

**CLONING AND EXPRESSION OF THE GENE FOR TRYPSIN
MODULATING OOSTATIC FACTOR AND ISOLATION OF A
NEW TRYPSIN INHIBITOR FROM *CALOPOGONIUM*
MUCUNOIDES SEEDS TO CONTROL THE
LARVAE OF *AEDES ALBOPICTUS* (SKUSE)**

Thesis submitted to the
University of Calicut for the partial fulfilment of the
requirements for the degree of
Doctor of Philosophy in Zoology

by

DEEPTHI M



**DEPARTMENT OF ZOOLOGY
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CERTIFICATE

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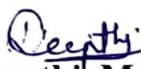


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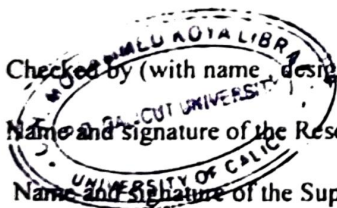


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ABBREVIATIONS

ABC	:	ATP-binding cassette
AChE	:	Acetylcholinesterase enzyme
Aea-TMOF	:	<i>Aedes aegypti</i> - Trypsin modulating oostatic factor
AOX1	:	Alcohol oxidase 1 promoter
API	:	Aspartyl protease inhibitor
APS	:	Ammonium per sulphate
ASV	:	Amplicon sequence variant
BBi	:	Bowman-Birk inhibitor
BSA	:	Bovine serum albumin
Bti	:	<i>Bacillus thuringiensis</i> subspecies <i>israeliensis</i>
CHIKV	:	Chikungunya virus
CLIP	:	Class II associated invariant chain peptide
CmTI	:	<i>Calopogonium mucunoides</i> trypsin inhibitor
CNBr	:	Cyanogen bromide
DDT	:	Dichloro diphenyl trichloroethane
DEAE	:	Diethyl ethanolamine
DEET	:	Dimethylphthalate 2-ethyl-1, 3-hexane diol and N, N-diethyl-m-toluamide
DENV	:	Dengue virus
DTT	:	Dithiothreitol
EDTA	:	Ethylene diamine tetra acetic acid

EEEV	:	Eastern equine encephalitis virus
FPLC	:	Fast protein liquid chromatography
GFP	:	Green fluorescent protein
GM	:	Genetically modified
GST	:	Glutathione S transferase
HDR	:	Homology-directed repair
IEC	:	Ion exchange chromatography
IGR	:	Insect growth regulator
IMAC	:	Immobilized metal affinity chromatography
IPTG	:	Isopropyl β - D-thiogalactopyranoside
JTAT	:	Jembrana trans-activator transcription protein
KTI	:	Kunitz-type inhibitor
LB	:	Luria Bertani
MAFFT	:	Multiple alignment using fast fourier transform
NTA	:	Nitriloacetic acid
OD	:	Optical density
OTU	:	Operational taxonomic unit
PBS	:	Phosphate buffer saline
PCoA	:	Principal component analysis
PCR	:	Polymerase chain reaction
PINII	:	Potato proteinase inhibitor II
PPI	:	Plant protease inhibitor
ScFv	:	Single chain variable fragment

SDS	:	Sodium dodecyl sulphate
SEC	:	Size exclusion chromatography
SINV	:	Sindbis virus
SIT	:	Sterile insect technique
SLEV	:	St. Louis encephalitis virus
SPI	:	Serine protease inhibitor
SUMO	:	Small ubiquitin-like modifier
TCA	:	Trichloro acetic acid
TEMED	:	Tetramethylene diamine
TMOF	:	Trypsin modulating oostatic factor
USUV	:	Usutu virus
WEEV	:	Western equine encephalitis virus
WHO	:	World health Organisation
WNV	:	West Nile virus
YFV	:	Yellow fever virus
ZIKV	:	Zika virus

ABSTRACT

Mosquitoes are one of the major insect vectors that transmit many infectious diseases. Many diseases like dengue fever, malaria, etc. are transmitted by mosquitoes. Conventional control methods using different classes of chemical insecticides resulted in development of resistance in mosquitoes. In addition, these chemicals cause severe environmental contamination and health hazards. Hence newer strategies with target specificity need to be developed. In this study, two methods for the control of *Aedes albopictus* larvae is described. In the first method, wild-type or mutated Trypsin Modulating Oostatic Factor (TMOF) peptide was expressed in *Escherichia coli* and used for the control of *Aedes albopictus* larvae. In addition, the impact of the TMOF treatment on the larval gut microbiota is also examined. The second method utilises a new trypsin inhibitor isolated from the seeds of *Calopogonium mucunoides* to control the larvae of *Aedes albopictus* mosquito.

Trypsin Modulating Oostatic Factor (TMOF) is a naturally occurring decapeptide which inhibits trypsin synthesis in mosquito. As trypsin is required for digestion of protein, the mosquito and mosquito larvae dies on treatment with TMOF. In this study, the gene coding for TMOF was cloned and expressed in *Escherichia coli*. Mutant TMOF was also developed and expressed in *Escherichia coli*. Bacterial cells expressing TMOF/mutant TMOF were lysed and used to prepare crude TMOF/mutant TMOF preparation. Second instar *Aedes albopictus* larvae were treated with the crude TMOF/mutant TMOF and significant mortality was observed within 48 hours of treatment. The LC_{50} (48 hours) for crude TMOF was 242.1 ± 6.04 $\mu\text{g/mL}$ and that for crude mutant TMOF was 236.54 ± 6.49 $\mu\text{g/mL}$. Crude TMOF/mutant TMOF were purified by nickel affinity chromatography. The purified TMOF/mutant TMOF was found to have an LC_{50} (48 hrs) of 2.13 ± 0.02 $\mu\text{g/mL}$ (56.89 ± 1.42 nM) and 1.91 ± 0.02 $\mu\text{g/mL}$ (50.93 ± 1.50 nM) respectively to *Aedes albopictus* larvae. The yield of purified TMOF/mutant TMOF was 3.12 and 2.98 μg per milligram of bacterial pellet. The purified mutant TMOF is 1.12 times better than the purified wild type TMOF as a larvicide to the larvae of *Aedes albopictus*. The crude TMOF and crude mutant TMOF were also toxic to *Culex quinquefasciatus* larvae but not to non-target organisms like *Drosophila melanogaster*, *Luprops tristis*, *Aplocheilus lineatus*. Analysis of the gut microbiota of *Aedes albopictus* larvae on treatment with recombinant TMOF/mutant TMOF revealed that there is a reduction in the gut bacterial population compared to untreated larvae. The impact of gut microbiota towards the TMOF toxicity need to be investigated. As the mechanism of action of TMOF/mutant TMOF

is receptor mediated and the receptor is only reported from mosquitoes, using recombinant TMOF/mutant TMOF as a larvicide is an ecofriendly method of mosquito control.

For the isolation of a new trypsin inhibitor, a total of 45 plant extracts were screened by trypsin inhibition assay. Using proteinase K the plant extracts with proteinaceous inhibitor was found and used to test *Aedes albopictus* larvae. The extract from the seeds of *Calopogonium mucunoides* showed the highest trypsin inhibition ($87.99 \pm 0.38\%$) and percentage mortality ($100 \pm 0.00\%$) towards *A. albopictus* larvae. Thus, the seed extract from *Calopogonium mucunoides* was used for the purification of the trypsin inhibitor by ammonium sulphate fractionation, size exclusion chromatography, ion exchange chromatography and trypsin affinity chromatography. On toxicity analysis the LC_{50} (48 hours) for *Aedes albopictus* larvae for crude, 0-30% ammonium sulphate fraction and size exclusion fraction were 0.50 ± 0.01 , 0.48 ± 0.01 , and 0.32 ± 0.00 $\mu\text{g/mL}$, respectively. The purified *Calopogonium mucunoides* trypsin inhibitor (CmTI) had a subunit molecular weight of 22 kDa. The yield of purified CmTI was 0.1 μg per gram of seed. For obtaining in large quantities, the CmTI gene can be cloned and expressed in a suitable host like bacteria or yeast, and heat killed cells expressing CmTI can be used as a mosquito larvicide.

Key words: Mosquito control, Cloning, *Aedes albopictus*, larvicide, trypsin inhibitor


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സംഗ്രഹം

നിരവധി പകർച്ചവ്യാധികൾ പരത്തുന്ന പ്രധാന കീടവാഹകരിൽ ഒന്നാണ് കൊതുകുകൾ. ഡെങ്കിപ്പനി, മലേറിയ, തുടങ്ങിയവ കൊതുകുകൾ വഴിയാണ് പകരുന്നത്. വ്യത്യസ്ത തരം രാസകീടനാശിനികൾ ഉപയോഗിച്ചുള്ള പരമ്പരാഗത നിയന്ത്രണരീതികൾ കൊതുകുകളിൽ പ്രതിരോധശേഷി വികസിക്കാനും ഗുരുതരമായ പരിസ്ഥിതി മലിനീകരണത്തിനും ആരോഗ്യ പ്രശ്നങ്ങൾക്കും കാരണമാകുന്നു. അതിനാൽ ലക്ഷ്യ നിർദ്ദിഷ്ടതയോടെയുള്ള പുതിയ നിയന്ത്രണ മാർഗങ്ങൾ വികസിപ്പിക്കേണ്ടതുണ്ട്. ഈ പഠനത്തിൽ, *ഈഡിസ് ആൽബോപിക്റ്റസ്* ലാർവകളെ നിയന്ത്രിക്കുന്നതിനുള്ള അത്തരം രണ്ട് രീതികൾ വിവരിച്ചിരിക്കുന്നു.

