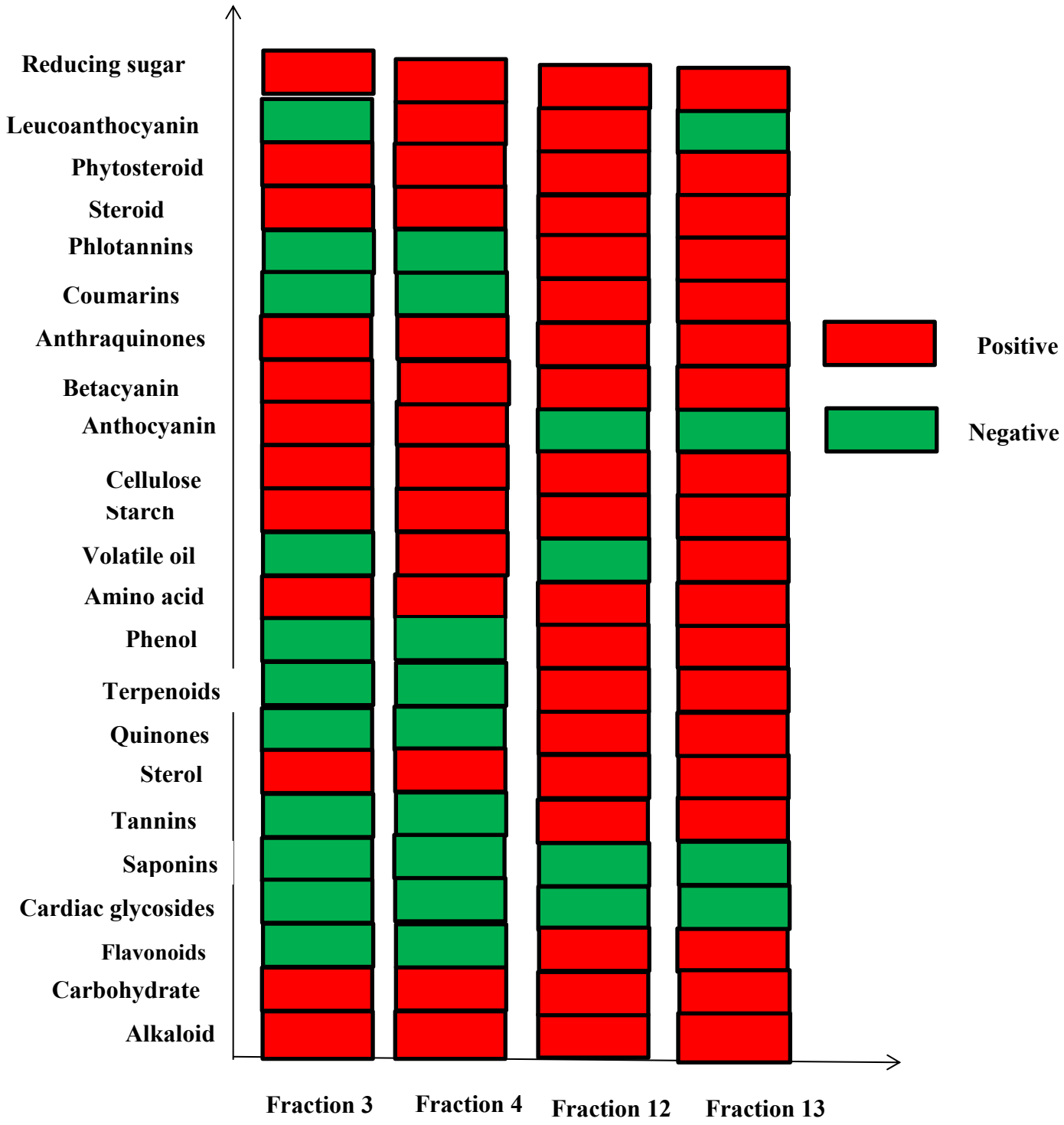


Figure 5.1: Qualitative phytochemical analysis of bioactive fractions

5.2: Fourier Transform infrared spectroscopy (FT-IR)

FT-IR analysis of bioactive fractions (3, 4, 12, and 13) in ethanol solvent helps in the identification of functional groups of active compounds present in each fraction. Each peak in the spectrum represents the specific peak value corresponding to the specific functional groups. The absorbance was noted from 400 to 4000 cm^{-1} wavelength. The results of FT-IR analysis confirmed the presence of alcohol, primary amine, aliphatic primary amine, secondary amine, alkane, carboxylic acid, alkene, anhydride, nitro-compound, conjugated anhydride, aromatic ester/aromatic amine, alkyl aryl ether, ester/tertiary alcohol, aliphatic ether, sulfoxide, aliphatic phosphate, aromatic phosphate, halo-compound, aliphatic-primary amine, alkyne, aldehyde, acid-anhydrides, amide, isothiocyanate, secondary alcohol, and nitrile present in all fractions (3, 4, 12, and 13).

The absorbance between 3124.8 -3732.9cm^{-1} confirmed the presence of alcohol. The absorption peak at 3403.8-3491.6 represents primary amine while absorption at 3353.2-3398.1 cm^{-1} represents aliphatic primary amine. The absorption range of secondary amine was 3336.7 cm^{-1} and peaks at 2954.5-2847.9 cm^{-1} exhibit alkanes. The absorbance between 3533.9-3641.6 cm^{-1} revealed the presence of amide and was observed in fractions 12 and 13. The absorption peak of aldehyde was between 263.9-2819.1 cm^{-1} and present in fractions 4 and 12. Carboxylic acid was observed in fractions 3, 12, and 13 with absorption peaks 3197.3-3284.7cm^{-1}. Alkene was present in fractions 3, 4, and 12 having absorbance at 704.5-3077.9 cm^{-1} respectively. Halo-compounds were present in fractions 3 and 4 with absorption peak 633.2-664.3cm^{-1} respectively, and absent in fractions 12 and 13. Alkyl–aryl ether was absent in fraction 13 while in remaining fraction absorption was 1064.5-1255.9 cm^{-1}. Aliphatic ether was present only in fraction 3 (1110.3cm^{-1}). Nitrile was present only in fraction 13 (2308.1cm^{-1}). Isothiocyanate was present only in a fraction 12 at an absorption peak of 2053.5 cm^{-1}. Aliphatic phosphate was present only in fraction 3 with an absorption peak of 99678 cm^{-1}. Sulfoxide with S=O stretch has a maximum absorption at 1061.6 in fraction 3. Alkyne was present only in fraction 4 with an absorption peak of 3268.3 cm^{-1}. Aromatic phosphates with P-C-O stretch have a maximum absorption at 858.4-898.1 cm^{-1} in fraction 3 while Nitro-compounds with N=O stretch at 1542.1-1558.9 cm^{-1} in fraction 3 and 4 respectively. Aromatic compounds were present in fractions 3 and 12 having an absorbance peak between 1280.1-1734.2 cm^{-1}. Anhydrides were present in fractions 3 and 4

having an absorbance peak observed between 1739.5-1766.3 cm^{-1}. Ester/ tertiary alcohol was present in fraction 3 at an absorption peak of 1164.6 cm^{-1}.

Table 5.2: FT-IR spectral peak values and functional groups obtained for fraction 3.

Peak No	Wavelength cm^{-1}/ Peak value	Stretching	Functional groups
1	3585.1	O-H	Alcohol
2	3548.8	O-H	Alcohol
3	3512.7	O-H	Alcohol
4	3491.6	N-H	Primary amine
5	3462.5	N-H	Primary amine
6	3443.2	N-H	Primary amine
7	3416.0	N-H	Primary amine
8	3366.4	N-H	aliphatic primary amine
9	3337.7	N-H	Secondary amine
10	3312.5	N-H	Secondary amine
11	3284.7	O-H	Carboxylic acid
12	3197.3	O-H	Carboxylic acid
13	3167.7	O-H	Alcohol
14	3077.9	C-H	Alkene
15	3028.3	C-H	Alkene
16	3006.9	C-H	Alkene
17	2951.8	C-H	Alkane
18	2891.5	C-H	Alkane
19	2849.3	C-H	Alkane
20	1766.3	C=O	Anhydride
21	1739.5	C=O	Conjugated anhydride
22	1542.1	N-O	Nitro compound
23	1280.1	C-O/C-N	Aromatic ester /Aromatic amine
24	1255.9	C-O	Alkyl aryl ether/aromatic ester
25	1164.6	C-O	Ester/tertiary alcohol
26	1110.3	C-O	Aliphatic ether
27	1061.6	S=O	Sulfoxide
28	996.8	P-O-C	Aliphatic phosphates
29	898.1	P-C-O	Aromatic phosphates
30	858.4	P-C-O	Aromatic phosphates
31	783.1	C-Cl	Halo compound
32	743.2	C-Cl	Halo compound
33	704.5	C=C	Alkene
34	664.9	C-Br	Halo compound
35	636.6	C-Br	Halo compound

Table 5.3: FT-IR spectral peak values and functional groups obtained for fraction 4.

Peak No	Wavenumber (cm^{-1})/ Peak value	Stretching	Functional groups
1	3732.9	O-H	Alcohol
2	3707.6	O-H	Alcohol
3	3638.5	O-H	Alcohol
4	3597.8	O-H	Alcohol
5	3557.4	O-H	Alcohol
6	3511.9	O-H	Alcohol
7	3479.2	O-H	Alcohol
8	3446.5	O-H	Alcohol
9	3423.3	N-H	Primary amine
10	3398.1	N-H	Aliphatic Primary amine
11	3375.9	O-H	Alcohol
12	3351.2	O-H	Alcohol
13	3324.9	N-H	Secondary amine
14	3308.3	N-H	Secondary amine
15	3268.3	C-H	Alkyne
16	3167.7	O-H	Alcohol
17	3145.5	O-H	Alcohol
18	3084.2	C-H	Alkene
19	3063.3	C-H	Alkene
20	2954.5	C-H	Alkane
21	2954.5	C-H	Alkane
22	2848.3	C-H	Alkane
23	2632.9	C-H	Aldehyde
24	2603.8	O-H	Alcohol
25	2485.4	O-H	Alcohol
26	1744.7	C=O	Acid Anhydrides
27	1558.9	N=O	Nitro-Compound
28	1509.8	N=O	Nitro-Compound
29	1470.4	C-H	Alkane
30	1064.5	C-O	Alkyl aryl ether
31	894.8	C=C	Alkene
32	633.2	C-Br	Halo Compound

Table 5.4: FT-IR spectral peak values and functional groups obtained for fraction 12

Peak .No	Wavelength (cm^{-1}) / Peak value	Stretching	Functional groups
1	3641.6	N-H	Amide
2	3555.9	N-H	Amide
3	3486.2	O-H	Alcohol
4	3444.7	N-H	Primary amine
5	3403.8	N-H	Primary amine
6	3353.2	N-H	Aliphatic primary amine
7	3329.9	N-H	Secondary amine
8	3296.6	O-H	Carboxylic acid
9	3272.7	C-H	Alkyne
10	3234.9	O-H	Alcohol/Carboxylic acid
11	3215.5	O-H	Alcohol/Carboxylic acid
12	3171.2	O-H	Alcohol
13	3070.3	C-H	Alkene
14	2964.1	C-H	Alkane
15	2876.9	C-H	Alkane
16	2819.1	C-H	Aldehyde
17	2053.5	N=C=S	Isothiocyanate
18	1734.2	C-H	Aromatic compound
19	1471.1	C-H	Alkane
20	1101.8	C-O	Secondary alcohol
21	1059.3	C-O	Alkyl aryl ether

Table 5.5: FT-IR spectral peak values and functional groups obtained for fraction 13.

Peak. No	Wave length (cm^{-1}) peak value	Stretching	Functional groups
1	3617.8	O-H	Alcohol
2	3591.2	O-H	Alcohol
3	3560.3	N-H	Amide
4	3533.9	N-H	Amide
5	3482.1	N-H	Primary amine
6	3377.7	N-H	Aliphatic primary amine
7	3357.7	N-H	Aliphatic primary amine
8	3336.7	N-H	Secondary amine
9	3251.2	N-H	Aliphatic primary amine
10	3208.5	O-H	Carboxylic acid
11	3181.4	O-H	Carboxylic acid
12	3124.8	O-H	Alcohol
13	2988.6	C-H	Alkane
14	2958.7	C-H	Alkane

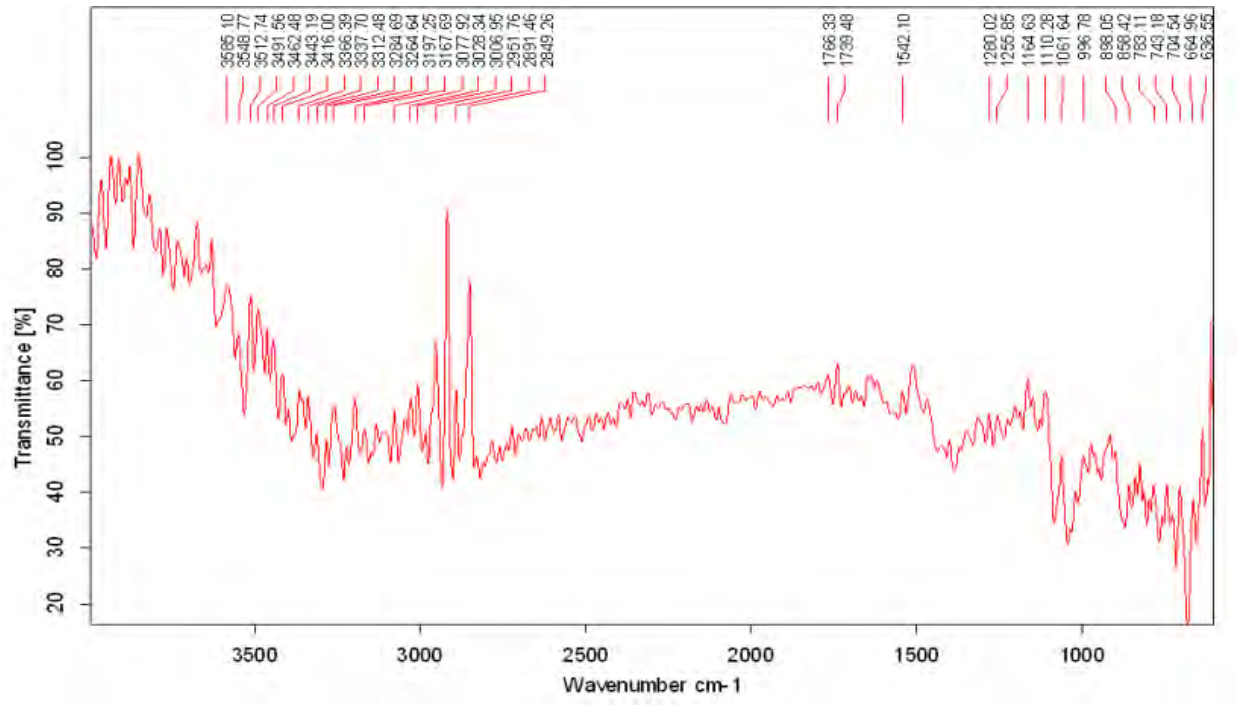


Figure 5.2: FT-IR spectrum of fraction 3.

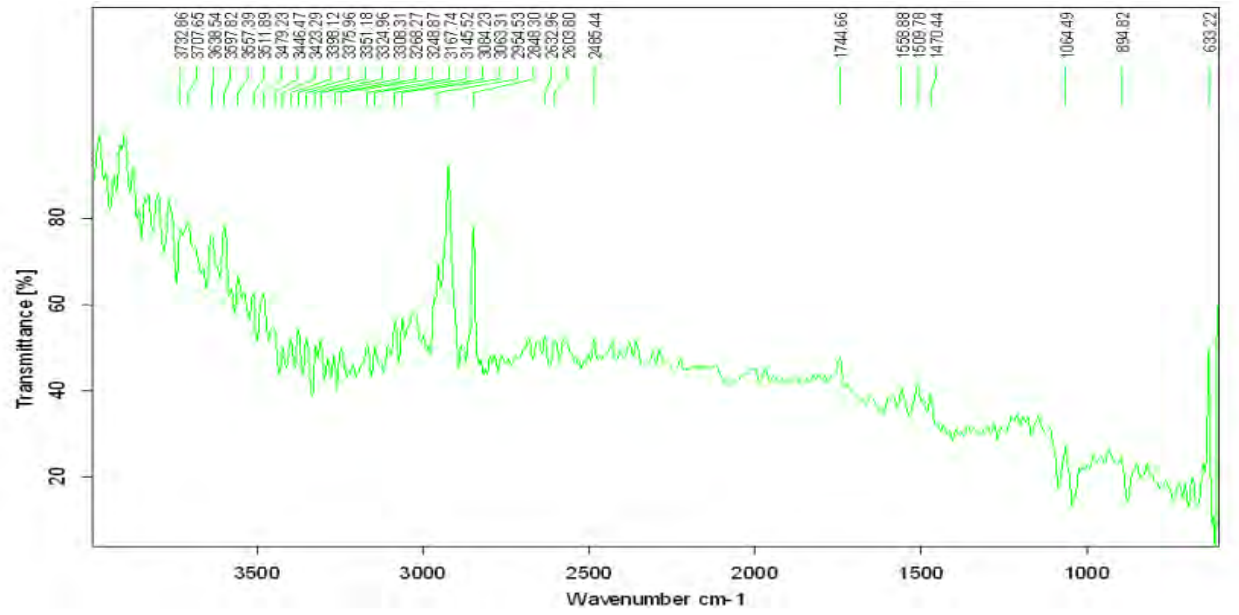


Figure 5.3: FT-IR spectrum of fraction 4.