ആദ്യത്തെ രീതിയിൽ വൈൽഡ്-ടൈപ്പ് അല്ലെങ്കിൽ മ്യൂട്ടേഡ് ട്രിപ്ലിൻ മോഡ്യുലേറ്റിംഗ് ഊസ്റ്റാറ്റിക് ഫാക്ടർ (TMOF) പെപ്റ്റൈഡ് ന്റെ ജീൻ ക്ലോൺ ചെയ്ത് എസ്പെറിച്ചിയ കോളിയിൽ ഉത്പാദിപ്പിച്ചു. കൊതുകിന്റെ കൂടലിൽ ട്രിപ്ലിൻ സംശ്ലേഷണം തടയുന്ന ഒരു ഡെകാപെപ്റ്റൈഡാണ് TMOF. പ്രോട്ടീൻ ദഹിപ്പിക്കാൻ ട്രിപ്ലിൻ ആവശ്യമുള്ളതിനാൽ, TMOF ഉപയോഗിക്കുമ്പോൾ കൊതുകും കൊതുകിന്റെ ലാർവകളും മരിക്കുന്നു. TMOF/മ്യൂട്ടൻ്റ് TMOF ഉത്പാദിപ്പിക്കുന്ന ബാക്ടീരിയ കോശങ്ങളെ ലൈസ് ചെയ്ത് ക്രൂഡ് TMOF/മ്യൂട്ടൻ്റ് TMOF തയ്യാറാക്കി. *ഈഡിസ് ആൽബോപിക്റ്റസ്* ലാർവകളിൽ ഉപയോഗിക്കുമ്പോൾ 48 മണിക്കൂറിനുള്ളിൽ ഗണ്യമായ മരണനിരക്ക് രേഖപ്പെടുത്തി. ക്രൂഡ് TMOF-ന്റെ LC_{50} (48 മണിക്കൂർ) മൂല്യം $242.1 \pm 6.04 \mu\text{g/mL}$ ആയിരുന്നു. ക്രൂഡ് മ്യൂട്ടൻ്റ് TMOF-ന്റെ LC_{50} (48 മണിക്കൂർ) മൂല്യം $236.54 \pm 6.49 \mu\text{g/mL}$ ആയിരുന്നു. *ഈഡിസ് ആൽബോപിക്റ്റസ്* ലാർവകളിൽ, വേർതിരിച്ച TMOF-ന്റെ LC_{50} (48 മണിക്കൂർ) മൂല്യം $2.13 \pm 0.02 \mu\text{g/mL}$ ഉം വേർതിരിച്ച മ്യൂട്ടൻ്റ് TMOF-ന്റെ മൂല്യം $1.91 \pm 0.02 \mu\text{g/mL}$ ഉം ആയിരുന്നു. *ഈഡിസ് ആൽബോപിക്റ്റസ്* ലാർവകൾക്കുള്ള ലാർവിസൈഡ് എന്ന നിലയിൽ വേർതിരിച്ച TMOF-നെക്കാൾ 1.12 മടങ്ങ് മികച്ചതാണ് വേർതിരിച്ച മ്യൂട്ടൻ്റ് TMOF. ക്വലെക്സ് ക്വിൻക്വെഫാസിയാറ്റസ് ലാർവകളിൽ ക്രൂഡ് TMOF നും ക്രൂഡ് മ്യൂട്ടൻ്റ് നും ദോഷഫലം ഉണ്ടെങ്കിലും ഡ്രോസോഫില മെലനോഗാസ്റ്റർ, ലൂപോഫ് ട്രിസ്റ്റിസ്, അപ്ലോചെലസ് ലീനിയറ്റസ് തുടങ്ങിയ ലക്ഷ്യമില്ലാത്ത ജീവികളിൽ ദോഷഫലം കണ്ടെത്തിയില്ല. റീകോമ്പിനൻ്റ് TMOF/മ്യൂട്ടൻ്റ് TMOF ഉപയോഗിച്ചുള്ള പരീക്ഷണത്തിൽ *ഈഡിസ് ആൽബോപിക്റ്റസ്* ലാർവകളുടെ കൂടൽ ബാക്ടീരിയയുടെ വിശകലനത്തിൽ, സാധാരണ ലാർവകളെ അപേക്ഷിച്ച് കൂടൽ ബാക്ടീരിയയുടെ വൈവിധ്യത്തിന് കുറവുണ്ടെന്ന് കണ്ടെത്തി. TMOF/മ്യൂട്ടൻ്റ് TMOF ന്റെ പ്രവർത്തന സംവിധാനം റിസപ്റ്റർ മധ്യസ്ഥത വഹിക്കുന്നതിനാലും റിസപ്റ്റർ കൊതുകുകളിൽ നിന്ന് മാത്രമേ റിപ്പോർട്ട് ചെയ്യപ്പെടുന്നതിനാലും, ലാർവിസൈഡായി റീകോമ്പിനൻ്റ് TMOF/മ്യൂട്ടൻ്റ് TMOF ഉപയോഗിക്കുന്നത് കൊതുകു നിയന്ത്രണത്തിന്റെ പരിസ്ഥിതി സൗഹൃദ രീതിയാണ്.

രണ്ടാമത്തെ രീതിയിൽ ഒരു പുതിയ ട്രിപ്ലിൻ ഇൻഹിബിറ്ററിനെ ഉപയോഗിക്കുന്നു. അതിനായി ട്രിപ്ലിൻ ഇൻഹിബിഷൻ അസ്സേ വഴി 45 സസ്യ സത്തുകൾ പരിശോധിച്ചു. പ്രോട്ടീനേസ് കെ ഉപയോഗിച്ച് പ്രോട്ടീനുകളായ ഇൻഹിബിറ്ററുകൾ ഉള്ള സസ്യ സത്തുകൾ കണ്ടെത്തി *ഈഡിസ് ആൽബോപിക്റ്റസ്* ലാർവകളിൽ പരീക്ഷിച്ചു. കാലോപോഗോണിയം മ്യൂക്കുനോയിഡസ് വിത്തുകളിൽ നിന്നുള്ള സത്തിൽ ഏറ്റവും ഉയർന്ന മരണനിരക്കും ($100 \pm 0.00\%$) ഏറ്റവും ഉയർന്ന ട്രിപ്ലിൻ ഇൻഹിബിഷനും ($87.99 \pm 0.38\%$) കാണിച്ചു. അങ്ങനെ, കാലോപോഗോണിയം മ്യൂക്കുനോയിഡസ് വിത്തുകളിൽ നിന്നുള്ള സത്ത് ട്രിപ്ലിൻ ഇൻഹിബിറ്ററിനെ വേർതിരിക്കാനായി

ഉപയോഗിച്ചു. വിഷാംശ വിശകലനത്തിൽ, ക്രൂഡ്, 0-30% അമോണിയം സൾഫേറ്റ്, ഫാക്ഷൻ, സൈസ് എക്സ്ക്ലൂഷൻ ഫാക്ഷൻ എന്നിവയ്ക്ക് *ഈഡിസ് ആൽബോപിക്റ്റസ്* ലാർവകളിൽ LC_{50} (48 മണിക്കൂർ) മൂല്യം യഥാക്രമം 0.50 ± 0.01 , 0.48 ± 0.01 , 0.32 ± 0.00 $\mu\text{g/mL}$ ആയിരുന്നു. ശുദ്ധീകരിച്ച ട്രിപ്പിൻ ഇൻഹിബിറ്ററായ CmTI യുടെ തന്മാത്രാ ഭാരം 22 kDa ആയിരുന്നു. വലിയ തോതിലുള്ള ഉത്പാദനത്തിനും ഉപയോഗത്തിനും, CmTI ജീൻ ബാക്ടീരിയ അല്ലെങ്കിൽ യീസ്റ്റ് പോലുള്ള അനുയോജ്യമായ ഒരു ഹോസ്റ്റിൽ ഉത്പാദിപ്പിക്കാൻ കഴിയും, കൂടാതെ അത്തരം കോശങ്ങളെ നിർവീര്യമാക്കിയ ശേഷം, കൊതുകുകൾക്ക് ഭക്ഷണം ആയി ഇത് ഉപയോഗിക്കാം. അങ്ങനെ ഈ രീതിയിൽ കൊതുക് നിയന്ത്രണ നടപടിയായി ഉപയോഗിക്കാം.