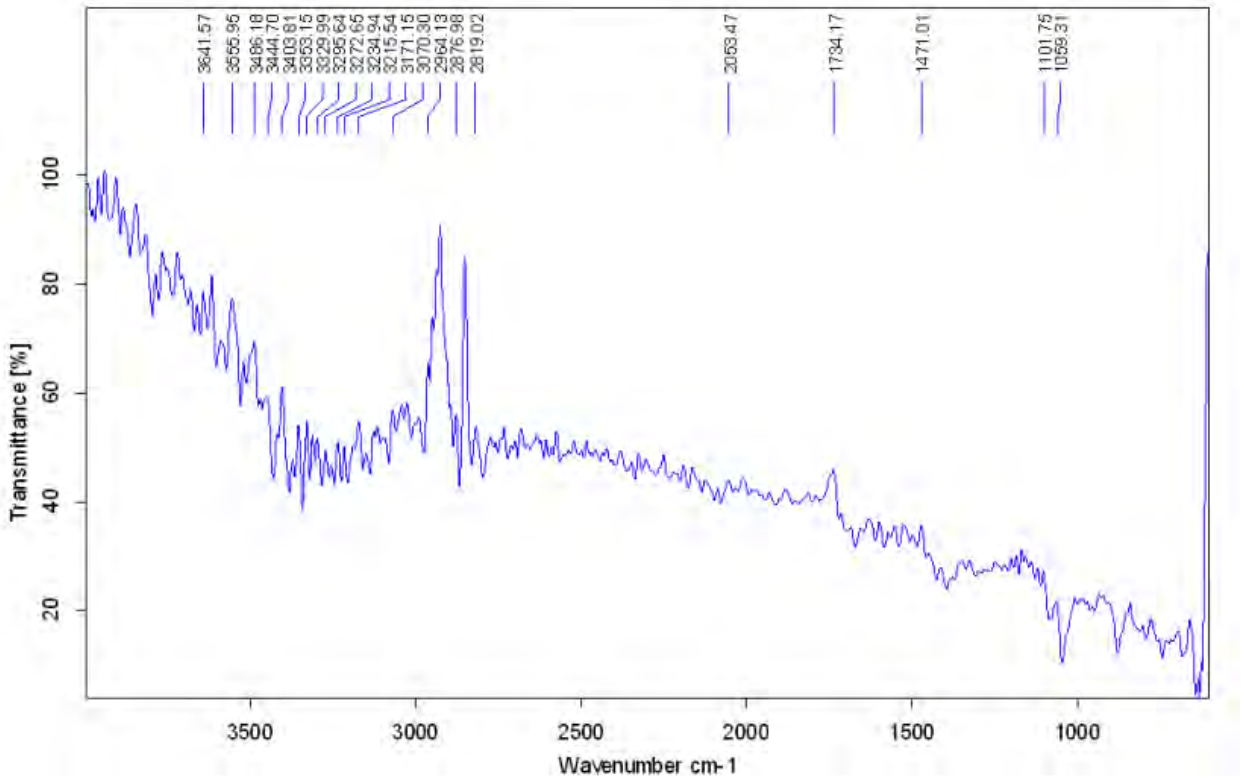


Figure 5.4: FT-IR spectrum of fraction 12.

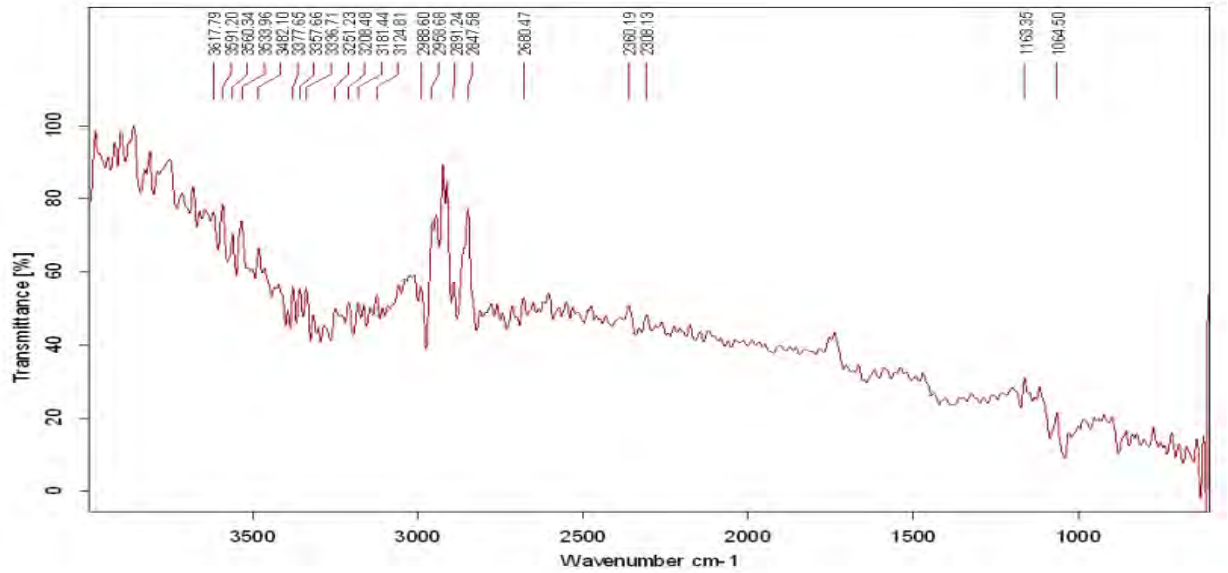


Figure 5.5: FT-IR spectrum of fraction 13.

5.3: UV-VIS Spectroscopic analysis:

Qualitative UV-Vis spectroscopic analysis of active fractions (3, 4, 12 and 13) from *M. piperita* showed a spectrum profile at 300 to 800nm due to the sharpness of peaks and proper baseline. The peak spectra of fraction 3 showed characteristic peaks at 666.8, 608.8, 535.6, 502.4, 402.9, 318.7 and 209.5 nm with the absorption of 0.331, 0.062, 0.051, 0.096, 1.020, 0.409 and 1.658 respectively. In case of fractions 4 observed peaks were 668.3, 633.6, 608.8, 532.9, 523.1, 502.4, 402.9, 344.9, 325.6, 311.7, 259.3, 238.5, 226.1 and 214.9 nm with absorption of 0.284, 0.059, 0.072, 0.093, 0.0803, 0.118, 0.875, 0.475, 0.492, 0.467, 1.129, 1.339, 1.254 and 0.558 respectively.

Peaks of fraction 12 were at 211.5, 236.7, 266.3, 291.3, 306.8, 327.8 and 383.6nm with absorption spectra of -0.655, 1.171, 0.767, 1.182, 1.205, 1.315 and 0.353 respectively. The observed peaks of fraction 13 were at 239.3, 264.2, 289.1, 331.4 and 357.72 nm with absorption of 0.031, 0.006, 0.166, 0.209 and 0.0734 respectively **(Table and figure 5.6-5.9).**

Table 5.6: UV-VIS spectrum values of fraction 3.

Sr. No	Wavelength (nm)	Absorbance
1	666.8	0.331
2	608.8	0.062
3	535.6	0.051
4	502.4	0.096
5	402.9	1.020
6	318.7	0.409
7	209.5	1.658

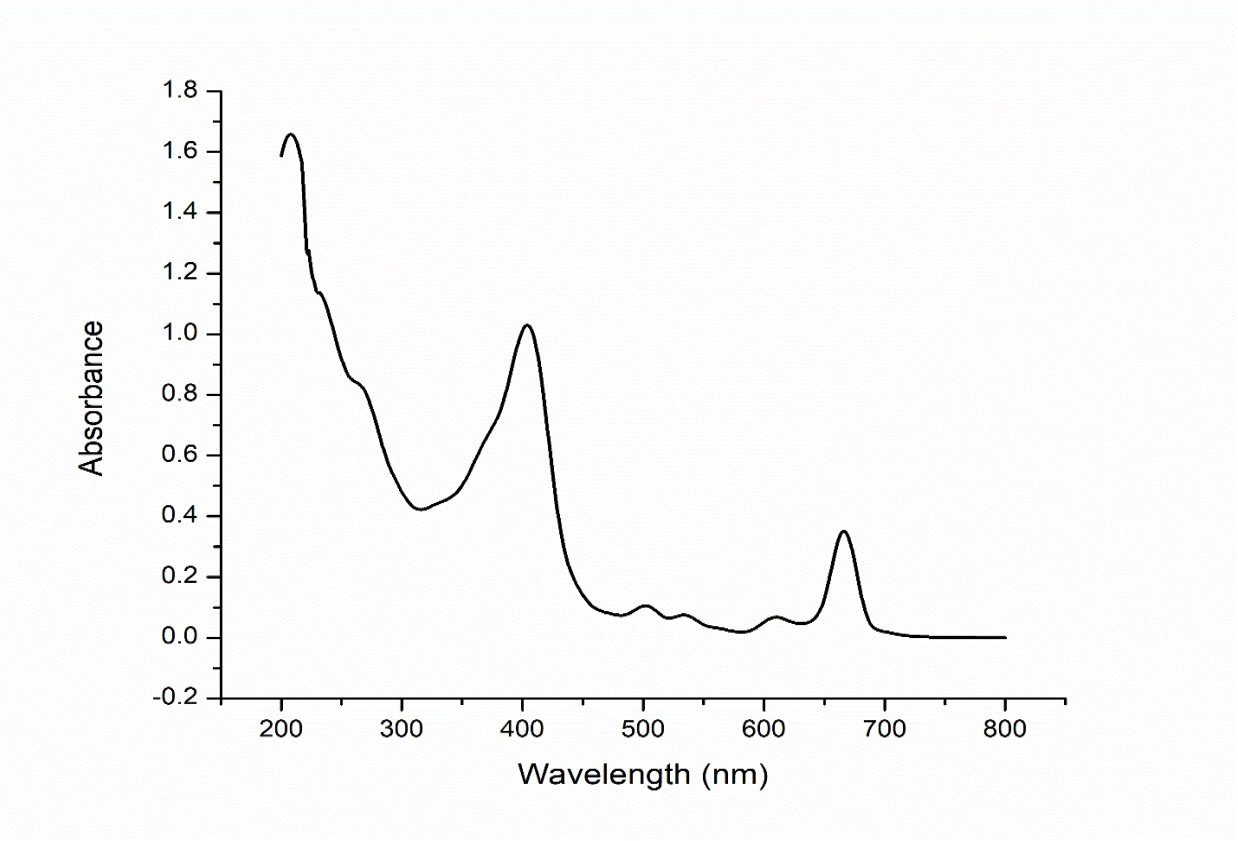


Figure 5.6: UV-VIS spectra of bioactive fraction 3.

Table 5.7: UV-VIS spectrum values of fraction 4.

Sr. No	Wavelength (nm)	Absorbance
1	668.3	0.284
2	633.6	0.059
3	608.8	0.072
4	532.9	0.093
5	523.1	0.0803
6	502.4	0.118
7	402.9	0.875
8	344.9	0.475
9	325.6	0.492
10	311.7	0.467
11	259.2	1.129
12	238.5	1.339
13	226.1	1.254
14	214.9	0.588

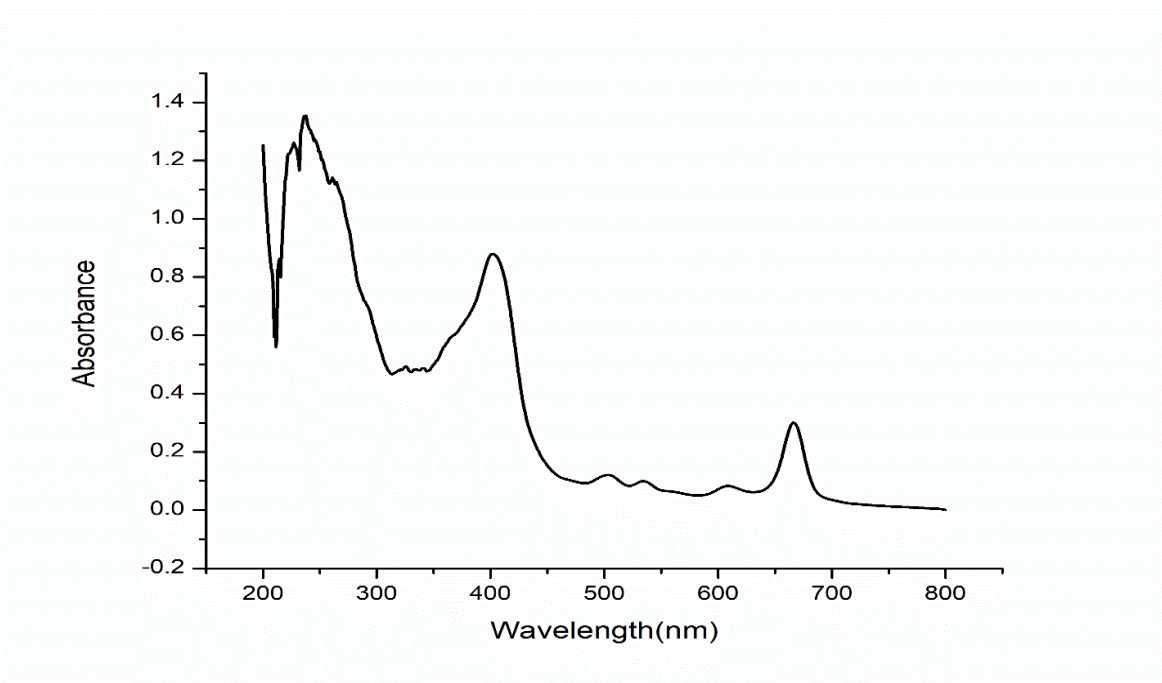


Figure 5.7: UV-VIS spectra of bioactive fractions 4.

Table 5.8: UV-VIS spectrum values of fraction 12.

Sr. No	Wavelength (nm)	Absorbance
1	211.5	-0.655
2	236.7	1.171
3	266.2	0.767
4	291.3	1.182
5	306.8	1.205
6	327.7	1.315
7	383.6	0.353

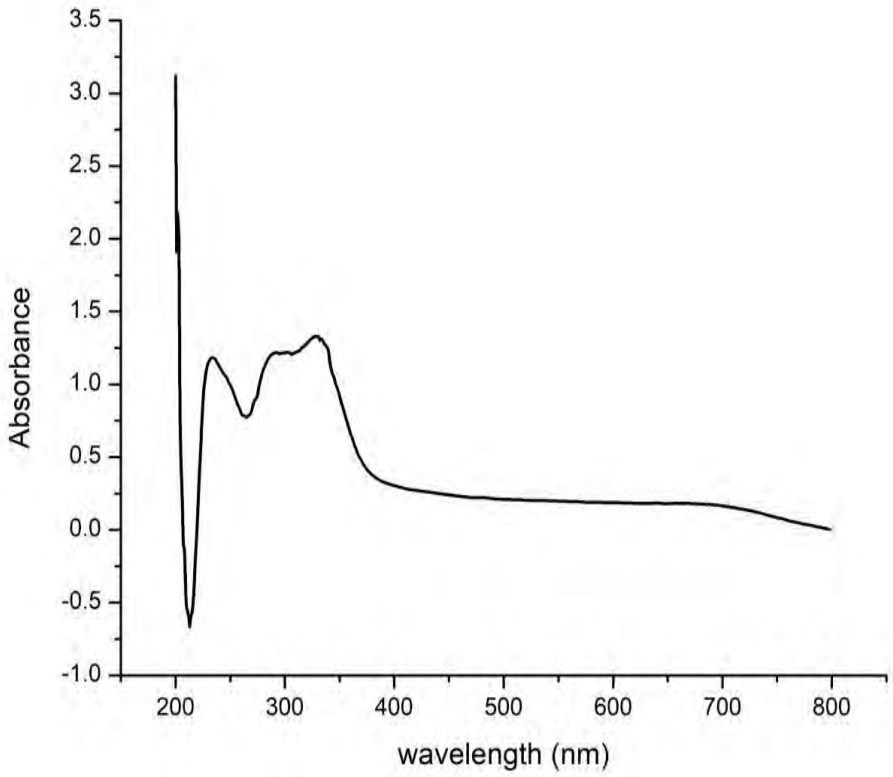


Figure 5.8: UV-VIS spectra of bioactive fractions 12.

Table 5.9: UV-VIS spectrum values of fraction 13

Sr. No	Wavelength (nm)	Absorbance
1	239.3	0.031
2	264.2	0.006
3	289.1	0.166
4	331.4	0.209
5	357.7	0.0734

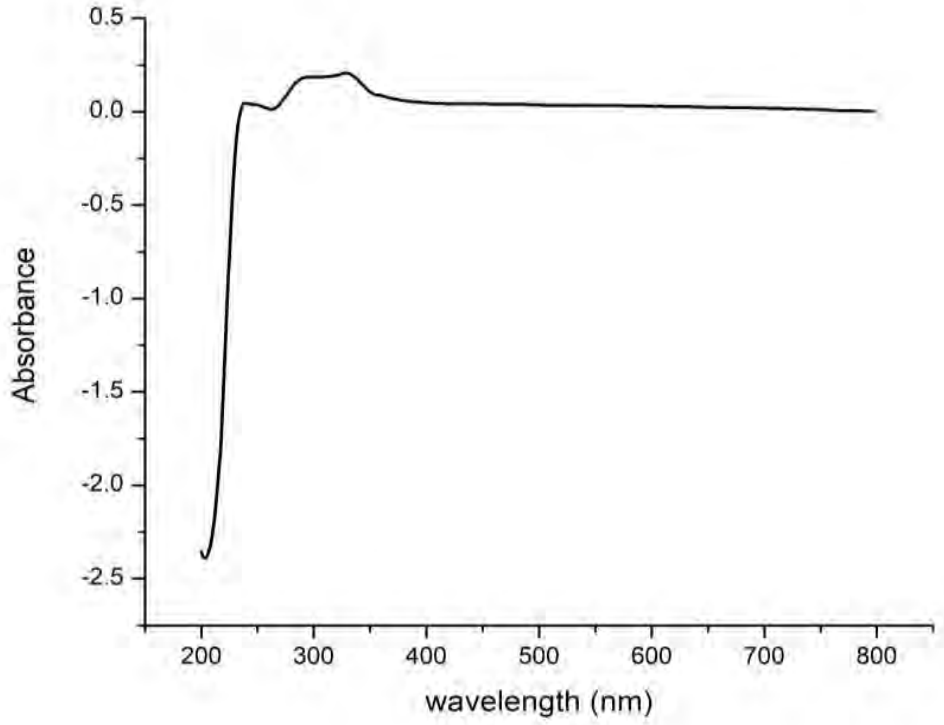


Figure 5.9: UV-VIS spectra of bioactive fractions 13.

5.4: Gas Chromatograph coupled Mass Spectroscopy (GC-MS)

5.4.1: GC-MS analysis of fraction 3

The GC-MS chromatograph of fraction 3 isolated from ethanolic leaves extract of *M. piperita* indicated twelve peaks which confirmed the presence of twelve bioactive compounds. The mass spectrum of each compound was characterized by comparison with the NIST library. Compound name, retention time, area percentage, molecular weight and a molecular formula of identified components are listed in **table 5.10**. The major bioactive compounds in fraction 3 are Ethanol (78.4%), 1, 2-Benzenedicarboxylic acid (60.21%), 1,2,4-Triazole (3.98%), Methoxyacetic acid (3.72%), Dodecamethyl (2.88%) , Carbazic acid (6.51%) and 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl (4.50%) but 1-Eicosanol (1.97%), decyl ester (1.57%), 3,4,5,6,9,10-hexahydro-3-methyl-14,16-bis[(trimethylsilyl)oxy] (1.31%), Nonyl nitrate (0.81%) and Ammonium oxalate (0.77%), are present in minor quantity. The GC-MS chromatogram of fraction 4 is represented in **figure 5.10**.

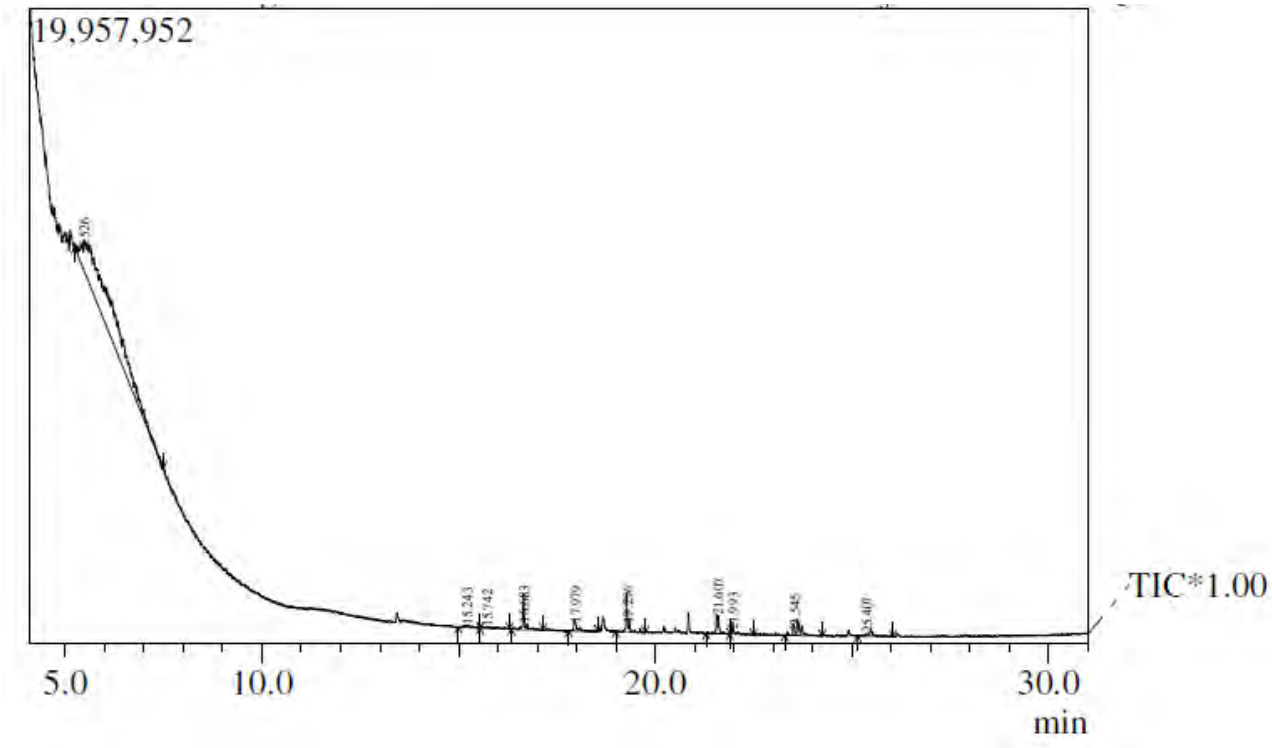
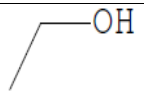
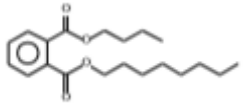
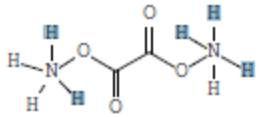
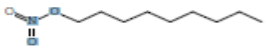
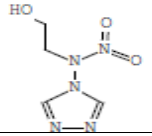
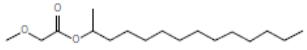
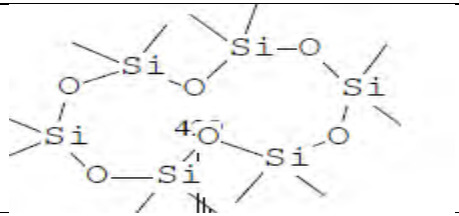
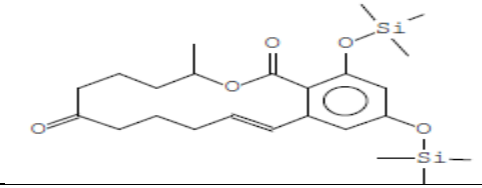
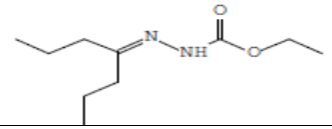
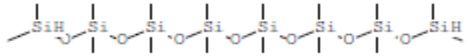
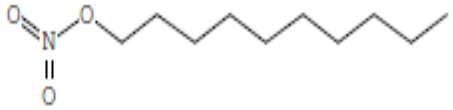


Figure 5.10: GC-MS chromatogram of fraction 3.

Table 5.10: Phytocomponents identified in fraction 3 by GC-MS peak report.

Peak #	Retention time	Area %age	Molecular Formula	Molecular Weight (g/mol)	Compound name	Structure
1	5.5	78.49	C_2H_6O	46	Ethanol	
2	10.1	60.21	$C_{20}H_{30}O_4$	334	1,2-Benzene dicarboxylicacid, butyl octyl ester	
3	15.2	0.77	$C_2H_8N_2O_4$	124	Ammonium oxalate	
4	15.7	0.81	$C_9H_{19}NO_3$	189	Nonyl nitrate	
5	16.7	3.98	$C_4H_7N_5O_3$	173	1,2,4-Triazole	
6	17.9	1.97	$C_{20}H_{42}O$	298	1-Eicosanol	
7	19.3	3.72	$C_{17}H_{34}O_3$	286	Methoxyacetic acid	

8	21.6	2.88	$C_{12}H_{36}O_6Si_6$	444	Dodecamethyl	
9	21.9	1.31	$C_{24}H_{38}O_5Si_2$	462	3,4,5,6,9,10-hexahydro-3-methyl-14,16-bis[(trimethylsilyl)oxy]	
10	22.9	6.51	$C_{10}H_{20}N_2O_2$	200	Carbazic acid	
11	23.5	4.50	$C_{16}H_{50}O_7Si_8$	578	1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl	
12	25.4	1.57	$C_{10}H_{21}NO_3$	203	decyl ester	

5.4.2: GC-MS analysis of fraction 4

The GC-MS chromatogram of fraction 4 isolated from ethanolic leaves extract of *M. piperita* revealed fifteen peaks at different retention times and confirm the presence of fourteen compounds **(Table 5.11).** Ethanol (85.49%), diethyl ester (2.07%), 5-Phenyl-3, 4-dihydro-2H-pyrrol (1.95%), 2-Tetradecanol (1.30%) , Eicosanoic acid (29.211%), ethoxyhydroxy (1.96%), Carbazic acid (2.01%), Isobornyl thiocyanoacetate (5.47%), 2-Pyrroline (6.54%), 4-Diazodamantanone (11.57%), and propanedioic acid (1.94%) are the major components of fraction 4. Other bioactive compounds are present in minor quantity include Propanamide (1.08%), Ammonium oxalate (0.94%), 1-Nitro-2-acetamido-1 (0.61%), 2-Heptanol (0.65%). The GC-MS chromatogram of fraction 4 is represented in **figure 5.11**.

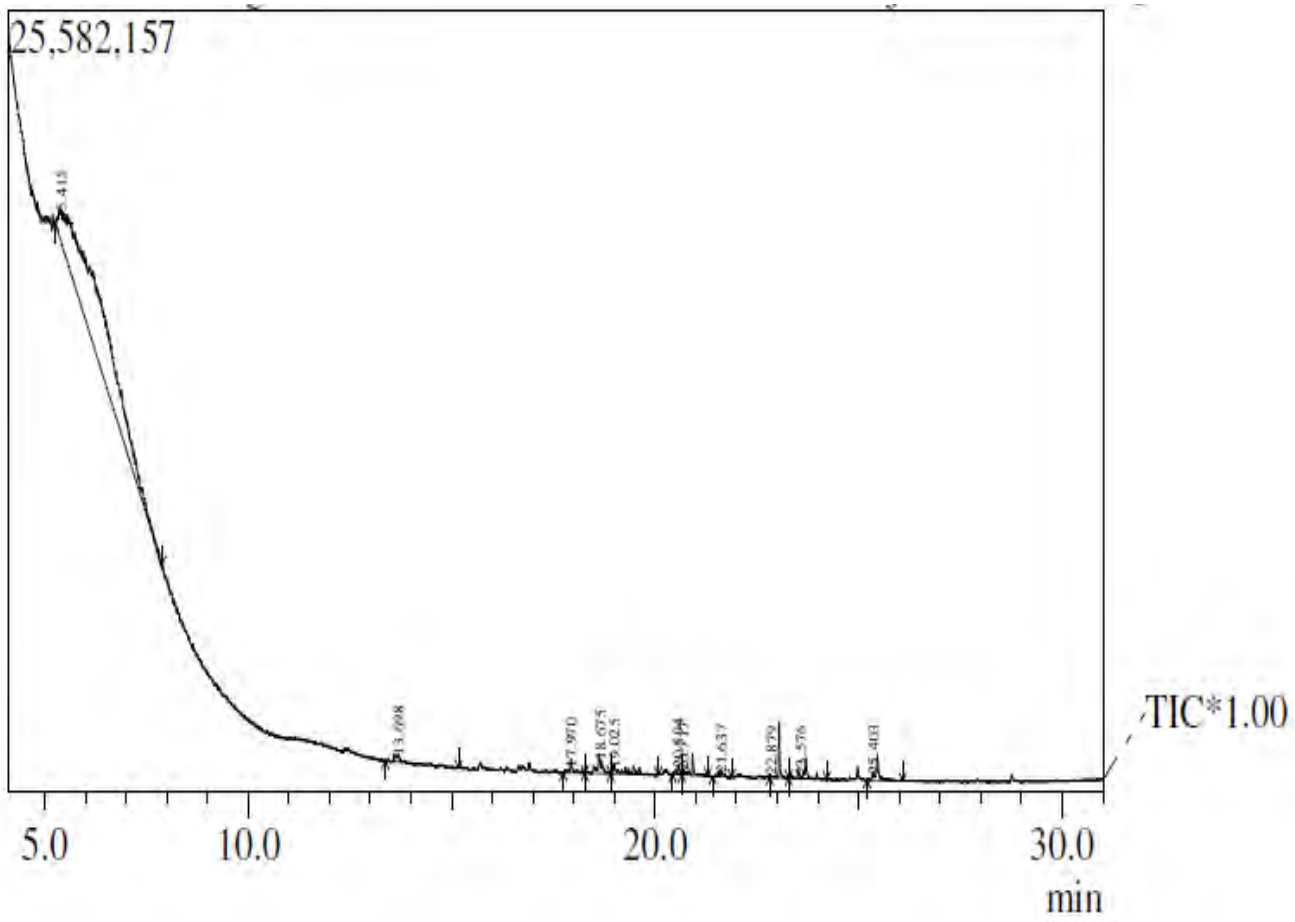
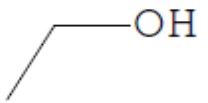
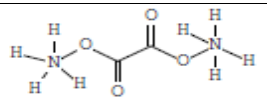
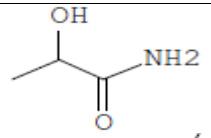
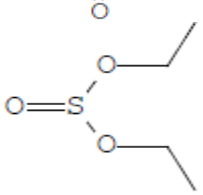
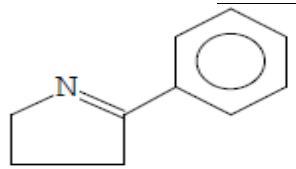
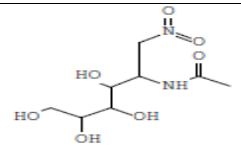
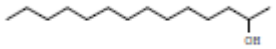
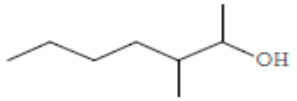
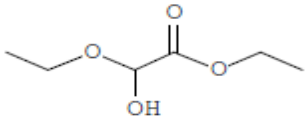
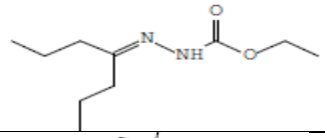
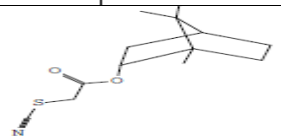
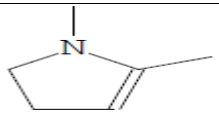
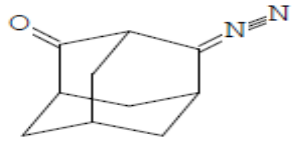
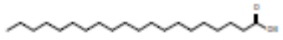
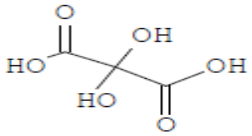


Figure 5.11: GC-MS chromatogram of fraction 4.

Table 5.11: Phytocomponents identified in fraction 4 by GC-MS peak report.