പ്രധാന വാക്കുകൾ: കൊതുക് നിയന്ത്രണം, ക്ലോണിംഗ്, *ഈഡിസ് ആൽബോപിക്റ്റസ്*, ലാർവിസൈഡ്, ട്രിപ്പിൻ ഇൻഹിബിറ്റർ

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GENERAL INTRODUCTION

0.1.1 Mosquito-borne diseases: public health impact and control measures

The outbreak of infectious diseases has been a common occurrence in different parts of the world. Direct or indirect contact, as well as some vehicles carrying the pathogens, can lead to its contraction. Vectors are organisms that act as carriers of various pathogens and transmit infectious diseases. Vector-borne disease accounts for more than 17% of global infectious diseases caused by parasites, viruses, or bacteria, with more than 700,000 deaths annually (WHO, 2024f). About 80% of the global population is at risk of any one or more vector borne diseases (WHO, 2024e). Dengue, malaria, filariasis, chikungunya, Lyme disease, etc. are some of the vector-borne diseases. Mosquitoes, tsetse flies, black flies, ticks, mites, reduviid bugs etc. are important vectors of infectious diseases.

Mosquitoes are vectors of many diseases including dengue, chikungunya, yellow fever, malaria and Zika etc. About 10% of the known mosquito species are recognised to have vectorial competence with public health relevance (Yee et al., 2022). The three main mosquito genera involved in disease transmission are *Anopheles*, *Aedes* and *Culex*. Malaria, the most common vector-borne disease is transmitted by female *Anopheles* mosquitoes. They transmit the protozoan *Plasmodium*, which is the causative organism of malaria. Malaria causes about 249 million cases globally and results in more than 608,000 deaths

annually (WHO, 2024f). The most prevalent viral infection transmitted by *Aedes* mosquitoes, specifically *A. aegypti* and *A. albopictus*, is the dengue virus. Dengue virus causes about 40000 deaths annually with more than 3.9 billion people in 132 countries are at risk of contracting dengue (WHO, 2024f). The mosquitoes in *Culex* and *Anopheles* genera can transmit nematodes like *Wuchereria bancrofti* which cause lymphatic filariasis that affects the lymphatic system and causes disability. The most important group of pathogens transmitted by mosquitoes is arboviruses. About 70% of known pathogenic arboviruses are transmitted by mosquitoes (Puig-Torrents & Diez, 2024; Young, 2024).

Aedes albopictus is one of the most invasive mosquito species (Bonizzoni M et al., 2013). Originating from Southeast Asia with the common name Asian tiger mosquito, *A. albopictus* have invaded almost all major continents. *Aedes albopictus* are transported globally via national and international trade through water, air, or road. Plant products and contaminated tyres are one of the major pathways enabling their widespread dispersal. The *A. albopictus* species has the ability to adapt to climatic changes and coexist with other vector species (Paupy et al., 2009; Medlock et al., 2012). They are opportunistic feeders, having a wide host range (Eritja et al., 2005). *Aedes albopictus* is a carrier of pathogens like viruses, bacteria, nematodes, etc. They harbour more than 22 kinds of arboviruses and are the primary vector for dengue after *A. aegypti* (European Union, 2016). *Aedes albopictus* transmits diseases like dengue, chikungunya, Zika, West Nile fever, etc. (Bonizzoni M et al., 2013).

Changes in environmental conditions coupled with anthropogenic interactions have caused increased incidence of infectious diseases. Mosquitoes share a major hold in transmitting various kinds of pathogens, and this makes the control of mosquito population, an important task for the maintenance of public health. Most of the diseases transmitted by mosquitoes are viral-borne and have no cure. So, prevention of chances to get infection is the primary option to get rid of such diseases. Usage of mosquito nets impregnated with insect repellents, window screens, mosquito repellents containing DEET, Picaridin or IR3535, use of mosquito coils and vaporisers and usage of clothes covering maximum body parts are ways of personal protection (WHO, 2024a). The conventional mosquito control methods use chemical insecticides like pyrethrins, organochlorides, organophosphates and carbamates. Pyrethroids are the most widely used insecticides for indoor residual spraying and the only synthetic insecticide currently used in insecticide treated nets and fabrics, with irritant or repellent activity on mosquitoes and less mammalian toxicity (WHO, 2020). One of the main organochlorides used since earlier times was DDT which was favoured for its cheap availability and higher efficiency. However, insecticides like DDT caused severe environmental issues and non-target toxicity. Continuous use of insecticides led to the development of resistance in mosquitoes, and the prolonged exposure to insecticides caused the mosquitoes to resist more than one class of insecticides (Şengül Demirak & Canpolat, 2022). Another adaptation of mosquitoes was in their increased activity of detoxifying enzymes, which are metabolic enzymes responsible for biodegradation of insecticides and elimination of their insecticidal

effects (Hemingway & Ranson, 2000). Use of plant products like essential oils as repellents became common as it has no severe side effects. In addition, insect growth regulators like pyriproxyfen have also been found to be effective against *Aedes aegypti* mosquitoes (Sihuincha et al., 2005).

Environment-friendly methods involving the use of insectivorous fishes, biopesticides, pheromones, sterilized males, refractory mosquitoes, endosymbiont, midgut symbionts, etc. are being developed with varying degrees of success and have been used for mosquito control (Tyagi, 2016). Another development in the field is the use of insecticide conjugated to nanoparticles. Silver nanoparticles coupled with plant products having mosquito larvicidal property have been reported (Benelli et al., 2017). The use of bioinsecticides, composed of botanical or plant-based compounds, has been a perfect alternative due to their minimal hazardous effects on human health and the environment (Şengül Demirak & Canpolat, 2022). The concept of Sterile Insect Technique (SIT) was reported in 1959 (Knipling, 1959) and was applied to mosquito control (Bellini et al., 2013). The SIT method was used to control *Aedes aegypti* and *A. albopictus* mosquito populations (Bouyer et al., 2020; Lees et al., 2021). Boosted SIT is another concept combining other control agents like pyriproxyfen in autodissemination with SIT for a better control (Bouyer & Lefrançois, 2014). A new formulation in the form of tablets containing a lectin preparation showed mosquitocidal activity against different developmental stages of *Aedes aegypti* mosquitoes (Silva et al., 2021).

The use of enzyme-inhibitor interaction is reported for managing various pests (Ryan, 1990). One such class of compounds is plant protease inhibitors, which are found to be effective against different pests as they inhibit gut proteases interrupting the digestion of proteins (Ryan, 1990; De Leo et al., 2002). Trypsin being the major protease in the mosquito gut, inhibiting it by trypsin inhibitors of botanical origin is a promising strategy. For this purpose, many trypsin inhibitors from plants are purified and characterised.

0.1.2 Trypsin Modulating Oostatic Factor and its application in mosquito control

Inhibition of yolk deposition in mosquitoes by ovarian humoral factors has been demonstrated (Meola & Lea, 1972; Else G James & Judson, 1972). Oostatic hormones from various insects were reported to have anti-gonadotropic activity (Huebner E. & Davey K.G., 1973; Liu & Davey, 1974; Davey & Kuster, 1981). The oostatic hormone from *Aedes aegypti* was purified, and the decapeptide (YDPAPPPPPP) was named as Trypsin Modulating Oostatic Factor (TMOF). Secreted primarily by ovaries 18 hours after a blood meal, this decapeptide interferes in trypsin biosynthesis in mosquito midgut. The TMOF peptide in the haemolymph binds to the ABC transporter, enters the midgut cells and blocks trypsin mRNA translation. The stoppage of trypsin synthesis prevents blood digestion and affects metabolic activities, including vitellogenesis. Absence of vitellogenin affects egg development, and unavailability of amino acids from protein digestion leads to the death of the organism. Thus, TMOF peptide can be used as a promising strategy for mosquito control.

Trypsin like enzymes are physiologically important in many organisms, including mosquito larvae (Y. J. Yang & Davies, 1971). The amount of trypsin and chymotrypsin in the larval gut increases from first instar to fourth instar and then decline as it enters pupation (Y. J. Yang & Davies, 1971). Larvicidal activity of TMOF peptide isolated from *A. aegypti* (Aea-TMOF) and its analogues was observed on feeding these to *A. aegypti* larvae along with Brewer's yeast. It was confirmed that the quantity of TMOF provided via feed had an inverse relation with survival of the larvae and the N-terminal four amino acids (YDPA) alone in TMOF are enough to produce 95% activity (Borovsky & Meola, 2004). Also, TMOF was expressed as a fusion protein with the coat protein of tobacco mosaic virus and fed to the *A. aegypti* larvae as yeast adsorbed virions. This caused inhibition of trypsin activity up to about 90 percent and resulted in larval starvation and mortality (Borovsky et al., 2006). Trypsin Modulating Oostatic Factor expressed in *Pichia pastoris* cells produced a synergistic effect with Cry proteins when fed to *A. aegypti* larvae (Borovsky et al., 2010). Expression of Aea-TMOF in the fungi *Beauveria bassiana*, an entomopathogenic fungi, resulted in increased virulence against larvae and adults of *Anopheles gambiae* causing mortality and reduced fecundity (Kamareddine et al., 2013). Genetically engineered *Chlorella desiccata* with *tmfA* gene expressing TMOF peptide when fed to the larvae of *A. aegypti* resulted in low survival and can cause mortality up to 96% (Borovsky et al., 2016). Also, heat killed *Saccharomyces cerevisiae* cells transformed with gene carrying *tmfA* and *gfp-tmfA* when fed to *A. aegypti* mosquito larvae, caused mortality to an extent of 95% (Borovsky et al., 2018). Using a multi copy integrating plasmid pICZB, multiple insertions of *tmfA* gene

was achieved in *Pichia pastoris* cells resulting in increased expression of TMOF leading to a higher mortality within 3 days (Borovsky et al., 2020).

0.1.3 Use of plant protease inhibitors for mosquito control

Proteases are hydrolytic enzymes that cleave peptide bonds in target proteins. They are found in a wide range of organisms and catalyse many metabolic processes like protein digestion for providing essential nutrients, such as amino acids, for the growth of organism. However, they need to be regulated; otherwise, continuous protease action evokes some other detrimental effects. Protease inhibitors are molecules that modulate protease activity, thereby regulating its activity. These inhibitors are broadly distributed across plants, animals, and microorganisms (Valueva & Mosolov, 2004; Christeller, 2005; Haq et al., 2004; Supuran et al., 2002; Mosolov & Valueva, 2005; Mosolov et al., 2001). In plants, protease inhibitors are typically found in storage organs or other aerial parts (De Leo et al., 2002) and are often induced in response to injury or attacks by insects or pathogens (Ryan, 1990). Plant protease inhibitors (PPIs) are key components of plant defence systems. They disrupt the digestive enzymes in the gut of herbivores, reducing herbivory. Plant protease inhibitors may be proteinaceous or non-proteinaceous in nature (Polya, 2003). Plant protease inhibitors are divided into four classes such as cysteine protease inhibitors, aspartyl protease inhibitors, metalloprotease inhibitors and serine protease inhibitors (Koiwa et al., 1997) based on the type of proteases they inhibit. Out of these, large numbers of PPIs are directed towards serine and cysteine proteases (Turk & Bode, 1991).

In insects also proteases play a major role in the growth, development and reproduction. Antinutritional effect of plant serine protease inhibitors against lepidopteran insect pests were reported in previous studies (Shukle & Murdock, 1983). Cysteine protease inhibitors have the ability to suppress various infectious nematodes like *Meloidogyne incognita*, *Globodera tabaccum*, and *G. pallida* (Williamson & Hussey, 1996). Similarly, cysteine protease inhibitors from pearl millet inhibit development of various pathogenic fungi, for example, filamentous fungus (*Trichoderma reesei*) (Joshi et al., 1998). A serine protease inhibitor from *Glycine max* has negative effects on the larvae of *Spodoptera littoralis* (El-latif, 2015); another protease inhibitor from *Cajanus platycarpus* was found to be active against *Helicoverpa armigera* (Swathi et al., 2015).

Transfer of cowpea trypsin inhibitor gene to tobacco offered resistance to various insect pests like *Heliothis*, *Spodoptera*, *Locusts* etc. (Hilder et al., 1987). This study led to the idea of development of transgenic plants with resistance to various pests. The potato proteinase inhibitor II (PINII) gene was transferred to Japonica rice varieties which resulted in the accumulation of PINII protein offering resistance to the pink stem borer, *Sesamia inferens* (Duan et al., 1996). The maize proteinase inhibitor and potato carboxypeptidase inhibitor were fused and introduced into rice plants, and the expressed gene confers resistance to *Chilo suppressalis* (Quilis et al., 2014). Protein engineering can be used as an effective approach for developing recombinant protease inhibitors with potent pesticidal activity. However, there are compensating physiological mechanisms such as overproduction of digestive proteases

adopted by insects against anti-nutritional dietary challenges (Zhu-Salzman & Zeng, 2015).

Trypsin inhibitors from various plants have been identified with potential for mosquito control, as trypsin is a major gut protease in mosquitoes (Pontual et al., 2014; Sasaki et al., 2015; Sara et al., 2020; N et al., 2021). A Kunitz type trypsin inhibitor has been purified from the *Cassia leiandra* seeds, having larvicidal activity on *A. aegypti* with an LC_{50} of 2.28×10^{-2} M (Dias et al., 2017), and from *Allium sativum* (Shamsi et al., 2018) with LC_{50} of 50.83 $\mu\text{g/mL}$. Another trypsin inhibitor purified from *Enterolobium contortisiliquum* seeds caused toxicity in *A. aegypti* larvae in response to prolonged exposure and had enhanced activity when combined with *Bti* toxins (Tabosa et al., 2020).

The present work describes two new methods for the control of *A. albopictus* larvae. The first method was using wild-type or mutated TMOF peptide expressed in *Escherichia coli*. In addition, the impact of the TMOF treatment on the gut microbiota of the mosquito larvae was also examined. The second method was using trypsin inhibitor isolated from the seeds of *Calopogonium mucunoides* to control the larvae of *A. albopictus* mosquito.

CHAPTER 1

**Cloning and expression of TMOF/mutant TMOF in
Escherichia coli and study its toxicity and impact on
gut bacterial composition in the larvae of
Aedes albopictus mosquito**

1.1 INTRODUCTION

Vector-borne diseases account for more than 17% of global infectious diseases caused by parasites, viruses or bacteria, with more than 700,000 deaths annually (WHO, 2024f). Malaria, lymphatic filariasis, trypanosomiasis, dengue and Zika are some of the major vector-borne diseases. Many organisms transmit infectious pathogens mechanically or biologically. Arthropods form a major group of biological vectors, including mosquitoes, flies, ticks and mites. Most of these biological vectors are haematophagous with blood-sucking adaptations to feed on the host. The ability of a vector species to acquire, maintain, and transmit specific pathogens depends upon various factors like the susceptibility of a vector to get infected, the ability of a pathogen to replicate within the vector, and the capacity of a vector to transmit this pathogen to new hosts (Holmes, 2023). This broad concept is described as the vectorial capacity of a vector species.

Mosquitoes are major biological vectors transmitting diseases like dengue, malaria, chikungunya, West Nile fever, Japanese encephalitis etc. About 10% of the known mosquito species are recognised to have vectorial competence, which is a fact of public health concern (Yee et al., 2022). Among mosquitoes, *Anopheles*, *Culex* and *Aedes* are the three main genera involved in disease transmission. *Aedes* group is a major vector of arthropod-borne viruses like dengue, Zika and chikungunya virus. The two major species of *Aedes* are *A. aegypti* and *A. albopictus*. *A. albopictus* is more aggressive in terms of its dispersal and nuisance behaviour compared to *A. aegypti*. The ability of *A. albopictus* to adapt to climatic change and increase their potential to spread and survive new

environments makes them the most invasive mosquito at present (Bonizzoni et al., 2013). Besides, they can coexist with other vector species and are opportunistic feeders with a wide host range, including humans, domestic and wild animals, reptiles, birds and amphibians (Turell et al. 1995 ; Eritja et al. 2005 ; Paupy et al., 2009; Medlock et al., 2012). These make the control of mosquitoes relevant for the sake of public health. Mosquito control using conventional insecticides such as organophosphates and organochlorides pose severe problems like the development of resistance and environmental health hazards. Thus, the development of new biorational strategies is required.

The effect of oostatic factors on egg development in many insects is reported. Trypsin modulating oostatic factor (TMOF) is a decapeptide isolated from *Aedes aegypti* mosquitoes. This ovarian peptide inhibits trypsin biosynthesis in gut of mosquitoes and is a part of organism's own way of enzyme regulation. Recent research using TMOF peptide had shown the toxicity of TMOF to mosquitoes like *Aedes aegypti*, *Anopheles gambiae*, *Culex quinquefasciatus*, etc. (Borovsky et al., 1990; Borovsky & Meola, 2004). These studies report that the TMOF peptide can be used for the control of mosquito larvae.

The TMOF peptide has been used as fusion protein with *Bti* toxins and conjugated with polyethylene glycol. In some other formulations, TMOF was adsorbed on yeast particles for toxicity analysis. Advanced studies by expressing TMOF in different hosts like yeast, algae and fungi, which are directly fed by the larvae, were also conducted (Kamareddine et al., 2013; Borovsky et al., 2016; Borovsky et al., 2018).

All these feeding experiments confirm the ability of TMOF as a biorational way of mosquito control.

In this work, TMOF was cloned and expressed in *Escherichia coli* cells and studied its toxicity to *Aedes albopictus* larvae. A mutant TMOF gene was also identified, expressed in *E. coli* and compared for its efficiency as a larvicide with that of wild type. The effect of recombinant proteins on the gut microbiota of *A. albopictus* was also studied, as gut microbiome has a prominent role in determining the vectorial competence.