Peak #	Retention time	Area %age	Molecular Formula	Molecular Weight (g/mol)	Compound name	Structure
1	5.4	85.49	C_2H_6O	46	Ethanol	
2	13.7	0.94	$C_2H_8N_2O_4$	124	Ammonium oxalate	
3	17.9	1.08	$C_3H_7NO_2$	89	Propanamide	
4	18.7	2.07	$C_4H_{10}O_3S$	138	Sulfurous acid, diethyl ester	
5	19.1	1.95	$C_{10}H_{11}N$	145	5-Phenyl-3,4-dihydro-2H-pyrrol, 1-Pyrroline	

6	20.6	0.61	$C_8H_{16}N_2O_7$	252	1-Nitro-2-acetamido-1	
7	20.7	1.30	$C_{14}H_{30}O$	214	2-Tetradecanol	
8	21.6	0.65	$C_8H_{18}O$	130	2-Heptanol,	
9	22.9	1.96	$C_6H_{12}O_4$	148	Acetic acid, ethoxyhydroxy	
10	23.6	2.01	$C_{10}H_{20}N_2O_2$	200	Carbazic acid,	
11	24.1	5.47	$C_{13}H_{19}NO_2S$	253	Isobornyl thiocyanoacetate	
12	25.0	6.54	$C_6H_{11}N$	97	2-Pyrroline	
13	25.4	11.57	$C_{10}H_{12}N_2O$	176	4-Diazodamantanone	

14	29.2	8.54	$C_{20}H_{40}O_2$	312	Eicosanoic acid	
15	28.5	1.94	$C_3H_4O_6$	136	Propanedioic acid	

5.4.3: GC-MS analysis of fraction 12

The GC-MS chromatogram of fraction 12 isolated from ethanolic leaves extract of *M. piperita* revealed fourteen peaks at different retention times and confirm the presence of fourteen compounds **(Table 5.12)**. Ammonium oxalate, 1-Pyrroline, Ethanol, Propanamide, octahydro-3,6,8,8-tetramethyl-, acetate, Isobornyl thiocyanoacetate, 2-Pyrroline, 2-Octanol, n-Hexadecanoic acid, Eicosanoic acid, 2-Propanol, 1-bromo- and Phytol was the most abundant components having an area percentage of 5.60%, 20.78%, 16.36%, 13.80%, 27.66%, 15.41% , 10.21, 7.64%, 15.38%, 8.54%, 19.28% and 12.65% respectively. Alpha-Bisabolol (0.39%) and 3-(3,7-dimethyl-2,6-octadienyl)-6-pentyl- (4.28%) are the minor components. The GC-MS chromatogram of fraction 12 is represented in figure 5.12.

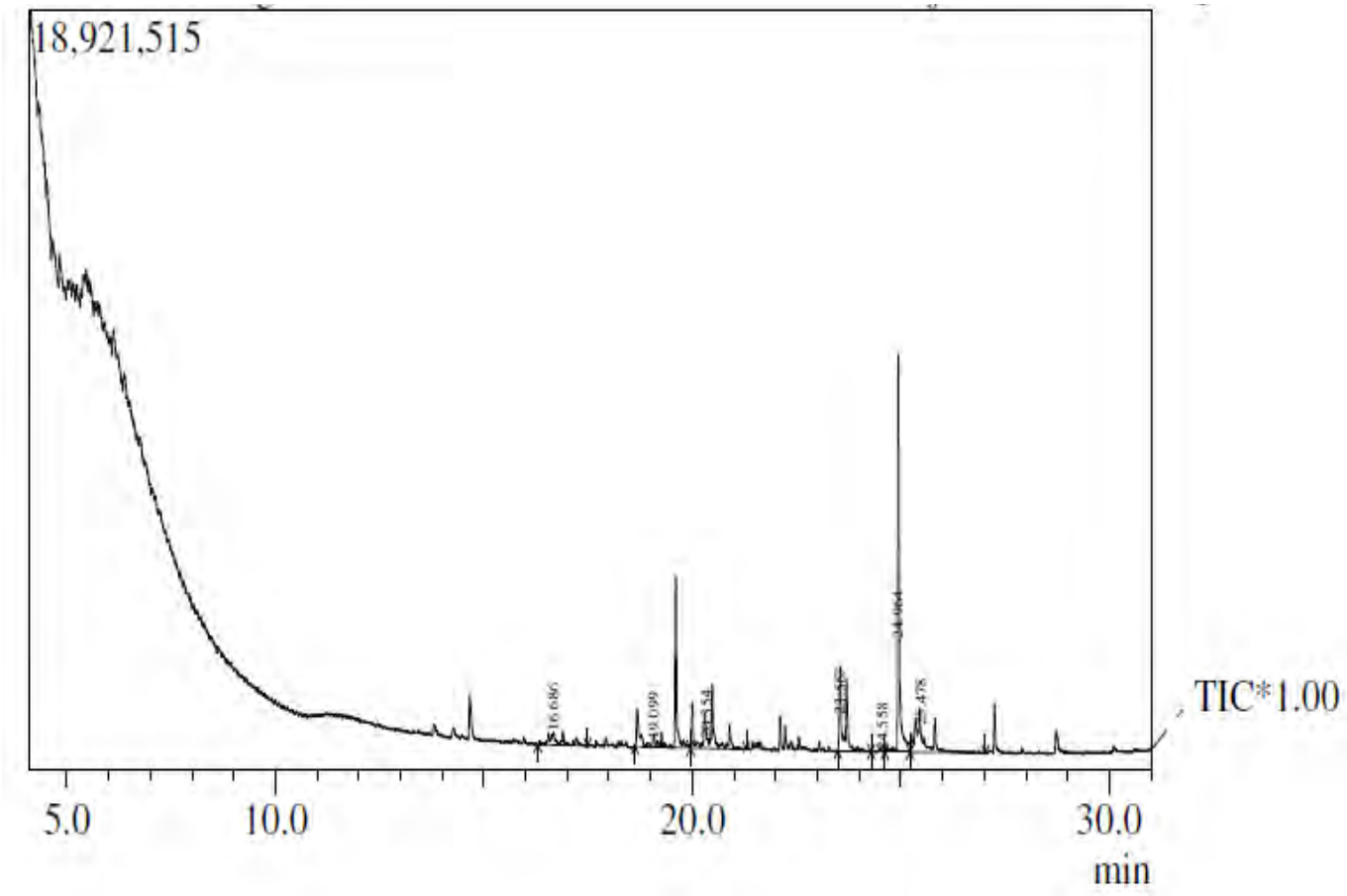
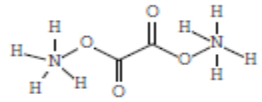
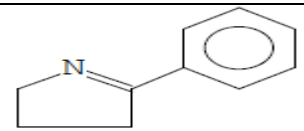
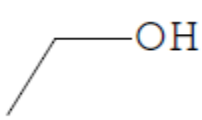
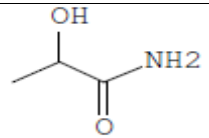
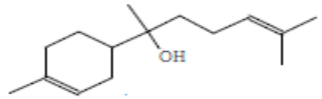
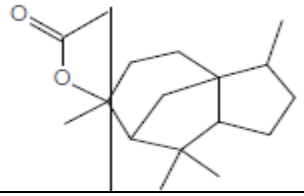
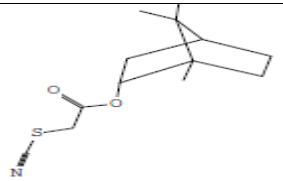
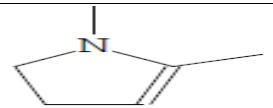
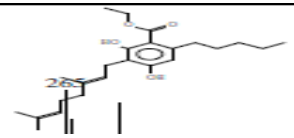
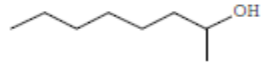
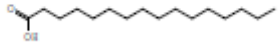
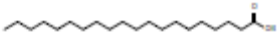
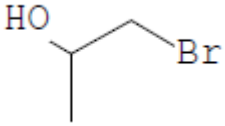
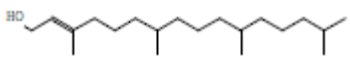


Figure 5.12: GC-MS chromatogram of fraction 12.

Table 5.12: Phytocomponents identified in fraction 12 by GC-MS peak report.

Peak #	Retention time	Area %age	Molecular Formula	Molecular Weight (g/mol)	Compound name	Structure
1	16.7	5.60	$C_2H_8N_2O_4$	124	Ammonium oxalate	
2	19.1	20.78	$C_{10}H_{11}N$	145	1-Pyrroline, 5-Phenyl-3,4-dihydro-2H-pyrrole	
3	20.3	16.36	C_2H_6O	46	Ethanol	
4	23.6	13.80	$C_3H_7NO_2$	89	Propanamide	
5	24.6	0.39	$C_{15}H_{26}O$	222	alpha.-Bisabolol	
6	24.9	27.66	$C_{17}H_{28}O_2$	264	octahydro-3,6,8,8-tetramethyl-, acetate,	

7	24.4	15.41	$C_{13}H_{19}NO_2S$	253	Isobornyl thiocyanoacetate	
8	25.6	10.21	$C_6H_{11}N$	97	2-Pyrroline	
9	26.1	4.28	$C_{24}H_{36}O_4$	388	Beta. Resorcylic acid, 3-(3,7-dimethyl-2,6-octadienyl)-6-pentyl-	
10	27.6	7.64	$C_8H_{18}O$	130	2-Octanol	
11	28.1	15.38	$C_{16}H_{32}O_2$	256	n-Hexadecanoic acid	
12	28.6	8.54	$C_{20}H_{40}O_2$	312	Eicosanoic acid	
13	29.1	19.28	C_3H_7BrO	138	2-Propanol, 1-bromo-	

14	29.8	12.65	$C_{20}H_{40}O$	296	Phytol	

5.4.4: GC-MS analysis of fraction 13

The GC-MS chromatogram of fraction 13 isolated from ethanolic leaves extract of *M. piperita* revealed twelve peaks at different retention times and confirm the presence of eleven compounds **(Table 5.13).** Benzene ethanol (23.04%), p-Xylene (52.29%), Ethanol (24.12%), N-[5-(5-phenyl-3,4-dihydro-2H-pyrrol-4-yl)nonan-5-yl]benzamide (29.451%, Ammonium oxalate (21.20%), Nonanoic acid (9.18%), Butanoic acid (12.89%) and Benzyl 1-benzyl-2-hydroxyethyl(methyl)carbamate (19.57%) are the major components. Heptanoic acid (7.90%), Benzimidazole (5.55%) , Silane (2.59%) and 3,5-bis-trimethylsilyl (5.72%) are the minor components. The GC-MS chromatogram of fraction 7 is represented in **figure 5.13.**

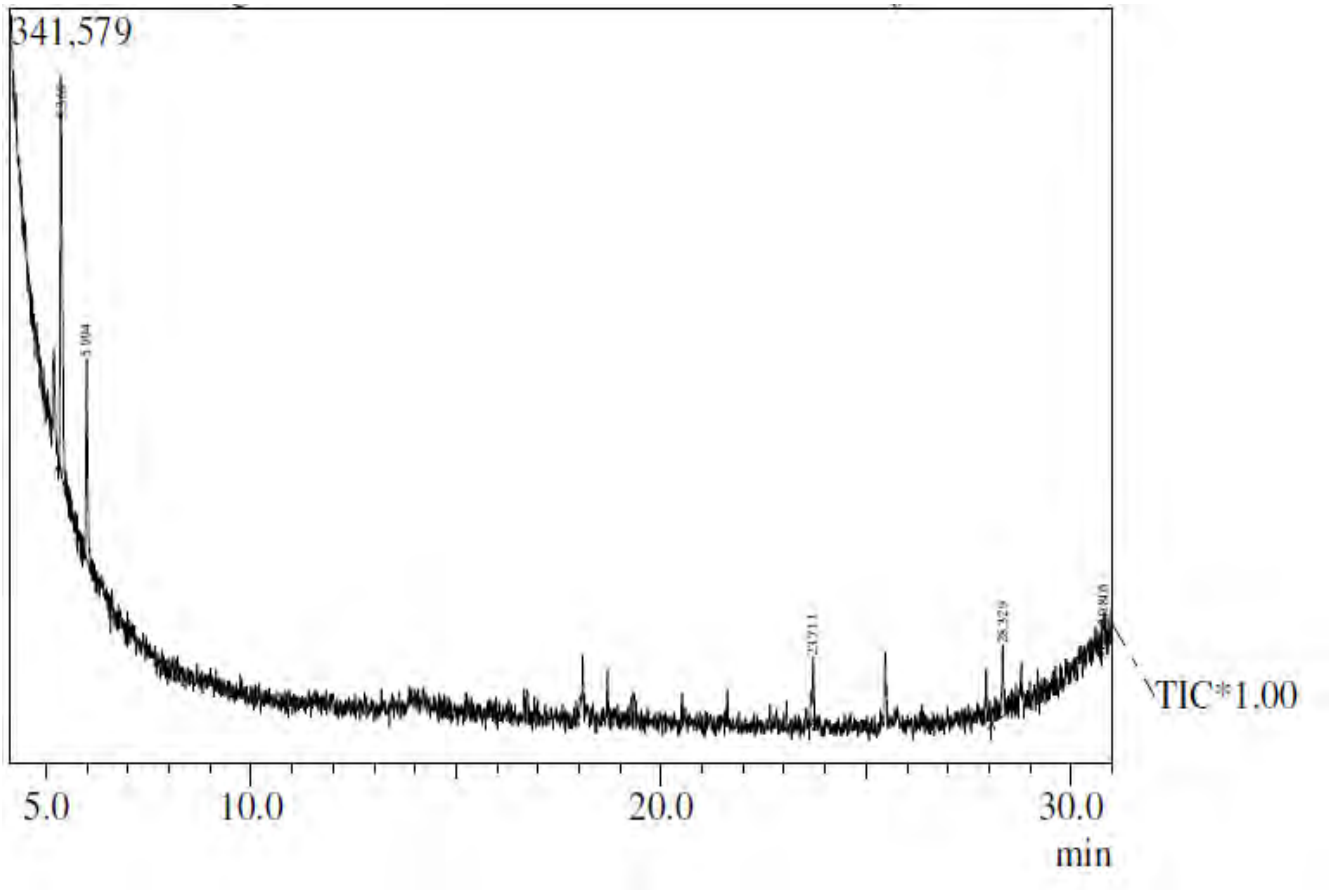
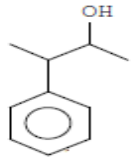
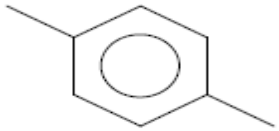
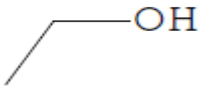
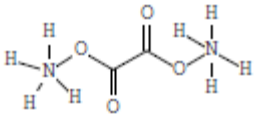
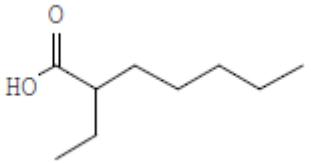


Figure 5.13: GC-MS chromatogram of fraction 13

Table 5.13: Phytocomponents identified in fraction 13 by GC-MS peak report.

Peak #	Retention time	Area %age	Molecular Formula	Molecular Weight (g/mol)	Compound name	Structure
1	5.2	23.04	$C_{10}H_{14}O$	150	Benzene ethanol	
2	5.4	52.29	C_8H_{10}	106	p-Xylene	
3	5.6	24.12	C_2H_6O	46	Ethanol	
4	5.9	21.20	$C_2H_8N_2O_4$	124	Ammonium oxalate	
5	23.7	7.90	$C_9H_{18}O_2$	158	Heptanoic acid	

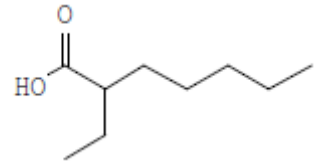
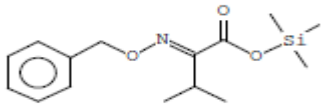
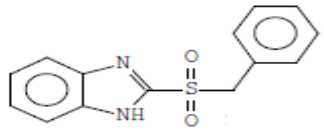
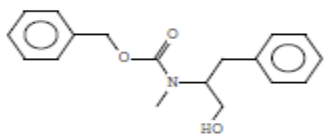
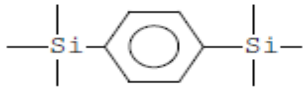
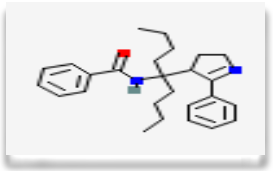
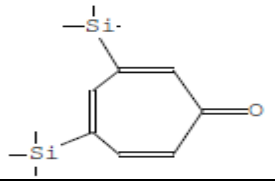
6	23.7	9.18	$C_{13}H_{26}O_2$	214	Nonanoic acid, methyl ester	
7	25.1	12.89	$C_{15}H_{23}NO_3S_i$	293	Butanoic acid	
8	27.5	5.55	$C_{14}H_{12}N_2O_2S$	272	Benzimidazole	
9	28.3	19.57	$C_{18}H_{21}NO_3$	299	Benzyl 1-benzyl-2-hydroxyethyl(methyl)carbamate	
10	30.7	2.59	$C_{12}H_{22}S_{i2}$	222	Silane	
11	29.5	25.67	$C_{26}H_{34}N_2O$	390.6	N-[5-(5-phenyl-3,4-dihydro-2H-pyrrol-4-yl)nonan-5-yl]benzamide	
12	30.8	5.72	$C_{13}H_{22}OS_{i2}$	250	3,5-bis-trimethylsilyl-	

Table 5.14: Biological activities of phytochemical compounds isolated from bioactive fractions (3, 4, 12 and 13) of *M. piperita* ethanolic leaves extract.