1.2 OBJECTIVES OF THE STUDY

- 1) To clone the gene coding for TMOF and express in bacteria and to study its effect on *Aedes albopictus* larvae
- 2) To mutate the gene coding for TMOF to change the amino acids in the peptide
- 3) To compare the effect of mutant TMOF to that of wild type TMOF for identifying better TMOF peptide sequence for mosquito control
- 4) To study the gut bacterial composition of larvae treated with recombinant TMOF peptide by 16S ribosomal RNA gene sequencing

1.3 REVIEW OF LITERATURE

1.3.1 Vector-borne diseases

Different parts of the world have witnessed an upsurge of infectious diseases. Vectors are described as any organism that can act as a carrier of infectious pathogens among different species of organisms (Kuno & Chang, 2005). Vector-mediated transmission is described as either mechanical or biological, which occurs by biting, penetration or through the gastrointestinal tract. In mechanical transmission, a vector simply transports a pathogen from contaminated sources without interfering with any biological activities of either. But in biological transmission, as the term indicates, a biological association develops between the carrier and the pathogen being transmitted. It occurs when the pathogen reproduces and develops to its mature infective stages within the vector and is being transmitted to the host. Arthropods are one of the major groups of biological vectors, including mosquitoes, flies, ticks, mites etc. Hemipteran bugs are another group of biological vectors. Among arthropods, hexapods are the major vectors, and they transmit pathogens by biting the host. The common feature of most of these biological vectors is their blood sucking nature. Female mosquitoes require a protein-rich blood meal for the egg development.

Vector-borne disease accounts for more than 17% of global infectious diseases caused by parasites, viruses or bacteria, with more than 700,000 deaths annually (WHO, 2024f). About 80% of the global population is at risk of any one or more vector-borne diseases (WHO, 2024e). Dengue, malaria, Lyme disease, Chagas disease, rickettsial pox etc. are

some of the vector-borne diseases. Dengue and malaria are transmitted by mosquitoes, Lyme disease by ticks, Chagas disease by hemipteran bugs and rickettsial pox by mites. Table 1.3.1 lists the ten most important vector-borne human diseases (Shaw & Catteruccia, 2019).

Table 1.3.1 List of major vector-borne human diseases

Human disease	Causative organism	Vector
Malaria	Protozoan: <i>Plasmodium</i>	Mosquito: <i>Anopheles</i>
Lymphatic filariasis	Nematodes: <i>Wuchereria bancrofti</i> , <i>Brugia malayi</i> , <i>Brugia timori</i>	Mosquito: <i>Culex</i> , <i>Aedes</i> , <i>Anopheles</i> (in Africa), <i>Mansonia</i>
Sleeping sickness (human African trypanosomiasis)	Protozoan: <i>Trypanosoma brucei</i>	Tsetse fly: <i>Glossina</i>
Chagas disease (American trypanosomiasis)	Protozoan: <i>Trypanosoma cruzi</i>	Reduviid bug: <i>Rhodnius</i> , <i>Triatoma</i>
Leishmaniasis	Protozoan: <i>Leishmania</i>	Sand fly: <i>Phlebotomus</i> , <i>Lutzomyia</i>
River blindness (Onchocerciasis)	Nematode: <i>Onchocerca volvulus</i>	Black fly: <i>Simulium</i>
Dengue	Flavivirus: DENV	Mosquito: <i>Aedes</i>
Yellow fever	Flavivirus: YFV	Mosquito: <i>Aedes</i> , <i>Haemagogus</i>
Zika	Flavivirus: ZIKV	Mosquito: <i>Aedes</i>
Chikungunya	Flavivirus: CHIKV	Mosquito: <i>Aedes</i>

Like all pathogens cannot thrive in different vector species, the vector species also cannot afford the needs required by every pathogen. There are adaptations developed by pathogens as well as the vectors. The pathogens make use of the blood feeding adaptations a vector organism possesses to get in and satisfy their intrinsic needs. The ecological conditions, behavioural patterns, requirement of nutrients, etc. contribute to the ways through which a vector is evolving. Vector competence refers to the ability of a vector species to acquire, maintain, and transmit specific pathogens and depends on various factors like the susceptibility of a vector to getting infected, the ability of a pathogen to replicate within the vector, and the capacity of a vector to transmit the pathogen to new hosts (Holmes, 2023). The introduction of infection by a vector between endemic and non-endemic areas is aided by mechanical translocation and subsequent adaptation (Mattar V & González T, 2016).

1.3.2 Major arthropod vectors

Arthropods are one of the most diverse group of organisms and are still evolving. Their ability to withstand and adapt to various conditions and host preferences has enabled them to act as successful vectors of infectious pathogens. Arachnids (ticks) and insects are the major arthropods with vector adaptations. Most of them are haematophagous, and during this time, transmission of pathogens to the host occurs. Mosquitoes and tsetse flies transmit pathogens primarily stored in the salivary glands while feeding on blood from a host; sand flies transmit pathogens via saliva, and in black flies, the pathogens are forced out of the vector head while chewing host skin. Ticks can sense body heat and odours and can attach to the human body. Fleas act as carriers of tapeworms and bacteria like *Yersinia pestis*, the causative organism of

plague. *Chrysomia* species and cockroaches are mechanical vectors for diarrhoea and dysentery (Europa, 1997). Table 1.3.2 lists the major arthropod vectors (WHO, 2024f).

Table 1.3.2 List of major arthropod vectors (Reproduced from WHO, 2024f)

Vector	Disease caused	Type of pathogen
<i>Aedes</i> mosquitoes	Chikungunya Dengue Lymphatic filariasis Rift Valley fever Yellow fever Zika	Virus Virus Parasite Virus Virus Virus
<i>Anopheles</i> mosquitoes	Lymphatic filariasis Malaria O'nyong'nyong virus	Parasite Parasite Virus
<i>Culex</i> mosquitoes	Japanese encephalitis Lymphatic filariasis West Nile fever	Virus Parasite Virus
Culicoides flies	Oropouche fever	Virus
Blackflies	Onchocerciasis (river blindness)	Parasite
Fleas	Plague Tungiasis	Bacteria Ectoparasite
Lice	Typhus Louse-borne relapsing fever	Bacteria Bacteria
Sandflies	Leishmaniasis Sandfly fever (Phlebotomus fever)	Parasite Virus
Ticks	Crimean-Congo haemorrhagic fever Lyme disease Relapsing fever (borreliosis)	Virus Bacteria Bacteria

Vector	Disease caused	Type of pathogen
	Rickettsial diseases	Bacteria
	Tick-borne encephalitis	Bacteria
	Tularaemia	Bacteria
Triatome bugs	Chagas disease (American trypanosomiasis)	Parasite
Tsetse flies	Sleeping sickness (African trypanosomiasis)	Parasite

A notable characteristic of arthropod disease vectors is the significant individual variations in their susceptibility to pathogenic infection (Baxter et al., 2017). Arthropod-borne viruses, or arboviruses, are a group of viruses that are specifically transmitted by arthropod vectors. The vectoral capacity enables the arthropods to be the carriers of a range of arboviruses. Seventy out of over 500 identified arboviruses are infecting humans and are mostly transmitted through mosquitoes, flies, ticks and midges (Puig-Torrents & Díez, 2024). Chikungunya, dengue, Powassan and St. Louis encephalitis are some arboviral infections. The increased prevalence and spread of arboviral diseases is driven by ecological and human demographic factors (Young, 2024).

1.3.3 Mosquitoes as vectors

Mosquitoes are small dipterans with a slender body and a pair of wings. The females are haematophagous while males are phytophagous. The blood that fed from the host provides the female mosquitoes a protein-rich meal for the production of eggs. The range of hosts varies in

different mosquito species, and humans are one of the common ones for most. The bloodsucking nature is supported by the modified mouth parts they possess and accounts for their ability as one of the most efficient biological vectors.

Mosquitoes are vectors of many infectious diseases including malaria, dengue, chikungunya, lymphatic filariasis, Zika and West Nile fever. Approximately 10% of the known mosquito species are recognised as potential or confirmed vectors of pathogens with public health relevance (Yee et al., 2022). Mosquito microbiota has as a key role in the development of pathogens in their gut, which leads to the development of resistance to pathogens and the cost of parasite infections in terms of survival rate (Clayton et al., 2013; Gabrieli et al., 2021). Malaria, a parasitic infection caused by *Plasmodium* and transmitted by Anopheline mosquitoes, causes about 249 million cases globally and results more than 608,000 deaths annually (WHO, 2024f). Dengue, the most prevalent viral infection transmitted by *Aedes* mosquitoes, causes about 40000 deaths annually, with more than 3.9 billion people in 132 countries are at risk of contracting dengue (WHO, 2024f). Since 2014, major outbreaks of dengue, malaria, yellow fever and Zika have affected populations, causing challenges to health systems of many countries while other diseases such as chikungunya and lymphatic filariasis resulted in chronic suffering, life-long morbidity, disability and occasional stigmatisation (WHO, 2024f).

Plasmodium is the parasitic protozoan transmitted by female *Anopheles* mosquitoes which is responsible for malaria, the most prevalent vector-borne disease (Shaw & Catteruccia, 2019). Out of the 400 species of

Anopheles mosquitoes present, about 40 species are known to have the status of a malaria vector (WHO, 2024d) including *Anopheles gambiae*, *Anopheles arabiensis* and *Anopheles stephensi*. Malaria can be cured if treated early. Infectious sporozoites stored in the salivary glands of mosquitoes enter the human blood stream while the vector feeds on blood. Within human body they undergo asexual replication, giving rise to trophozoites that develop into invasive gametocytes in the liver and RBCs. The gametocytes then enter the mosquito body and complete the sporogonic cycle to give rise to infectious sporozoites (Zekar & Sharman, 2025).

Lymphatic filariasis or elephantiasis, is a nematode infection caused by *Wuchereria bancrofti* transmitted through *Culex* and *Anopheles* mosquitoes although transmission through *Aedes* is reported in some areas. The immature filarial larvae transmitted by mosquitoes enter the human body, mature and reach the lymphatic system where they reproduce and give rise to microfilariae, the infectious forms (WHO, 2024c). They are taken by mosquitoes while feeding on blood, and the cycle continues. Filariasis affects the lymphatic system, causes abnormal enlargement of body parts and imparts pain, disability and social stigma (WHO, 2024c). There are reports indicating the role of *Aedes notoscriptus* in the transmission of the bacterium *Mycobacterium ulcerans* causing Buruli ulcer (Mee et al., 2024; Buultjens et al., 2024).

Arboviruses are the most important pathogens transmitted by mosquitoes. More than 70% infectious arboviruses are transmitted by mosquitoes (Puig-Torrents & Díez, 2024; Young, 2024). An extension in the geographical range of arboviruses is supported by increased

human movement and urbanisation, environmental adaptation of the mosquito vectors with climate change, and the uncontrolled expansion of mosquito populations (WHO, 2024b). The spread of mosquito-borne viruses is dependent on the presence of a suitable mosquito vector. In addition, the knowledge of the mosquito species distribution and their vector competence are crucial factors in estimating the risk of mosquito-virus emergence to new areas or their maintenance in particular regions (Schulz & Becker, 2018). *Aedes* and *Culex* are the most relevant arbovirus-transmitting mosquito group. Within the *Aedes* genus, *Aedes aegypti* and *A. albopictus* particularly serve as vectors for a range of viruses, including chikungunya virus (CHIKV), dengue virus (DENV), and zika virus (ZIKV). Additionally, *A. aegypti* is also a vector for yellow fever virus (YFV). The *A. aegypti* species originated in Africa and spread to the Americas between the 15th and 18th centuries through trade ships, and to Asian countries in the 19th century (Brown et al., 2014). *Aedes albopictus*, originally from Southeast Asian forests, spread to islands in the Pacific and Indian oceans and to Europe (Medlock et al., 2012) and America (Carvalho et al., 2014) around the 1980s. *Aedes aegypti* as well as *A. albopictus* used to feed on animal blood before their expansion to other geographical areas. However, currently they prefer human blood (Tempelis et al., 1970; Kamgang et al., 2012; Sivan et al., 2015). Predicted distribution of *Aedes* vector species shows that they are now present in almost all continents and expand rapidly to new geographical areas (Kraemer et al., 2015). Among the *Culex* genus, *C. pipiens* is the main vector of West Nile virus (WNV), Eastern equine encephalitis virus (EEEV), Western equine encephalitis virus (WEEV), St. Louis encephalitis virus (SLEV), Sindbis virus (SINV), and Usutu

virus (USUV). *Culex pipiens* is native to North Africa, West Asia and Europe. Similar to *Aedes*, trading via ships and worldwide movement of the human population contributed to worldwide distribution of *C. pipiens* (Haba & McBride, 2022). Most of the *Culex* species feed both on human hosts and other vertebrates. *Culex pipiens* predominantly blood feeds on birds (42.5–70.5%), playing an important role in the spread of WNV from secondary reservoirs in the avian-to-mammal cycle (Sawabe et al., 2010; Osório et al., 2012; Tiron et al., 2021).

1.3.4 *Aedes albopictus* vector status

Aedes albopictus is the most invasive mosquito at present (Bonizzoni M et al., 2013). *Aedes albopictus*, originally from Southeast Asia, spread to islands in the Pacific and Indian oceans, and further to Europe and America (Medlock et al., 2012; Carvalho et al., 2014). *Aedes albopictus* is currently considered as ecotonal and found in degraded and managed landscapes and densely populated urban areas, typically characterised by little vegetation and abundant artificial breeding sites and human hosts (Paupy et al., 2009; Reiskind et al., 2017; Hendy et al., 2020; Yeo et al., 2023). With the common name as ‘Asian tiger mosquito’, *A. albopictus* is aggressive in terms of its dispersal, nuisance and ability to transmit various germs. The competence and ecological plasticity enable them to thrive in tropical as well as temperate conditions. They can lay desiccation-resistant eggs, thus tide over extreme conditions. The ecological plasticity of *A. albopictus* varies in their feeding behaviour and breeding sites. In addition, their ability to adapt to climatic change increases their potential to spread and survive in new environments and coexist with other species of vectors (Paupy et al.,

2009; Medlock et al., 2012). *Aedes albopictus* are called as opportunistic feeders (Turell et al., 2005) owing to the wide host range, including human, domestic and wild animals, reptiles, birds and amphibians (Eritja et al., 2005). *Aedes albopictus* is a carrier of a variety of pathogens like bacteria, nematodes and viruses. It harbours more than 22 kinds of arboviruses and is a primary vector for dengue after *A. aegypti* (European Union, 2016).

Aedes albopictus is a known vector of emerging and re-emerging arboviruses like ZIKV, DENV, CHIKV that produced outbreaks in several countries, raising an unprecedented number of clinical cases (Gutiérrez-López et al., 2023; Fritzell et al., 2018). This ability of *A. albopictus* to carry different kinds of viruses and feed on multiple hosts make them more competent as a vector.

1.3.5 Mosquito control measures

The increased incidence of infectious diseases results from changes in environmental conditions, travel and transport, chaotic urbanisation and insufficient vector control measures. Geographical conditions and uncleanliness are also factors in favour of increasing mosquito population. Vector control is of paramount importance, as no treatment is available for many of the vector-borne diseases. In the case of mosquitoes, it is a requisite in light of their medical and veterinary importance. Most of the diseases transmitted by mosquitoes are viral-borne and have no complete cure. So, prevention of chances to get infection is the primary option to get rid of such diseases. The mosquito control measures include physical, chemical and biological methods.

The first line of defence to mosquito populations lies in understanding the vector populations, their life cycle and behavioural patterns. The most convenient control measure for mosquito population is in the management of mosquito larval growth by reducing the sources of larval growth. Physical methods target the elimination of mosquito breeding sites and advise the usage of personal protection measures. Proper disposal of waste, cleaning domestic and outdoor water containers and removal of artificial habitats for mosquitoes should be done to eliminate mosquito breeding sites (WHO, 2024a). Usage of mosquito nets impregnated with insect repellents, use of mosquito coils and vaporizers, window screens and usage of clothes covering maximum body parts can be taken for personal protection (WHO, 2024a). Plant based essential oils mostly produced from members of the Lamiaceae, Poaceae, Rutaceae and Myrtaceae families and synthetic products like indalone, dimethylphthalate 2-ethyl-1, 3-hexane diol and N,N-diethyl-m-toluamide (DEET) are commonly used insect repellents (Tyagi, 2016). The oviposition trap invented and used for the surveillance of adult *Aedes* vectors was modified to render it lethal to larvae of *A. aegypti* (Lok et al., 1977). Practically, oviposition traps can destroy progeny by using fine nylon netting. Modified traps with insecticide treated oviposition substrate can kill the eggs, larvae if any hatched and the adults which came into contact with the insecticide (Lok et al., 1977; Zeichner & Perich, 1999).

Biological control uses certain organisms, natural products and enemies for mosquito control. Biological agents, such as bacteria, fungi, viruses, predaceous insects such as dragonflies, notonectids and copepods, and

larvivorous fishes, can limit the larval population to an extent without harm to the environment (Service M W, 1983; Tyagi, 2016). *Bacillus thuringiensis* has been used as a larvicide against mosquitoes. It is not toxic to humans but may cause lethal effects in some other non-target species. Formulations from *B. thuringiensis* and *B. sphaericus* were used against *Aedes albopictus*, *Culex quinquefasciatus*, *C. gelidus* and *C. malayi* (Baruah & Das, 1994). *Beauveria bassiana* and *Metarhizium anisopliae* are ascomycete fungi found to be effective in controlling adult and larval *Aedes* mosquitoes (Scholte et al., 2004). *Poecilia reticulata*, *Tilapia mossambica*, *Sarotherodon niloticus*, *Astynax fasciatus*, *Betta splendens*, *Oreochromis mossambicus*, *Lepisosteus tropicus*, *Brycon guatemalensis*, *Ictalurus meridionalis* and *Cyprinus carassius* are larvivorous fishes that feed on *Aedes* populations (Han et al., 2015). Shrimps of the species *Machrobrachium pantanalense*, *M. amazonicum*, *M. brasiliense* and *M. jelskii* are reported to have larvicidal effect on *Aedes aegypti*, *Culex quinquefasciatus* and *Anopheles darlingi* (W. M. D. Coelho et al., 2017). Usage of phytochemicals were found effective against different mosquito species (Sukumar et al., 1991; Shaalan et al., 2005). The organic solvent extracts from plants using acetone, chloroform, petroleum ether, methanol etc. were found to be good enough in laboratory trials, but need to be developed into better formulations for field applications.