Sr. No.	Compound name	Compound nature	Biological activity	Reference
1	Ethanol	Alcoholic	Anti-Infective Agents, Central Nervous System Depressants, antiseptic drug, neurotoxin. Disinfectant	Dudley *et al.,* 2004
2	Ammonium oxalate	ammonium salt	Anticoagulants, to preserve blood outside the body.	*Suresh et al*., 2015
3	Eicosanoic acid	straight-chain saturated fatty **acid**	Antifungal & Antibacterial	Lohani *et al*., 2013
4	Carbazic acid	Monocarboxylic acid and a one-carbon compound	Antioxidant and antimicrobial agent	Elokhina *et al*., 2000
5	Propanamide	Monocarboxylic acid amide and a primary fatty amide	Anticancer Drug	Kamal *et al*., 2013
6	Butanoic acid	Fatty acid ester	Antioxidant and antimicrobial agent	Ali *et al*., 2019

7	Phytol	Diterpene	Anti-cancer, anti-inflammatory, hypocholesterolemic, nematicide, anticoronary, antiarthritic, hepatoprotective, anti-androgenic.	Sermakkani and Thangapandian, 2012
8	1, 2-Benzenedicarboxylic acid	aromatic dicarboxylic acid	Antioxidant and antimicrobial agent	Thangakrishnakumari *et al*., 2013
9	N-[5-(5-phenyl-3,4-dihydro-2H-pyrrol-4-yl)nonan-5-yl]benzamide	Heterocyclic organic compound	anti-inflammatory, anti-bacterial and antifungal	Srinivasan *et al.,* 2013
10	isobornylthiocyanoacetate	Ester	anti-inflammatory, anti-microbial, cytotoxic activity	Kanthal *et al*., 2014
11	2-pyrrolline	Methyl ketone	Antioxidant, pesticide, analgesic activity	Kantilal *et al*., 2011
12	Nonanoic acid	a-linolenic acid	Anticane	Sandosh *et al*., 2013
13	n-hexadeconic acid	Fatty acid/Palmitic acid	Antioxidant, hypocholesterolemic nematicide, pesticide, lubricant, antiandrogenic, flavor, antimicrobial,	Sermakkani and Thangapandian, 2012

MOLECULAR DOCKING:

NS3 protease's three-dimensional crystal structure has been designated for docking. To justify the antilarval potency, the twelve compounds from **fraction 3 (figure 5.14)** were tested based on binding affinity with NS3. The best docked ligand-protein complex, as defined by best binding affinity values, hydrogen bond number, and hydrogen bonding residues of all the compounds.. The hydrogen bonds of compound 1, 2-Benzenedicarboxylic acid with NS3 protease are shown in 2D and 3D interaction diagrams. For 1, 2-Benzenedicarboxylic, the key contact residues for docked legends were (Lys73, lle165, Lys74, Asn167, Val154, Ala164, Asn152, Met49, Leu76, Leu149, Leu85, Gly148, Trp83, Val146, Val147, Ala166, Gly88, Gly87). With the NS3 protease, the 1, 2-Benzenedicarboxylic determines their connection with the protein by a variety of chemical forces, i.e. bonds.

Four hydrogen bonds appear to be complex: one between the compound's acidic hydrogen and the protein's electronegative component, two between the electronegative oxygen and the positive part of the remnant protein, and a fourth between the compound's electronegative nitrogen and the residual protein. Many other significant electrochemical forces, such as Van der Waals, carbon hydrogen contact, covalent binding, and so on, are also involved. The process of covalent inhibition is irreversible. The irreversible inhibitors work in concert with their specific targets in a time-dependent way, and the reaction eventually comes to a halt before reaching equilibrium.. Based on electrochemical contact forces, this research reveals that the 1, 2-Benzenedicarboxylic acid and the protein can have a strong association. This showed that 1, 2-Benzenedicarboxylic acid and protein bind to the NS3 receptor effectively. Molecular docking has contributed in the development of novel small drug-like scaffolds with the best binding selectivity and affinity for the target.

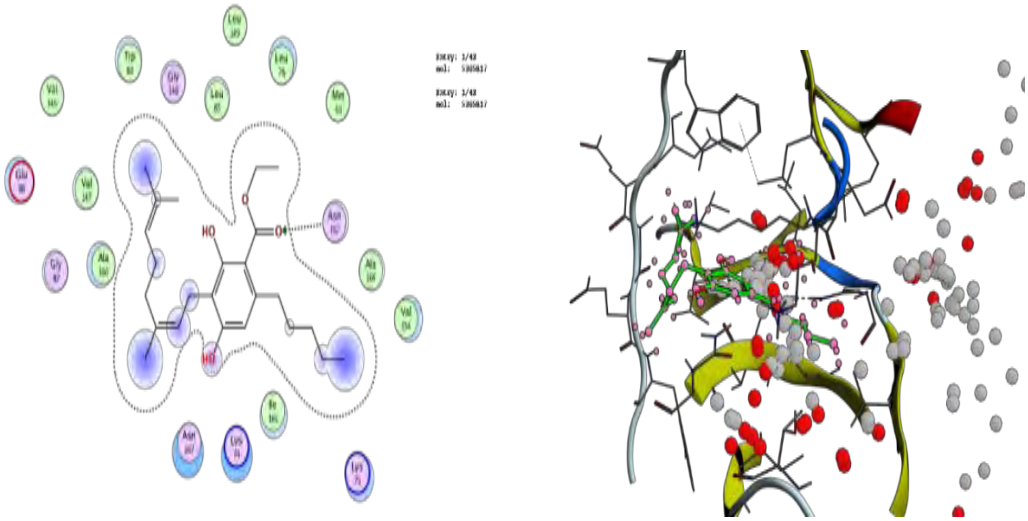


Figure 5.14: 2D and 3D interaction of compound 1, 2-Benzenedicarboxylic with the target protein (PDB ID: 2FOM).

For docking, the NS3 protease (three-dimensional crystal structure) is chosen. The fifteen compounds from **fraction 4 (figure 5.15)** were evaluated for antilarval efficacy based on binding affinity with NS3. The best docked ligand-protein complex, as determined by the highest binding affinity values, number of hydrogen bonds, and hydrogen bonding residues. The chemical with the greatest E value (7.6336) kcal/mol, Eicosanoic acid produced substantial results. The hydrogen bonds of compound Eicosanoic acid with NS3 protease are depicted in 2D and 3D interaction diagrams. For Eicosanoic acid, the key contact residues for the docked legends were (Thr120, Val72, Leu76, Lys73, Asp75, Lys74,Asn152, Gly153, Val154, lle123, Asn167, Ala164, Ala166, Gly148, Leu149, lle165, Val147, Trp83). With the NS3 protease, the substance Eicosanoic acid specifies their interaction with the protein through a variety of chemical factors, i.e. bonds. Four hydrogen bonds appear to be elaborate: one between the compound's acidic hydrogen and the electronegative part of the residual protein, two between the electronegative oxygen and the positive part of the residual protein, and a fourth between the electronegative nitrogen of the residual protein.

Many other significant electrochemical forces, such as Van der Waals, carbon hydrogen contact, covalent binding, and so on, are also involved. The process of covalent

inhibition is irreversible. The irreversible inhibitors work in concert with their specific targets in a time-dependent way, and the reaction eventually comes to a halt before reaching equilibrium. This research suggests that the Eicosanoic acid and the protein can have a strong relationship based on electrochemical interaction forces. This demonstrated that the chemical Eicosanoic acid binds to the NS3 receptor very well. Molecular docking has aided in the discovery of new tiny drug-like molecules.

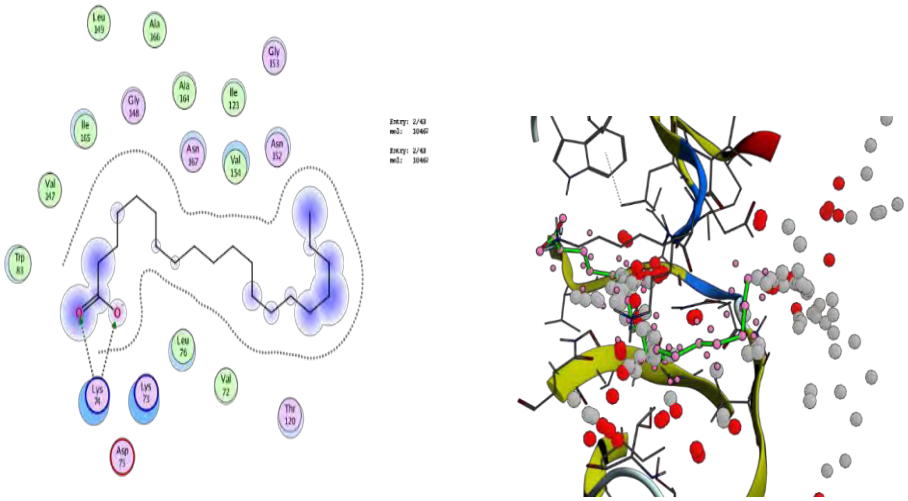


Figure 5.15: 2D and 3D interaction of compound with the Eicosanoic acid target protein (PDBID: 2FOM).

The enzyme NS3 protease (three-dimensional crystal structure) was chosen as the target for the docking study because it plays a key role in antilarval effectiveness. To justify the antilarval potency, the fourteen compounds from **fraction 12 (figure 5.16)** were assessed based on binding affinity with NS3. The best docked ligand-protein complex, as defined by best binding affinity values, hydrogen bond number, and hydrogen bonding residues. Phytol had the greatest E value (-6.9107) kcal/mol of all the substances, indicating that it was the most effective.

The hydrogen bonds of compound Phytol with NS3 protease are depicted in 2D and 3D interaction diagrams. (Asn167, lle165, Lys74, Ala166, Ala164, Leu149, Gly148, Asn152, Val147, Val146, Leu76, Trp83, Leu85, Glu88) were the essential contact

residues for the docking legends for Phytol. The interaction of the substance Phytol with the NS3 protease is defined by a variety of chemical forces, i.e. bonds.

Four hydrogen bonds appear to be complex: one between the compound's acidic hydrogen and the protein's electronegative component, two between the electronegative oxygen and the positive part of the remnant protein, and a fourth between the compound's electronegative nitrogen and the residual protein. Many other significant electrochemical forces, such as Van der Waals, carbon hydrogen contact, covalent binding, and so on, are also involved. The process of covalent inhibition is irreversible.

.The irreversible inhibitors work in concert with their specific targets in a time-dependent way, and the reaction eventually comes to a halt before reaching equilibrium. This research suggests that Phytol and the protein can have a strong relationship based on electrochemical contact forces. This demonstrated that the chemical Phytol has a good affinity for the NS3 receptor. The use of molecular docking has aided in the discovery of innovative tiny drug-like scaffolds with the highest binding selectivity and affinity for target.

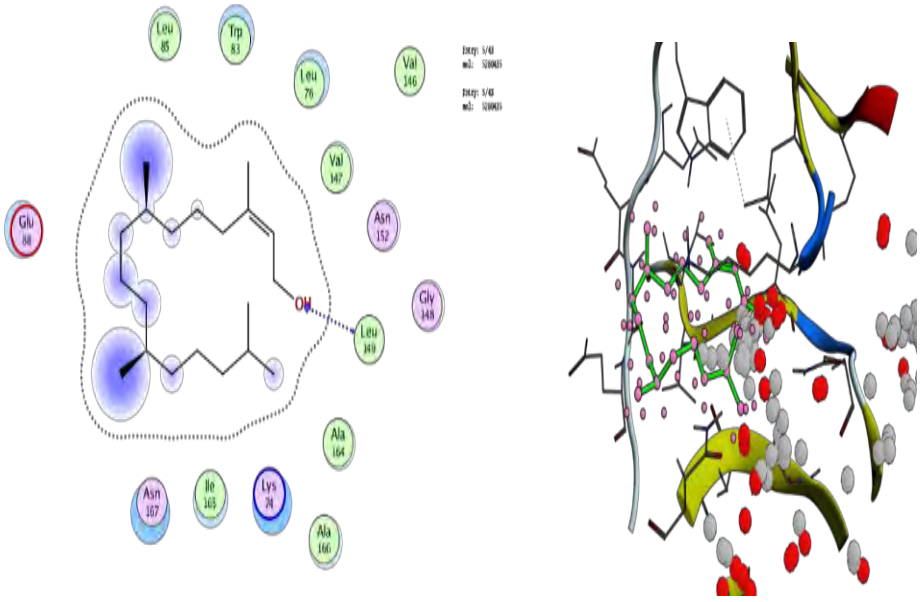


Figure 5.16: 2D and 3D interaction of compound Phytol with the target protein (PDBID: 2FOM).

The enzyme NS3 protease (three-dimensional crystal structure) was chosen as the target for the docking study because it plays a key role in antilarval effectiveness. To justify the antilarval potency, the twelve compounds from **fraction 13 (figure 5.17)** were assessed based on binding affinity with NS3. The best docked ligand-protein complex, as defined by best binding affinity values, hydrogen bond number, and hydrogen bonding residues. N-[5-(5-phenyl-3,4-dihydro-2H-pyrrol-4-yl)nonan-5-yl]benzamide has the greatest E value (-6.9107) kcal/mol of all the compounds studied.

The hydrogen bonds of compound N-[5-(5-phenyl-3,4-dihydro-2H-pyrrol-4-yl)nonan-5-yl]benzamide with NS3 protease are shown in 2D and 3D interaction diagrams. For N-[5-phenyl-3,4-dihydro-2H-pyrrol-4-yl)nonan-5-yl]benzamide, the significant contact residues were (Asn167, lle165, Lys74, Ala166, Ala164, Leu149, Gly148, Asn152, Val147, Val146, Leu76, Trp83, Leu85, Glu88). The NS3 protease's interaction with the molecule N-[5-(5-phenyl-3,4-dihydro-2H-pyrrol-4-yl)nonan-5-yl]benzamide is defined by a variety of chemical forces, i.e., bonds.

Four hydrogen bonds appear to be complex: one between the compound's acidic hydrogen and the protein's electronegative component, two between the electronegative oxygen and the positive part of the remnant protein, and a fourth between the compound's electronegative nitrogen and the residual protein. Many other significant electrochemical forces, such as Van der Waals, carbon hydrogen contact, covalent binding, and so on, are also involved. The technique of covalent inhibition is an irreversible inhibition.

The irreversible inhibitors work in concert with their specific targets in a time-dependent way, and the reaction eventually comes to a halt before reaching equilibrium. This research suggests that the N-[5-(5-phenyl-3,4-dihydro-2H-pyrrol-4-yl)nonan-5-yl]benzamide and the protein can have a strong relationship based on electrochemical contact forces. The chemical N-[5-(5-phenyl-3,4-dihydro-2H-pyrrol-4-yl)nonan-5-yl]benzamide binds well to the NS3 receptor, as evidenced by this. The use of molecular docking has aided in the discovery of innovative tiny drug-like scaffolds with the best binding selectivity and affinity for the target.

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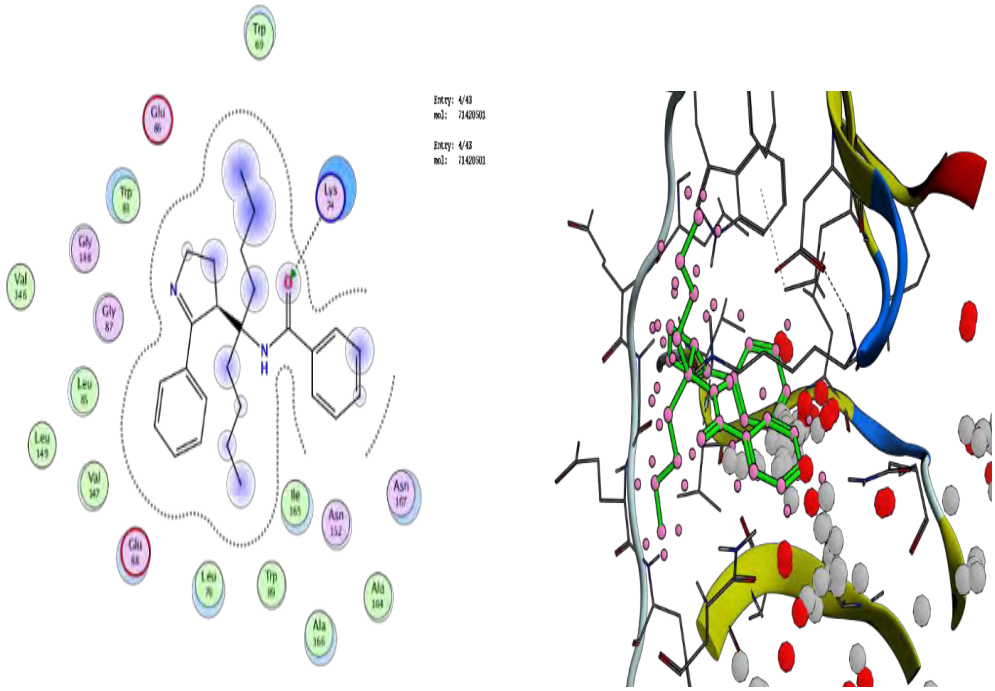

Figure 5.17: 2D and 3D interaction of compound N-[5-(5-phenyl-3,4-dihydro-2H-pyrrol-4-yl)nonan-5-yl]benzamide with the target protein (PDB ID:2FOM).