The chemical method includes the usage of certain chemical insecticides. Four classes of insecticides are mainly used in mosquito control programs, which include pyrethroids (e.g., deltamethrin, permethrin, cypermethrin, lambda-cyhalothrin), organochlorines (e.g.,

DDT), organophosphates (e.g., malathion, fenitrothion), and carbamates (e.g., propoxur, bendiocarb) (WHO, 2018). But this traditional strategy raised the tolerance level of the mosquitoes and triggered the development of resistance. In addition, the environmental contamination by such chemicals lead to ecological issues and health hazards to humans and livestock. Pyrethroids were the most widely used insecticides for indoor residual spraying and in insecticide treated nets and fabrics, which has irritant or repellent activity on mosquitoes and cause less mammalian toxicity (WHO, 2020). Dichlorodiphenyltrichloroethane (DDT), the first synthetic organochloride used for residual spraying, targets sodium channels in mosquitoes, and its use is favoured by low cost and high effectiveness (Şengül Demirak & Canpolat, 2022). Organophosphates and carbamates are two other insecticides sharing similar mode of action by inhibiting acetylcholinesterase (AChE) enzyme. Inhibition of AChE prevents the breakdown of the neurotransmitter acetylcholine, resulting in neuromuscular overstimulation and death of insects (Mengle & Casida, 1958; Fukuto, 1990; Fournier & Mutero, 1994). Though organophosphates and carbamates were in use in parallel with pyrethroids and organochlorides, their non-target toxicity and cost of production were comparatively high, limiting their usage.

1.3.6 Challenges in mosquito control measures

Common practices like indoor residual spraying and insecticide-coated nets etc. are not very effective for all mosquitoes. These were ideal for *Anopheles* species, but not for *Aedes*. The variations in the ecology of mosquito species evoke differences in their behavioural patterns. So,

individual ecology does matter in the control of specific species. In addition, the presence of high-transmission hotspots and heterogeneity make mosquito management a more difficult task (Acevedo et al., 2015). Insect traps used widely for mosquito control are no more satisfactory, as they are affected by wind, making them an unstable option.

Continuous application of chemical insecticides led to the development of resistance and cross-resistance in mosquitoes against such chemicals. Thus, insecticide resistance has become a major obstacle in the control of mosquito populations. At least 50 mosquito species have developed resistance to one or more insecticides since 1947 (Brown, 1983). Many species also developed irritability toward DDT and other such insecticides. Insecticide resistance is mostly inferred from the ability of an insect to survive the exposure to a standard dose of insecticide, either by physiological or behavioural adaptations (WHO, 2016).

In mosquitoes, alterations of target site nerve receptors (e.g., mutations in *kdr*, *Rdl* and *Ace-1R* genes) and detoxification due to increased or modified enzyme activities (e.g., monooxygenases (P450s), glutathione-S-transferases and carboxylesterases) were the two major mechanisms that contributed to developing insecticide resistance (WHO, 2018; Şengül Demirak & Canpolat, 2022). Significant expansion of knockdown resistance mutation restricted the usage of pyrethroids in mosquito control (Gan et al., 2021). Gene mutation in AChE was responsible for the development of resistance to organophosphates and carbamates (Şengül Demirak & Canpolat, 2022). The increased activity of detoxifying enzymes, which are metabolic enzymes responsible for

biodegradation of insecticides, helped in the elimination of their insecticidal effects (Hemingway & Ranson, 2000). Later, some formulations using different chemical insecticides were put into use. However, resistance development interferes in the activity since prolonged exposure to various insecticides caused the mosquitoes to resist more than one class of insecticides (Şengül Demirak & Canpolat, 2022). A gradual prevalence of resistance developed in endemic regions of mosquito population where insecticides extensively used to counter disease outbreaks, later expanding to non-endemic regions, advancing the adaptations of mosquito populations to survive. On the contrary, the toxicity of insecticides continued to evoke genetic impairments in human and other non-target populations.

1.3.7 Recent advances in mosquito control

Current research on mosquito control is now focused on understanding the mosquito resistance to synthetic insecticides and developing novel strategies to overcome the resistance issues. In this direction natural compounds are attractive as they are in general less toxic than the synthetic ones. Environment-friendly methods involving use of biopesticides, pheromones, sterilized males, refractory mosquitoes, endosymbiont, midgut symbionts, etc. can be use with varying degrees of success (Tyagi, 2016). There are compounds, such as insect growth regulators, that mimic insect hormones and can be used for mosquito control. Advances in nanotechnology and genetic engineering offer new techniques that are more target-specific.

Insect growth regulators (IGRs) are compounds that either imitate or interfere with natural hormones, thereby disrupting insect development (Pener & Dhadialla, 2012). Insect growth regulators like methoprene, diflubenzuron, pyriproxyfen and novaluron have been found active against many species of mosquito larvae including *Aedes aegypti*, *Culex quinquefasciatus* etc. (Baruah & Das, 1996; Ansari et al., 2005; Sihuincha et al., 2005; Arredondo-Jiménez & Valdez-Delgado, 2006). The field trials with IGRs were done in some mosquito breeding habitat and found to be effective in eliminating populations of *A. aegypti* and *C. quinquefasciatus*. (Baruah & Das, 1996; Ansari et al., 2005; Arredondo-Jiménez & Valdez-Delgado, 2006). However, recent reports suggests that mosquitoes have developed resistance against some of these IGRs and necessitates requirement of new tools (Dennehy et al., 2010).

Biopesticides are further categorised into four subgroups, namely; microbial biopesticides, plant incorporated protectants, botanical pesticides and pheromones (Chengala Laxmishree & Singh Nandita, 2017). The use of bioinsecticides, composed of botanical or plant-based compounds, has been a perfect alternative due to their minimal hazardous effects on human health and environment (Şengül Demirak & Canpolat, 2022). Another technique developed is by making use of nanoparticles. Green synthesis of metallic nanoparticles using biological extracts from plants and microbes has been reported (P. Singh et al., 2016). Preparation of microbicide silver nanoparticles coupled with plant products having mosquito larvicidal property can be used for limiting mosquito population (Benelli et al., 2017). Zinc oxide

nanoparticles with *Syzigium cumini* seed extract as reducing agent was found to have ovicidal effect on *Aedes aegypti* (Roopan et al., 2019).

The concept of Sterile Insect Technique (SIT) can be applied for mosquito control (Bellini et al., 2013) especially in the control of *Aedes aegypti* and *A. albopictus* populations (Bouyer et al., 2020; Lees et al., 2021). Boosted SIT is another concept combining pyriproxyfen autodissemination with SIT for a better control (Bouyer & Lefrançois, 2014). The incompatible insect technique by *Wolbachia* infection when combined with sterile insect technique resulted in nearly elimination of *A. albopictus* population in field (Zheng et al., 2019). A new formulation in the form of tablets containing a lectin preparation showed mosquitocidal activity against different developmental stages of *Aedes aegypti* mosquitoes, and this formulation method is proposed as a new control strategy for *A. aegypti* populations (Silva et al., 2021). A technique that involves the use of genetically modified (GM) mosquitoes for the purpose of vector control is another potential option. Genetic modification of mosquitoes can cause reduction in the wild mosquito populations and may reduce their efficiency to transmit various pathogens. Transgenically modified *Aedes aegypti* with a dominant lethal gene was released into wild mosquito populations in Brazil (B. R. Evans et al., 2019). This resulted in mixing of the populations with wild type and released modified strains along with a rare viable hybrid offsprings with robust power to reproduce. This reports reminds the necessity of taking regular monitoring and ethical considerations that need to be taken into account (Javed et al., 2022).

Another development is in the manipulation of mosquito gut microbiota. The gut microbiota has significant role in pathogen transmission and the mosquito biology (Douglas, 2011). Bacteria such as *Wolbachia*, are reported to shorten the life span of some mosquito species (McMeniman et al., 2009). Their infection also reduces the ability of dengue, chikungunya and Zika virus to infect *A. aegypti*, reducing pathogenic transmission (Moreira et al., 2009; Dutra et al., 2016). The insect reproduction can be regulated by *Wolbachia* infection through cytoplasmic incompatibility in offspring where the uninfected female eggs cannot survive when fertilized by infected male sperm (Jiggins, 2017). The use of insecticides or antibiotics have been found to affect the bacterial community of *A. albopictus* gut modifying the abundance of bacteria. Such alterations are found to affect the pathogen transmission (Guégan et al., 2018; Juma et al., 2020). *Aedes aegypti* mosquitoes without gut microbiota produce fewer eggs, live longer with low metabolic rate and exhibit lower competence for dengue virus (Harrison et al., 2023).