DISCUSSION

Secondary metabolites with thought-provoking biological functions abound in plants. Overall, secondary metabolites are a significant cause with a wide range of structural and functional features (De-Fatima *et al*., 2006). Antibiotics have traditionally been derived from natural compounds derived from microbial sources. Screening medicinal plants for active compounds has become crucial due to the increasing acceptability of herbal medicine as an alternative method of health treatment (Nair and Chanda, 2006; Khalaf *et al*., 2008). Phytochemical characterization (UV-VIS, FT-IR, and GC-MS) is a helpful method for qualitative analysis of diverse secondary metabolites. Different analyses showed that all of the bioactive fractions (3, 4, 12, and 13) isolated from the ethanolic leaves extract of *M. piperita* contained a variety of secondary metabolites, including alkaloids, carbohydrates, sterol, amino acids etc.

Cardiac glycosides and Saponins were absent in all fractions. Tannins, quinones, terpenoids, coumarins, and phlobatannins were not found in fractions 3 and 4, but they were found in fractions 12 and 13. Only fraction 4 contained the volatile oil, which was absent in the other three fractions. Fractions 3 and 4 contained anthocyanin, whereas fractions 12 and 13 did not. Fractions 4 and 12 have leucoanthocyanins, but fractions 3 and 13 did not. Tannins, quinones, terpenoids, coumarins, and phlobatannins were not found in fractions 3 and 4, but they were found in fractions 12 and 13. Only fraction 4 contained the volatile oil, which was absent in the other three fractions. Fractions 3 and 4 contained anthocyanin, whereas fractions 12 and 13 did not. Fractions 4 and 12 have leucoanthocyanins, but fractions 3 and 13 did not (Table and figure 5.1).

Comparable phytochemicals are stated by Kalimuthu and Prabakaran (2013) in phytoconstituents analysis of *Ceropegia pusilla*, Jabin and Nasreen (2016) in leaves extract of *Lawsonia inermis, Punica granatum, Syzygium cumini, Capsicum annum, Jatropha curcas*, *Murraya koenigii* and Uzer *et al.,* (2005) in extracts of *Magnifera indica, Vernonia amygdalina, Carica papaya*, and *Psidium guajava*. Phytochemical analysis of different parts of medicinal plants including *Ipomea aquatic* (leaves), *Ricinus communis* (roots), *Bryophyllum pinnatum (leaves), Tinospora cordifolia (leaves),*

Xanthium strumarium (leaves), Terminalia bellerica (leaves), Tinospora cordifolia (stem) and Oldenlandia corymbosa (whole plant) was directed by Parekh and Chanda (2007) and confirmed the presence of tannins, saponins, steroids, terpenoids, glycosides, flavonoids and alkaloids as in accordance with consequences described in the present study.

Secondary metabolites in plants, such as flavonoids, tannins, and aromatic chemicals, help to defend against microbes. Saponins, alkaloids, tannins, flavonoids, phenolic chemicals, and terpenoids are found in plant extracts and have antiplasmodic, bacteriocidal, and analgesic properties (Nithyadevi and Sivakumar, 2015). Flavonoids are a type of polyphenolic chemical that acts as an anti-inflammatory and anti-microbial agent by reducing the growth of bacteria that are resistant to antibiotics (Kalimuthu and Prabakaran, 2013).

Flavonoids are antioxidants with anti-inflammatory properties, free radical scavengers with high water solubility that helps to avoid oxidative cell damage, and anti-cancer agents (Njoku and Akumefula, 2007). Many organisms are poisoned by alkaloids, which have a bitter taste (Purohit, 2017). Tannins are complex biological compounds produced as a defensive ingredient by most plants. Tannins contain antibacterial, antidiarrheal, astringent, antioxidant, and anti-inflammatory properties and are commonly employed as tanning agents (Killedar and More, 2010). Terpenoids are volatile organic molecules with antibacterial, antifungal, anticancer, and cytotoxic properties (Sridhar *et al*., 2005). They are also utilized as a defense against a variety of insects, fungus transmitted by insects, and harmful endophytic fungi (Langenheim, 1994).

At 300 to 800 nm, UV-VIS spectroscopic examination of bioactive fractions was done. The absorption of 0.331, 0.062, 0.051, 0.096, 1.020, 0.409, and 1.658 was observed in the peak spectra of fraction 3 at 666.8, 608.8, 535.6, 502.4, 402.9, 318.7, and 209.5 nm, respectively. The measured peaks for portions 4 were 668.3, 633.6, 608.8, 523.1, 502.4, 402.9, 344.9, 325.6, 311.7, 259.3, 238.5, 226.1 and 214.9 nm, respectively, with absorption of 0.284, 0.059, 0.072, 0.093, 0.0803, 0.118, 0.875, 0.475, 0.492, 0.467, and 1.129. Fraction 12 had absorption spectra of -0.655, 1.171, 0.767, 1.182, 1.205, 1.315, and 0.353 with peaks at 211.5, 236.7, 266.2, 291.3, 306.8, 327.7, and 383.6 nm. Fraction

13 has absorption peaks of 0.031, 0.006, 0.166, 0.209, and 0.0734 at 239.3, 264.2, 289.1, 331.4, and 357.7 nm, respectively (Table and figure 5.6-5.9).

Ibrahim *et al*., 2008 observed identical absorption spectra of *Mentha spicata* plant extract at 666 nm and the nearest remaining peaks wavelength. Makky *et al*., (2012) found the closest absorption peaks in leaf extract of *Meizotropis pellita* at 660nm and on chosen red seaweed by Dasgupta *et al*., (2013) at 669.1 nm (*Acanthophora specifera*). Fractions 4, 12, and 13 have nearly identical UV absorption spectra, with comparable absorption peaks. The absorption spectra between 300 and 350 reveal information regarding the nature of flavonoids, according to Abirami and Rajendran (2012). The present study also reports absorption spectra in the range of these wavelengths, confirming the existence of flavonoids and their derivatives.

The identification of functional groups of active chemicals contained in each fraction is aided by FT-IR analysis of bioactive fractions (3, 4, 12, and 13) in ethanol solvent. Each peak in the spectrum represents the exact peak value corresponding to the particular functional groups. Between 400 and 4000 cm^{-1} of wavelengths were used to measure the absorbance. The FT-IR analysis revealed the occurrence of alcohol, primary amine, aliphatic primary amine, secondary amine, alkane, carboxylic acid, alkene, anhydride, nitro-compound, conjugated anhydride, aromatic ester/aromatic amine, alkyl aryl ether, ester/tertiary alcohol, aliphatic ether, Sulfoxide, aliphatic phosphate, aromatic phosphate,

The presence of alcohol was confirmed in all fractions (3, 4, 12, and 13) by absorbance values ranging from 3124.8 to 3732.7. Primary amine has an absorption peak at 3403.81-3491.56 cm^{-1}, while aliphatic primary amine has an absorption peak at 3353.2-3398.1cm^{-1}. Secondary amine has an absorption range of 3336.7cm^{-1}, with alkane maxima at 2954.5-2847.6 cm^{-1}. FT-IR study of *Senna auriculata*, *Phyllanthus amarus*, *Solanum torvum*, and *Phyllanthus maderaspatensis* in various solvents was carried out by Ashokkumar and Ramaswamy (2014), exhibited the existence of alkanes, alkenes, alkyl halides, alcohols, phenols, aldehydes, amides, amines, carboxylic acids, ketones, esters and ethers in methanolic extract. In the current work, similar functional groups were discovered in *M. Piperita* ethanolic leaves extract. These functional groups were also

discovered in several medicinal plant extracts by Packialakshmi and Naziya (2014). OH groups were identified more frequently in bioactive fractions than any other functional group. Because the OH group has a high capacity for hydrogen binding, its presence indicates that the ethanolic leaves extract of *M. pierita* has a higher potential for growth inhibitory effects against microorganisms (Nithyadevi and Sivakumar 2015; Prasanna and Anuradha, 2016).

The GC-MS method is a useful technique for obtaining more precise information on the qualitative analysis of plant extracts. The Maas spectrum reveals the composition and structure of active chemicals, while the relative concentration of each component is shown by the height of various peaks (Sakthivel *et al*., 2010). In this study, GC-MS analysis on the bioactive fractions derived from ethanolic leaf extracts of *M. piperita* (GC-2010, Shimadzu). Table 5.10–5.13 lists the discovered components compared to the NIST library along with the compound name, retention duration, area percentage, molecular weight, and a molecular formula (Gomathi *et al*., 2015).

The GC-MS analysis confirmed that ethanol and ammonium oxalate was found in all fractions (3, 4, 12, and 13). Fractions 3 and 4 contain carbazic acid. In addition, fractions 4 and 12 contain isobornyl thiocyanoacetate. Fractions 3 and 12 contain eicosanoic acid. Fractions 4 and 12 were found to contain propanamide. Fraction 12 contains the n-Hexadecanoic acid and phytol. Fraction 13 contains Butanoic acid, while fractions 4 and 12 contain 2-pyrroline.The GC-MS analysis confirmed that ethanol and ammonium oxalate were found in all fractions (3, 4, 12, and 13). Fractions 3 and 4 contain carbazic acid. In addition, fractions 4 and 12 contain isobornyl thiocyanoacetate. Fractions 3 and 12 contain eicosanoic acid. Fractions 4 and 12 were found to contain propanamide. Fraction 12 contains the n-Hexadecanoic acid and phytol. Fraction 13 contains Butanoic acid. Fractions 4 and 12 contain 2-pyrroline.

N-Hexadecanoic acid has antioxidant, hypocholesterolemic, nematicide, pesticide, lubricant, antiandrogenic, flavour, and antibacterial characteristics (Palmitic acid) (Saikia and Parthasarathy, 2010). Anti-inflammatory, anti-microbial, and cytotoxic properties of isobornylthiocyanoacetate Antioxidant, insecticide, and analgesic action are all properties of 2-pyrrolline. In contrast to Octadecanoic acid, which has not yet been identified,

phytol is a diterpene with anti-cancer, anti-inflammatory, hypocholesterolemic, nematicide, anti-coronary, anti-arthritic, hepatoprotective, anti-androgenic, anti-cancerous, anti-inflammatory, anti-microbial, and anti-nociceptive properties (Sermakkani and Thangapandian 2012; Al-Tameme *et al*., 2015). Phytol has good immunostimulant qualities, according to Singaravadivel and Santhanaraj (2016).

Janakiraman *et al.,* (2012) reported antibacterial activity of phytol against *Mycobacterium tuberculosis* and *Staphylococcus aureus*, respectively. Ammonium oxalate in methanolic leaves extract, phytol in methanol and ethyl acetate leaves extract, and isobornylthiocyanoacetate in ethyl acetate extract of Broussonetia luzonica were reported by Kumaresan *et al.,* (2011). In the leaves extract of *Eichhomia crassipes*, Prabakaran and Mani (2017) found n-Hexadecanoic acid, ethanol, phytol, 2-pyrroline, and Eicosanoic acid. Jayapria and Shoba (2015) found similar chemicals in the methanolic leaves extract of *Justicia adhatoda*. Merlin *et al*., (2009) found 5-Phenyl-3,4-dihydro-2H-pyrrol and acetic acid in the ethanolic leaves extract of *Calotropis procera*, as well as 2-Heptanol, 2-pyrrolline, and Propanamide in the chloroform extract. The availability of the entire genome sequence brings up new avenues for therapeutic target identification (Affonso *et al*., 2013). Various enzymes, such as the NS3 protease, are being studied as therapeutic targets. The 3D structure of the NS3 protease provides a solid foundation for developing novel parasite-specific inhibitors (Narayanan and Velmurugan, 2013).

Covalent molecular docking can be utilized to characterize the covalent interaction (between inhibitors and biological targets) during computer-aided drug design processes (Brooijmans and Kuntz, 2003). The enzyme NS3 protease was selected as a target for the docking study since it is crucial for larvicidal action. Each molecule and its target is connected by two hydrogen bonds. Because they enable protein ligand binding and are essential for protein folding, hydrogen bonds are important in docking. The ligand protein interacting complex has additional forces, such as Vander wall forces, alkyl, and Pi-alkyl interactions (Salentin *et al*., 2014). The formation of a strong cohesive environment by Vander wall forces stabilizes the created complex. Charge transfer is aided by alkyl and Pi-alkyl interactions, which assist enter the medication into the

receptor's binding site (Arthur *et al*., 2019). The 1, 2-benzenedicarboxylic acid of F3, eicosanoic acid of F4, Phytol of F12, and N-[5-phenyl-3, 4-dihydro-2H-pyrrol-4-yl) nonan-5-yl] benzamide of F13 showed the highest binding affinity for the target NS3 protease, according to silico molecular docking studies. All fractions of F3, F4, F12, and F13 exhibited good efficiency against Culex quinquefasciatus, according to the findings.

According to the current investigation, ethanolic extract is a strong therapeutic agent. Ethanol has a high capacity for isolating bioactive components from *M. piperita* leaves extract, which are responsible for a wide range of biological activities. The components included in *M. piperita* extract are vital for human health since they provide numerous health advantages when consumed. More study is needed to limit the use of synthetic medicine formulations in favor of natural goods, which have proved to have fewer side effects. As a result, it is suggested that more research be done on the purification and separation of specific phytochemical ingredients in order to determine their therapeutic, biological activity, and characterization for medicinal and food applications. It provides the possibility for different therapy regimens based on this extract to be developed. The use of FT-IR and GC-MS can be improved to achieve better identification, and results reveal that *M. piperita* leaf extract has antibacterial and insecticide properties. These biologically active elements could be extracted further and used in the formulation of traditional pesticides for the management of a variety of insect pests. The extract of the fractions (F3, F4, F12, and F13) displayed substantial Larvicidal activity, according to our findings. According to the silico molecular docking studies, the 1, 2-benzenedicarboxylic acid of F3, eicosanoic acid of F4, Phytol of F12, and N-[5-phenyl-3, 4-dihydro-2H-pyrrol-4-yl) nonan-5-yl] benzamide of F13 had the highest binding affinity for the target NS3 protease. According to the data, all fractions of F3, F4, F12, and F13 showed good effectiveness against larvicidal. *M. piperita* could be a good option for botanical pesticide manufacturing based on the availability of these phytochemicals in bioactive fractions. The phytochemical profile of *M. piperita* leaf extract reveals a variety of bioactive compounds that can be investigated further for medicinal applications.

CHAPTER 5

GENERAL DISCUSSION

GENERAL DISCUSSION

Mosquitoes are widespread throughout the planet, with the exception of the Antarctica regions that are permanently frozen (Bibi, 2019). Pakistan is one of the subtropical countries where vector-borne diseases are a problem for example malaria, dengue fever, yellow fever, etc. that can be lethal due to its geography and environment (Ashfaq *et al*., 2014**;** Paksa *et al*., 2019). *Anopheles*, *Aedes*, and *Culex* are the three genera of mosquitoes belonging to Culicidae that are most common (Tandina *et al*., 2014).

There are several mosquito species found throughout the world. Neotropical species make up 31% of the known species, followed by Oriental (30%), Afrotropical (22%), and Australasian (20%) (Foley *et al*., 2007). The Nearctic region has the fewest species (5% of the total). Pakistan has 134 different types of mosquitoes spp. all of which belong to the Anophelinae and Culicinae subfamilies. Pakistan, like other Asian countries, is undergoing significant climate change that encourages epidemics of diseases spread by mosquitoes (Manzoor *et al*., 2020).

Morphological traits also contribute to the issue cannot be used to distinguish between the presence of cryptic species. A supplementary approach to figuring out the evolutionary relationships between species that have proven challenging to ascertain by morphological traits, developmental phases, and sexual dimorphism is molecular systematics (Clemens, 2011). DNA barcoding was created in 1993 in order to standardize the use of molecular markers for both species identification and taxonomic allocation through phylogenetic inference based on genetic variability. In conjunction with traditional taxonomy, the cytochrome oxidase c subunit I (COI) gene has been widely utilized for molecular identification and is an effective tool for identifying different mosquito species (Muñoz *et al*., 2021).

Morphology means the study of the size, shape, and structure of animals and of the relationships of their constituent parts. Because of technology, it is now possible to determine the polymorphism of a number of conventional taxonomic and systematic features, allowing for the identification of sibling/cryptic species complexes. The cytogenetic and molecular descriptions of such difficult and conventional species have changed the phylogeny and systematics of Anophelines (Chhilar, 2014). The accuracy of the geometric morphometric approach for classifying mosquitoes has been established. They make it possible to quickly

and accurately identify mosquito species, including closely related species and genera. When molecular identification is impossible due to a lack of morphological proficiency, morphometric identification may be beneficial (Martinet *et al*., 2021). Aslam Khan documented 134 mosquito species from the country, including 89 species from East Pakistan (now Bangladesh) and 91 from West Pakistan. This was the first attempt to describe the Culicidae fauna in Pakistan (Aslam khan, 1972). There are *Culex* (252, 66) and *Anopheles* species (42, 34) that have been recognized as living on the Indian subcontinent as well as in Pakistan.

A stereoscope was used in my research to identify numerous mosquito species. The following parameters were used in the morphometric analysis: complete body, proboscis, palpi, wings, foreleg, middle leg, and hind leg length. The tables (2.6-2.32) below provide morphometric measurements of the body parts of 27 mosquitoes obtained from eleven districts in Punjab (Northern (Rawalpindi, Gujar Khan, Gujrat, Chakwal, Gujranwala and Lahore) and Southern (Dera Ghazi khan, Rajanpur, Rahim yar Khan, Multan, and Bahawalpur) were found to be part of the current study. These species belonged to the five genera of *Anopheles (An. culicifacies, An. stephensi, An. sinensis, An. subpictus, An. maculatus, An. splendidus koidzum,* and *An. aconitus*, *Culex (Cx. quinquefasciatus, Cx. raptor, Cx. tritaeniorhynchus, Cx. bitaeniorhynchus, Cx. vagans Cx. vishnui, Cx. fatigans, Cx. pipiens, Cx. gelidus,* and *Cx. fuscocephala,*), *Aedes (Ae. aegypti and Ae. albopictus), and Armigeres* (*Ar. kuchingiensis, Ar. subalbatus, and Ar. flavus),* and *Manosoni (Ma. uniformis).*

Aedes albopictus and *Aedes aegypti*, which appear to be the main vector of recent dengue virus epidemics, were primarily found in District Lahore, Gujar Khan, and Multan when trying to determine the vector of dengue illnesses (Jabeen *et al*., 2021). In order to assess the biodiversity of mosquito species, a study was conducted in the area of Lahore. The species from the four genera *Anopheles*, *Culex*, *Armigeres*, and *Aedes* include *An. barriensis*, *Cx. pipiens*, *Cx. vishnui*, *Cx. pseudovishnui*, *Cx. fatigans*, *Cx. barraudi*, *Cx. fuscocephala*, *Cx. epidesmis*, *Ar. subalbatus*, *Ae. aegypti*, and *Ae. micropterus* (Low *et al*., 2013; Baboo *et al*., 2021).

The relative abundance and distribution status were also noted in the current study. There were 18 dominating, 4 subdominant, and 5 satellite species in order of relative abundance. There were three recurring, one frequent, four rare, and five sporadic species in

terms of distribution status. *Cx. pipiens* (27.5%) has the highest relative abundance, followed by *Cx*. *quinquefasciatus* (22.3%), *Ar. subalbatus* (16.3%), and *Ar. obturbans* (11.1%). With *Cx. bitaeniorhynchus* having the lowest relative abundance (1.3%) among the remaining species, they are all much rarer. However, Sharma *et al*. (2010) found that the same species from India has a significant amount of 16S rRNA diversity. The ongoing issue of animal mitochondrial and nuclear discordance can gain support from this disagreement (Toews and Brelsford, 2012). Prior studies on *Ae. aegypti* and *Ae. Albopictus* revealed wide spread genetic variation (Kamgang *et al*., 2011) providing vital information on population links and origins as well as understandings of their role in dengue transmission, when research included each species whole distribution, *Ae. albopictus* was less variable than *Ae. aegypti*, a finding consistent with *Mousson et al.* (2005). Both species demonstrated significant barcode variation inside Pakistan. Our review revealed that *Ae. albopictus* was more commonly distributed in Punjab than *Ae. aegypti*. According to Akhtar *et al*. (2012), *Ae. aegypti* larvae dominated (65%) in samples from water pots inside of households in Lahore. The influence of sampling method cannot be completely dismissed (Raharimalala *et al*., 2012), because *Ae. albopictus* current dominance in Pakistan shows a global trend towards its production and a decline in *Ae. aegypti* (Bagny *et al*., 2009).

There are 24 species of Anophelines present in Pakistan (Mahmood *et al*., 1984). The subgenus *Cellia* has a monophyletic ancestry is home to the majority of the species (bootstrap value: 97). The consequences approve with the most recent evolutionary tree for the relationships among anophelines, which was described by Harbach in 2004 and in other studies (Sallum *et al*., 2000). The current study examined the molecular systematics of two *Aedes* (*Ae. agypti* and *Ae. albopictus*) and one *Anopheles* species (*An. gambiae*).

Regarding these issues, natural products (NP), typically secondary metabolites produced by bacteria, plants, or fungus, are seen to be a possible substitute for synthetic insecticidal substances. In the agricultural industry, where there is a large need for alternative techniques and consequently a great growth in interest in biopesticides, the generally lower toxicity and environmental impacts of these substances have been addressed (Engdahl *et al*., 2022)

Chemical pesticides have a restricted use indoors due to their high toxicity and unfavorable ecological effects. The presence of exposed pesticide residues in food and their

toxicity to species other than those intended for use are two of the main issues (Arnason *et al*., 1989; Abudulai *et al*., 2001). However, these chemical pesticides are currently permitted for crop and soil treatment due to the lack of any effective alternatives (Alshehry *et al*., 2014). Phytophagous insects mostly rely on the volatiles of the plants to locate and distinguish their host pant. Because of this, non-host volatile emissions from plants can be employed as an alternate control technique to ward off insect pests (Mauchline *et al*., 2008).

These secondary metabolites are involved in numerous biological processes and come in a variety of groups. For example, flavonoids are polyphenolic chemicals that have anti-inflammatory, anti-microbial, antioxidant, and anti-cancerous properties (Njoku and Akumefula, 2007). Even at low concentrations, alkaloids are harmful to many organisms and have insecticidal characteristics, but their exact mechanisms of action vary (Gupta *et al*., 2010). Tannins are biological compounds that also have antioxidant, anti-inflammatory, anti-diarrheal, and antibacterial properties (Killedar and More, 2010). Terpenoids are volatile substances with cytotoxic, antibacterial, antifungal, and anticancer properties (Rajeswari *et al*., 2011).

Utilizing synthetic insecticides in mosquito nets, textiles, and indoor sprays is the most popular method of preventing mosquitoes from biting their human hosts. The use of chemical tactics has given optimism for stopping the spread of disease in endemic areas, but the emergence of insecticide resistance has been a significant obstacle to lowering the disease burden. Over time, mosquito populations have increased and reemerged due to the unchecked use of insecticides (Şengül and Canpolat, 2022).

Since medicinal plants have gained relevance in recent years are a rich source of potential medications for therapeutic activities (Yadav and Agarwala, 2011). These plants contain bioactive substances (flavonoids, alkaloids, tannins etc.) that have a specific physiological impact on the human body (Edeoga *et al*., 2005). These secondary metabolites were discovered to have an inhibitory impact and to operate as a defence mechanism against insects and bacteria. They are produced during the metabolic processes of the live organism (Vaghasiya *et al*., 2011).

Since the previous few decades, significant efforts have been undertaken to develop natural, environmentally friendly products such as microbial sprays, insect growth regulators, and botanical pesticides (Blaske and Hertal, 2001). In the past, a variety of medicinal plant

species have been used to assess environmental impact and toxicity of natural goods. The larvicidal activities of *Cx. quinquefasciatus* were assessed using the leaf and seed extracts of *Jatropha curcas*, the bark extract of *Melia azedarach,* the rhizome extract of *Curcuma* longa, the leaves extract of *Nerium indicumi*, and the crude seed extract of *Annona* (Nisar *et al*., 2012; Acda, 2014). Four instars of Larvae did not breach the barrier after curcuma longa extracts were administered to the *Cx. quinquefasciatus* infestation (Manzoor *et al*., 2011).

Additionally, the influence of antioxidant activities of crude extracts of *Azadirachta indica*, *Phyllanthus niruri*, *Andrographis paniculata*, and *Leucaena leucocephala* against *Ae. agypti* and *An. subpictus* was examined (Bakaruddin and Ab Majid, 2019). Numerous studies using crude leaf extract have shown that these plants reduce survival of mosquito larvae by altering their breeding places. These plants include *vetiver oil*, *lemon grass*, *Acassia* leaves, and *Taiwania cryptomerioides* (Chang *et al*., 2001), as well as *Calotropis procera* (Singh *et al.,* 2002), *Eucalyptus globulus*, *Rosmarinus officinalis*, *Cinammomum camphora*, *Coleus amboinicus, isoborneol*, and *Cumbopogon wintrianusjowitt* are a few of the herbs that have been used in traditional medicine (Singh *et al*., 2004), *Euphorbia kansuii, Lysitoma seemnii, Tabebina guaycan, Pseudotusuga menziesii, Diospyros sylvatica, and Curcuma aromatic* (Shi *et al*., 2008).

In current research, leaves extract of six tropical plants *i.e. M. piperita* L*., O. basilicum* L*., P. glabrum* L*., M. philippensis* L*., C. aruntium* L*., F. officinalis* L. were studied for their cytotoxicity, antilarval, and antioxidant study against four instars larvae of *Cx. quinquefasciatus* using ethanol and water as solvents. The mean mortality of instars larvae was estimated using five different doses (80, 160, 240, 320, and 400ppm) in fifteen-day experiment. In a time-dependent manner, (320 and 400ppm) concentrations were found to significantly effect on larvae mortality when compared to the control (F (df) = 520.9 (5); P< 0.05), and they also varied considerably from one another (F (df) = 480.4 (5); P 0.05). Furthermore, every plant showing varied antilarval activity in a dose and time-dependent way (F (df) = 460.8 (5); P< 0.05). As ethanolic and aqueous plant extract concentrations increases, so did the number of mortalities occur.

The effect of hours, concentrations, and plants on the mean mortality of all instar's larvae is denoted in figure (3.2-3.17). At the conclusion of the trial, *M. piperita* was determined to be the most effective plant, out of those that were examined with a significant

(P<0.05) decline in the mortality of instar larvae (93%). But *O. basilicum* (78%)*, P. glabrum* (62%) found to be significant (P< 0.05), but *M. philippensis* (42%), *C. aruntium* and *F. officinalis* (31%) have no significant impact on larval mortality. Additional Chi-square test results, the significance level of plants treated with larvae of all instars, and their LC_{50} and LC_{90} values are shown in (Table 2). In comparison to controls, the ethanolic extract has significant results than aqueous (Figure 3.2-3.17).

When mosquito larvae were exposed to plant extract, behavioral changes were also seen. For example, in the beginning of the trial, mosquito larvae stayed immobile and motionless for a short period of time after being subjected to the larvicidal bioassay and they gradually started to deviate from their regular behaviour and exhibit some anomalous reactions as compared to controls. Larval mortality was seen because they showed signs of weakness and shaking before becoming inactive and sluggish and finally died. Similar alterations in behaviour have previously been seen in larvae (Edori and Dibofori-Orji, 2016). According to Meshram *et al*. (2019), these abnormal behavioral patterns in larvae are brought on by exposure to the alkaloids that were extracted from *Epipremnum aureum* extract. The sensory system and cuticle's physiology are affected by this exposure.

The biochemical analysis of the experimental larvae showed that the levels of carbohydrates, proteins, and lipids were much lower in the extract-treated larvae as compared to control (Huang *et al*., 2004). At 400ppm concentration, plant extracts contain some harmful elements that disrupt the biochemistry of larvae and result in substantial mortality. Changes in energy absorption, peroxidation, and insecticidal distress could be observed (Qureshi *et al*., 2015). These effects are in response to Tenebrio molitor's stated decrease in oocyte haemolymph and fat components that it had previously shown to Malathion (Sharma *et al*., 2011). Microscopic observations showed that the plant extracts had a fatal effect on them, causing sluggish spinning, a lack of stability, and gut rupture. This can be brought on by a decline in the glucose level in relation to control. Additionally, it is suggested that it may be because the insecticidal pressure produced by these extracts reduced scavenging presentation, limited food material depletion, and deprived body wall guts (Lohar and Wright, 1993).

The present study of ethanolic extract of *M. piperita*, which exhibits high radical scavenging activity, is consistent with the earlier study published by Deeksha and

Arunachalam (2019). Fe^{3+} is changed into Fe^{2+} for the purpose of extracts' reducing power; Fe^{2+} then combines with $FeCl_2$ to produce complex ferrous. Similar to the current work, Kifayatullah *et al*. (2015) found that the reduction power of ethanolic extracts of *Pericampylus glaucus* (Lamk) rose as the extract concentration increased. Eshwarappa *et al.* (2014) found that *S. cumini* methanol extract showed comparable potential to the normal ascorbic acid utilized and better reducing power. The phosphate/Mo (V) combination was formed in the phosphomolybdenum technique of TAC measurement because antioxidant mediators present in the extract. The strongest antioxidant come from ethanolic leaf extract of *M. piperita* and *O. basilicum*. The overall antioxidant activity of *M. piperita* ethanolic leaves extract, according to Munira *et al*. (2018), is comparable to that of conventional catechin.

The LC_{50} values for the *F. glomerata* extract were 454.3 g/ml with a significant value of P=0.147, indicating moderate toxicity (Nguta *et al*., 2012). Diverse collection sites, plant tissues, harvesting times, and solvent extraction observed that toxicity displayed considerable variability (Bussmann *et al*. (2011). This characteristic diversity has permissible us to do more study on naturally active extracts against fleas (*Xenopsylla cheopis*), mosquitoes (*Ae. aegypti*), ticks (*Ixodes scapularis*), bacteria harming the health of the forest, and other living things (Johnston *et al*., 2001; Dietrich *et al*., 2006). In the present experiment, the plant extracts from *M. piperita, O. basilicum,* and *P. glabrum* showed acute toxicity toward brine shrimp. These preliminary studies demonstrate the efficacy of crude leaf extract from *M. piperita* as a potential larvicidal, insecticidal, and antioxidant agent that might be utilized to manage mosquito's larvae by focusing on their breeding places.

To determine the chemical components and biological activity of *M. piperita,* phytochemical characterization was done using FT-IR, GC-MS, and UV-VIS Spectroscopy. The UV-VIS spectrum showed two absorption peaks at 209.6nm (2.9) and 282.8nm (0.8) respectively. Similar absorption peaks have previously been seen in many plant leaves extracts (Rani *et al*., 2016; Rajeshkumar and Jayaprakash, 2016). The FT-IR results supported the existence of the previously described halo compounds, alcohol, alkanes, aromatic rings, aldehydes, ether, and amines (Ashokkumar and Ramaswamy, 2014; Packialakshmi and Naziya, 2014; Nithyadevi and Sivakumar, 2015). The beneficial chemicals T-Butyl hydrogen phthalate **(13.9%),** 2H-Pyran-2,4(3H)-dione,3-ethyl-5,5-dimethyl -6-phenyl **(13.2%),** Mono-(2-ethylhexyl) ester **(12.5%),** Olean-12-en-28-oic acid, 2.beta., 3.beta.,23-trihydroxy-methyl ester **(10.7%),** 1,2-Benzene dicarboxylic acid **(10.4%)** ,

and 8-Octadecen-1-ol acetate **(9.6%)** were found in *M. piperita* leaf extracts after GC-MS analysis. These substances have been shown to have antimicrobial, antioxidant, anti-fibrinolytic, and hemolytic activity in the past (Gomathi *et al*., 2015), as well as antiandrogenic, hypocholesterolemia, flavouring agents, nematicides, lubricants, pesticides activities (Sermakkani and Thangapandian, 2012; Al-Tameme *et al*., 2015; Kadhim, 2016).

For a docking study, the enzyme NS3 protease is crucial for larvicidal effectiveness was chosen as the target. In order to demonstrate their larvicidal capabilities, 10 different chemicals were used as ligands in the docking process. The target protein and the best-docked molecules are 1, 2-Benzenedicarboxylic acid and 3-ethyl-5, 5-dimethyl-6-phenyl. Each substance and the target protein have two hydrogen bonds. Hydrogen bonds play a crucial role in docking due to their role in facilitating protein-ligand binding for protein folding (Salentin *et al*., 2014)**.** In addition to these, the complex of ligand-protein interactions also includes Vander wall forces, alkyl, and Pi-alkyl interactions. Vander wall forces establish a solid cohesive environment, which stabilizes the developed complex. Alkyl and Pi-alkyl interactions are essential for charge transfer because they aid in putting the medication in the receptor's binding site (Arthur and Uzairu, 2019).

According to a phytochemical study of the plant, most effective plant leaves include a number of important bioactive components that can be utilized as natural insecticides. Therefore, additional research is being done to isolate, characterized, and identify bioactive chemicals from *M. piperita* leaf extract using column chromatography as well as to characterized them using qualitative phytochemical analysis (Vaghasiya *et al*., 2011). As plants are a rich source of biologically active compounds like flavonoids, alkaloids, tannins, carbohydrates, quinine, etc. that has many physiological effects on living cells. Phytochemical characterization from various plant parts has led to the discovery of some highly active drugs (Edeoga *et al*., 2005**;** Abdulhamid *et al*., 2017). These are plant metabolic byproducts that act as a defence against insects and microorganisms as well as having a growth-inhibiting impact (Vaghasiya *et al*., 2011). Since Plants are a rich source of natural bioactive substances have been increasingly popular in recent years offer an eco-friendly alternative to synthetic pesticides (Yadav and Agarwala, 2011).

Chromatographic techniques are frequently used to identify and measure particular bioactive compounds from the mixture (Church, 2005). Column chromatography is more

affordable than other analytical techniques, prevents cross-contamination, and simplifies the removal and degradation of stationary phases. Thin layer chromatography is an additional analytical technique for identifying substances and evaluating their purity (Abdulhamid *et al*., 2017). The current work used column chromatography with a step gradient approach in a variety of solvents to separate the *M. piperita* crude ethanolic leaves extract (15gm). The obtained fractions in various eluents were further characterized using thin-layer chromatography (TLC). In a larvicidal bioassay with a concentration range of 80-400ppm, 15 fractions (2–15) were collected and examined for their larvicidal, biochemical, antioxidant, and cytotoxic properties.

All used fractions had different effects on the four instars larvae of *Cx. quinquefasciatus* as compared to control. In comparison to the other fractions (2–15) used, fraction 13 was found to be the most effective, significantly ($P< 0.05$) reducing the larval population and causing a 95% mortality rate in *Cx. quinquefasciatus* with LC_{50} values of 1342.8 and 1420.1ppm, respectively, while also causing 80% (LC_{50}=578.3 ppm) decline in the population of mosquito larvae. The discoveries of the current research are consistent with those of Meshram *et al.* (2019). When compared to the standard alkaloid nicotine and the chemically synthesized larvicidal monocrotophose, they found that the alkaloid fractions (a8 and a31) isolated from *Epipremnum aureum* significantly induced repellent activities, behavioral changes, and caused larval mortality. The results of the present study are in accordance with Meshram *et al*., (2019). They described that when compared with the standard alkaloid Nicotine and chemically synthesized larvicides Monocrotophose, alkaloid fractions (a8 and a31) isolated from *Epipremnum aureum* significantly induce repellent activities, behavioral changes and caused larval mortality.

Against mosquito larval stages, fractions 3 and 4 showed 82% mortality. A study carried out by Doolittle *et al*. supports these findings (2007) are also comparable with a study focused by Maistrello *et al*. (2003). The highly poisonous compound turmerone from turmeric extract kills larvae by attacking their nervous system and disrupting their breathing system (Raje *et al*., 2015). The remaining fractions (5, 6, 9, 11, and 15) were found to be least effective against mosquito larvae and had no significant effect on larval population with lower LC_{50} values namely 1987.3, 1765.3, 1619.6, 1502.3, and 1273.1ppm respectively. According to Maistrello *et al*. (2003), the unequal drop in mosquito larvae population may be brought on by the harmful effects of bioactive substances that alter the physiological

conditions in the gut microenvironment. The extract treated larvae had much lower lipids, proteins, and carbohydrates values than the control group (Huang *et al*., 2004). After being showing to Malathion, Tenebrio molitor's oocyte haemolymph and lipid constituents decreased, causing these results (Sharma *et al*., 2011).

The current study found that fractions 3, 4, 12 and 13 had the best antioxidant activity based on the DPPH experiment and the lowest IC_{50} values (7.6, 8.2, 8.3, and 8.1), respectively. These four active fractions showed ethanol solvent elution, demonstrating relatively polar molecules. The findings showed that antioxidant power decreased with increasing extract content in each fraction. The highest reducing power and total antioxidant values are found in fractions 3, 4, 12 and 13 (250±0.85, 255±0.65, 265±0.69, 275±0.94), (70±0.54, 72±0.34, 77±0.21, 85±0.25), respectively. That is slightly lower but statistically similar to the reducing power of standard Ascorbic acid (3.9±1.04) on same concentrations. The ferric reducing antioxidant potential could be graded in following order; F13>F12>F4>F3>F11>F10>F9>F8>F7>F6>F5>F15>F14>F2>F1. Polar solvent was used to elute the fractions with the greatest reducing potential. When compared to the typical ascorbic acid (59.3), F13 had the highest total antioxidant capacity (85±0.25) g/ml, followed by F12, F4 and F3 (77±0.21, 72± 0.34 and 70±0.54) g/ml.

In the current analysis, the resulting fractions LC_{50} ranged from 5.1 to 85.2ppm for all fractions (Table 4.6). The fraction (F2) has the highest LC_{50} value (85.2), while the fraction (F13) has the lowest (5.12). The relative LC_{50} values for fractions (F2-F15) were 13.4, 6.5, 5.9, 45.7, 43.1, 41.5, 40.6, 39.1, 38.6, 35.8, 7.5, 5.1, 55.1, and 47.8ppm. The p-value for F3, F4, F12, and F13 fraction was significant ($P<0.005$). The toxicological impact of the plant extract is comparable to that of a normal medicine (tricaine methanesulfonate). The results imply that analgesic activities due to the occurrence of saponins, alkaloids, tannins, flavonoids, polysteroles, phenolic compounds, and terpenoids. These substances serve as a defence mechanism against microorganisms (Nithyadevi and Sivakumar, 2015).

According to Sasidharan *et al*. (2011) with some modifications, preliminary phytochemical characterization of the bioactive fractions 3, 4, 12 and 13 was done. The bioactive fractions were the subjects of preliminary phytochemical analysis, which revealed the presence of a variety of secondary metabolites, including alkaloids, carbohydrates, sterol, amino acids, starch, cellulose, flavonoids, Phenol, etc. in each fraction. All fractions lacked

cardiac glycosides and saponins. While found in fractions 12 and 13, tannin, quinones, terpenoid, coumarins, and Phlobatannins were absent from fractions 3 and 4. Only fraction 4 contained the volatile oil; the other three fractions did not. While they were lacking in fractions 12 and 13, anthocyanins were present in fractions 3 and 4. While it was lacking in fractions 3 and 13, leucoanthocyanin was present in fractions 4 and 12. (Table 5.1 and figure 5.1). Many plant extracts have been reported to contain similar phytochemicals in the past (Ayoola *et al*., 2008; Yadav and Agarwala, 2011; Kalimuthu and Prabakaran, 2013; Jabin and Nasreen, 2016).

The bioactive fractions were analyzed using UV-VIS spectroscopy from 200 to 800nm, and although having various absorption spectra, all of the fractions showed a consistent absorption peak at 608.50. According to Jain *et al*. (2016), the *Mentha spicata* plant extract has identical absorption spectra at 665nm and the closest remaining peak wavelength. Rajeshkumar and Jeyaprakash as well as Rani *et al*. (2016) both noted the closest absorption peaks. In the present experiment, the absorption spectra between 300 and 350nm reveal information on the nature of flavonoids, comparable spectra are also detected.

Similarly, FT-IR analysis of bioactive fractions was performed (Shimadzu ETIR spectrophotometer) from 400 to 4000cm^{-1} and absorption spectra were recorded. The FT-IR analysis revealed the occurrence of alcohol, primary amine, aliphatic primary amine, secondary amine, alkane, carboxylic acid, alkene, anhydride, nitro-compound, conjugated anhydride, aromatic ester/aromatic amine, alkyl aryl ether, ester/tertiary alcohol, aliphatic ether, Sulfoxide, aliphatic phosphate, aromatic phosphate present in all bioactive fractions. Previously the presence of these compounds has been confirmed in many studies (Packialakshmi and Naziya, 2014; Nithyadevi and Sivakumar 2015; Sithara *et al.,* 2017). According to Ashokkumar and Ramaswamy (2014), OH group has the hydrogen binding capacity and the common presence of this group in all bioactive fractions indicates the higher binding capacity of *M. piperita* leaves extracts imparts growth inhibitory activities against microorganisms.

The Maas spectrum is an effective tool for knowing the structure of active substances (Prabakaran and Mani, 2017). It was confirmed by the GC-MS analysis that all bioactive fractions F3, F4, F12, F13 contained ethanol and ammonium oxalate. Carbazic acid is present in fractions 3 and 4. Additionally, Isobornyl thiocyanoacetate is present in fractions 4 and 12.

Eicosanoic acid is present in fractions 3 and 12. Propanamide was discovered to be present in fractions 4 and 12. Phytol and n-hexadecenoic acid are present in fraction 12. Butanoic acid is present in fraction 13, while 2-pyrroline is present in fractions 4 and 12.

The biological actions of the phytochemical substances found in this study are described in prior reports (Casuga *et al.,* 2016). PN-Hexadecanoic acid is one of the compounds with bioactive properties that have been discovered. It has antibacterial, antioxidant, antifibrinolytic, antiandrogenic, hypocholesterolemia, flavouring agent, nematicides, lubricating, pesticidal, and hemolytic properties (Sermakkani and Thangapandian 2012; Gomathi *et al.,* 2015). Hexadecanoic acid methyl ester has a hypocholesterolemia, flavoring agent, antioxidant and pesticidal properties. 3, 7, 11, 15-Tetramethyl-2-hexadecen-1-ol has antimicrobial, anti-cancerous, anti-inflammatory, antioxidant activities and 1, 2-Benzenedicarboxylic acid diisooctyl ester have antifouling and antimicrobial activities. Biological activities of phytol are anti-cancerous, anti-inflammatory, anti-microbial and anti-nociceptive (Al-Tameme *et al.,* 2015). Among the commonly identified compounds *i.e.* n-Hexadecenoic acid, Hexadecenoic acid methyl ester, 3, 7, 11, 15-Tetramethyl-2-hexadecen-1-ol, 1, 2-Benzenedicarboxylic acid diisooctyl ester, Phytol and Octadecanoic acid have certain antimicrobial and pesticidal activities. However, one of the compounds, 3, 7, 11, 15-Tetramethyl-2-hexadecen-1-ol is present common in all bioactive fractions. For the control of different insect pests these biologically active constituents can be further eluted and might be utilized for the formulation of traditional pesticides.

The availability of complete genome sequence opens up new possibilities for identifying medicinal targets (Affonso *et al*., 2013). Many enzymes are being investigated as potential therapeutic targets, including the NS3 protease. The 3D structure of NS3 protease provides a strong platform for the creation of new parasite-specific inhibitors (Narayanan and Velmurugan, 2013). In computer-aided drug design processes, the covalent interaction (between inhibitors and biological targets) is characterized by covalent molecular docking (Brooijmans and Kuntz, 2003). For larvicidal action, the enzyme NS3 protease was designated as a target for the docking study.

Each molecule and its target are connected by two hydrogen bonds. Protein ligand binding and hydrogen bonds are essential for protein folding in docking. The ligand protein interacting complex has additional forces such as Vander wall forces, alkyl, and Pi-alkyl interactions (Salentin *et al*., 2014). The constructed complex is stabilized by Vander wall forces produce a strong cohesive environment. Alkyl and Pi-alkyl interactions facilitate charge transfer help the drug enter the binding site of the receptor (Arthur *et al*., 2019). In silico molecular docking experiments, the 1, 2-benzenedicarboxylic acid of F3, eicosanoic acid of F4, phytol of F12, and N- [5-phenyl-3, 4-dihydro-2H-pyrrol-4-yl] nonan-5-yl] benzamide of F13 had the best binding affinity for the target NS3 protease. The results showed that all fractions F3, F4, F12, and F13 were effective against *Cx. quinquefasciatus.* These biologically active constituents can be further eluted and might be utilized for the formulation of traditional pesticides which can be used for the control of different insect pests. It is suggested that the *M. piperita* could be a good source of larvicides.

CHAPTER 6

GENERAL CONCLUSION

GENERAL CONCLUSION

Mosquitoes are a monophyletic taxon of the order Diptera, suborder Nematocera, and family Culicidae. The Culicidae is responsible for the spread of infections, for example the west Nile virus, yellow fever, and filariasis etc. All of Pakistan provinces have different mosquito biodiversity. The greatest subtropical region, however, is Punjab, has diversity of mosquito species. It has five genera: *Anopheles*, *Culex*, *Armigeres*, *Aedes*, and *Mansonia*. For that purpose, an increasing number of entomological and field level surveys are required. To establish accurate morphological identification up to the species level, biometric research is required. A helpful and trustworthy method for classifying mosquito species is morphometric and molecular analysis. Out of six experimental plants, the crude leaves extract of *M. piperita* showed the most cytotoxicity, larvicidal, and antioxidant activities. The most bioactive fractions (3, 4, 12 and 13) of *M. piperita* were also tested for biological activities (cytotoxicity, larvicidal, and antioxidant) after being extracted using column chromatography technique. These bioactive fractions were shown to have larvicidal potential against the four instars of *Cx. quinquefasciatus* larvae. These fractions were qualitatively observed by preliminary phytochemical screening, UV-VIS spectroscopy, FT-IR, GC-MS, and molecular docking study. It revealed the presence of a number of bioactive compounds, including 1, 2-benzenedicarboxylic acid, eicosanoic acid, Phytol, and N- [5-phenyl-3, 4-dihydro-2H-pyrrol-4-yl] benzamide, which has the antioxidant, antibacterial, anti-inflammatory, and anti-cancerous effects. This bioactive component in *M. piperita* leaf extracts implies that it has some pesticidal , insecticidal and antimicrobial activity used for the formulation of pesticides that can be used to control various insect pests. Furthermore, this plant is easily accessible and low-cost in all areas of Pakistan. These discoveries may help in the development of a safe substitute for toxic chemical pesticides that present in the environment. The commercially available substances (1, 2-benzenedicarboxylic acid and Phytol) could be utilized in the formation of natural larvicidal products that are both cost-effective and have industrial applications for the control of insect pests.

CHAPTER 7

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RESEARCH ARTICLE

Biocidal action, characterization, and molecular docking of *Mentha piperita* (Lamiaceae) leaves extract against *Culex quinquefasciatus* (Diptera: Culicidae) larvae

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Abstract

Mosquitoes are found in tropical and subtropical areas and are the carriers of a variety of diseases that are harmful to people's health. *e.g.*, malaria, filariasis, chikungunya, dengue fever, etc. Although several insecticides are available, however, due to insect resistance and environmental hazards, more eco-friendly chemicals are needed for insect control. So, the current research was planned to explore the prospective of *Mentha piperita* to be used for the formulation of larvicides against mosquito *Culex quinquefasciatus*. The ethanolic and water extracts of *M. piperita* leaves were prepared using the soxhlet apparatus. The extracts were dried and subjected to prepare five concentrations multiple of 80 ppm. Each concentration was applied for its larvicidal efficaçy setting an experiment (in triplicate) in plastic containers of 1000 ml with extracts, 30 larvae of all four instars separately, and fed with dog biscuits along with controls. Observations were taken after each 12 hrs. till 72 hrs. The antioxidant perspective of *M. piperita* was determined by DPPH radical scavenging, total antioxidant capacity, and ferric reducing power assays. Using brine shrimp lethality bioactivity, the cytotoxic study was perceived. Standard techniques were used to classify the *M. piperita* extract using preliminary qualitative and quantitative phytochemicals, UV-Vis spectroscopy, FT-IR, and GC-MS analysis. *M. piperita* ethanolic leaves extract after 24 hrs. of exposure in 400 ppm showed 93% (LC_{50} = 208.976 ppm) mortality in ethanolic extract and 80% (LC_{90} = 246.900 ppm) in the water extract. In treated larvae, biochemical examination revealed a substantial ($P<0.05$) decrease in proteins, carbohydrates, and fat contents. The ethanol extract of *M. piperita* was the most efficient, killing brine shrimp nauplii in 50% to 90% of cases. TAC (125.4 3.5gAAE/mg DW) and FRP (378.1 1.0gAAE/mg DW) were highest in the ethanolic extract of *M. piperita*. The presence of medicinally active components such as alkaloids, carbohydrates, flavonoids, and others in *M. piperita* leaves extract in ethanol was discovered. The UV-Vis spectrum showed two peaks at 209.509 and 282.814 nm with the absorption of 2.338 and 0.796 respectively. The FT-IR consequences exhibited the