1.3.8 TMOF: history and relevance

Inhibition of yolk deposition in mosquitoes by ovarian humoral factors have been demonstrated (Meola & Lea, 1972; Else G James & Judson, 1972). In *Rhodnius*, the oostatic hormone is secreted by four pairs of abdominal neurosecretory cells, and they inhibit juvenile hormone action to deposit vitellogenin in follicle cells (Huebner E. & Davey K.G., 1973; Liu & Davey, 1974; Davey & Kuster, 1981). In mosquitoes, the oostatic hormone makes direct action on the ovary (Meola & Lea, 1972;

Borovsky, 1985). The blood meal of mosquitoes induces the de-novo synthesis of trypsin like enzymes in the midgut (Borovsky & Schlein, 1988). Thus, the availability of nutrients affect the egg development (Borovsky, 1988; Borovsky et al., 1990). The purified oostatic hormone from *A. aegypti* caused inhibition of vitellogenesis and egg development, and the activity of the hormone increased to its maximum after 48 hours of blood meal (Borovsky, 1985). Injections of partially purified oostatic hormone inhibited egg development, proteolytic enzyme activity and blood digestion in the midgut of *A. aegypti* and found to have similar effects in *C. quinquefasciatus*, *C. nigripalpus* and *Anopheles albimanus* indicating its non-specific activity in mosquito species (Borovsky, 1988).

The Trypsin Modulating Oostatic Factor (TMOF), a decapeptide from *A. aegypti* described as Aea-TMOF, was purified, and the sequence is Tyr-Asp-Pro-Ala-Pro-Pro-Pro-Pro-Pro (YDPAPPPPPP) with a molecular weight of 1047.6 Da (Borovsky et al., 1990). The N terminus of the peptide is important for the biological activity of the peptide (Borovsky et al., 1993; Borovsky & Meola, 2004). Feeding experiments on the larvae of *A. aegypti* confirmed that the tetrapeptide YDPA could achieve 95% biological activity of Aea-TMOF (Borovsky & Meola, 2004). The secretory peptide from the ovarian follicles starts to synthesise after 24 hours of blood meal, reaching its peak at 36 hours and then gradually declining, regulating the rate of trypsin biosynthesis in the midgut (Borovsky, Song, et al., 1994). The regulation is aided by the capacity of TMOF peptide and its analogues to traverse the gut

epithelium into haemolymph and binds to a gut receptor (Borovsky, Powell, et al., 1994; Borovsky & Mahmood, 1995). This has implications for developing a biorational insecticide.

The mosquito TMOF was found to have inhibitory role in trypsin biosynthesis of other arthropods like *Ctenocephalides felis*, *Stomoxys calcitrans*, *Musca domestica*, *Culicoides variipennis* and *Lutzomyia anthophora* (Borovsky et al., 1990; Borovsky et al., 1993). This suggests the possibility of existence of similar peptides in other organisms. A trypsin modulating oostatic factor was purified from the fleshfly, *Neobellieria bullata*, described as Neb-TMOF is a hexapeptide (NPTNLH) having trypsin synthesis inhibitory activity in the midgut (Bylemans et al., 1994). This caused reduction in the concentration of vitellogenin in haemolymph and retards oocyte growth (Bylemans et al., 1994). The Neb-TMOF unlike the TMOF from *A. aegypti*, is reported to have prothoracicostatic activity, as it can regulate ecdysone synthesis in *N. bullata* and *Calliphora vicina* (Huaa et al., 1994). However, this peptide is having a narrow functional cross activity in other insect species suggesting higher specificity of action (Bylemans et al., 1996). Another Oostatic factor discovered from *N. bullata* is Neb-colloostatin, a cleaved peptide from collagen-like precursor, has no direct effect on trypsin or ecdysone synthesis but inhibits the yolk deposition in penultimate oocytes in pre-vitellogenic ovaries (Bylemans et al., 1995).

1.3.9 TMOF as a mosquito larvicide

The resistance of orally administered TMOF to proteolytic degradation in the insect midgut suggests their potential applicability as biorational insecticidal agents (Borovsky & Mahmood, 1995). The TMOF and its analogues were given with blood enema to *Aedes aegypti* and led to inhibition of trypsin synthesis and egg development (Borovsky & Mahmood, 1995). Application of Aea-TMOF (TMOF isolated from *Aedes aegypti*) was studied in other arthropods as well.

The larvicidal effect of Aea-TMOF and its 29 analogues was studied by Borovsky and Meola by feeding experiments on the larvae of *A. aegypti* and they found the tetrapeptide YDPA could achieve 95% biological activity of Aea-TMOF (Borovsky & Meola, 2004). The LC₅₀ values obtained from feeding experiments using Aea-TMOF with Brewer's yeast on first instar larvae of *Aedes taeniorhynchus*, *Culex quinquefasciatus*, *C. nigripalpus* and *Anopheles quadrimaculatus* show higher values compared to that of *Aedes aegypti* (Borovsky & Meola, 2004). The mechanism of action of TMOF is preventing translation of trypsin mRNA. *Plutella xylostella* larvae were treated with synthetic analogues of TMOF and mortality up to 96% was observed within three days (Borovsky & Nauen, 2006).

The first subspecies of *Bacillus thuringiensis* used for the control of mosquitoes was *Bacillus thuringiensis israeliensis* (Bti) (Goldberg & Margalit, 1977). Larvicidal activity of different formulations of TMOF were tested (Misni et al., 2010). *Aedes aegypti* larvae treated with

TMOF- yeast wet paste and TMOF- yeast dried powder led to 44.3% and 60% mortality, respectively, by 24 hours. Trypsin modulating oostatic factor (TMOF) with *Bti* was formulated as rice husk, wettable powder and mosquito fudge cubes and the rice husk form was the most effective with 95- 97 % mortality within one hour (Misni et al., 2010). Synergistic activity of Cry11Aa toxins and TMOF expressed in *Pichia pastoris* cells reported 87.5% mortality in *A. aegypti* larvae within 5 days (Borovsky et al., 2010). *Pichia pastoris* cells expressing GST-Cry4Aa-TMOF produced 72% larval mortality in *A. aegypti* reporting the synergistic activity of Cry4Aa and TMOF (Borovsky et al., 2011). Conjugated polyethylene glycol with TMOF was found to have enhanced insecticidal activity against the larvae of *A. aegypti*, *A. albopictus*, *Heliothis virescens* and *Helicoverpa zea* (Jeffers et al., 2011). These laboratory trials paved the way for using TMOF as an insecticidal preparation. In addition, efforts were made to express TMOF in cells like yeast and algae, which are natural food for mosquito larvae.

1.3.10 Cloning and expression of TMOF

Mosquito larvae feed on microorganisms and other organic materials available in the natural aquatic system. Nutrient-rich diets based on microorganisms have been used for raising *A. aegypti* in laboratory conditions, and they have been found to feed on bacteria like *Escherichia coli*, *Staphylococcus aureus*; yeast such as *Saccharomyces cerevisiae*, *Pseudopzyna* sps; algae like *Chlorella* species; and cyanobacteria like *Arthrospira platensis* (Souza et al., 2019).

Nauen et al., (2001) describe the inhibition of trypsin synthesis in fourth instar *Heliothis virescens* larvae when fed with Aea-TMOF (Nauen et al., 2001). The transgenic expression of TMOF was first carried out in tobacco plants, where transgenic tobacco plants were produced by incorporating 6 TMOF peptides spaced by dibasic arginine residues (Tortiglione et al., 2002). A mortality of 20-33% was reported in *H. virescens* larvae when fed the transgenic plants containing TMOF (Tortiglione et al., 2002). The report supports the possibility of lepidopterans having TMOF-like hormones in their system involved in metabolic processes. The Aea-TMOF was fused with the coat protein of tobacco mosaic virus and expressed in *Nicotiana tabacum* leaves. *Aedes aegypti* larvae was treated with the purified coat protein-TMOF virion and it resulted in stunted growth and mortality of the larvae (Borovsky et al., 2006).

Aea-TMOF was cloned and expressed in the yeast *Pichia pastoris* and cyanobacterium *Anabaena* but, in the latter, low rate of expression reported (0.5 to 1µg in 10⁸ cells) (Borovsky et al., 2010). Trypsin modulating oostatic factor when expressed along with *Bti* toxin in *P. pastoris* has synergistic activity against *A. aegypti* larvae (Borovsky et al., 2010; Borovsky et al., 2011; Y. Lau et al., 2011). Trypsin modulating oostatic factor expressed in *P. pastoris* was made into insecticidal formulations against *A. aegypti* (Misni et al., 2010). The entomopathogenic fungus *Beauveria bassiana* has been used in the biological control of *Culex tarsalis*, *C. pipiens* and *Anopheles albimanus* (Clark et al., 1968). The Aea-TMOF expressed in the *B. bassiana* strains that were infected to *Anopheles gambiae* showed an increased virulence

and resulted in significant reduction in the fecundity of target mosquitoes (Kamareddine et al., 2013).

The *Aedes aegypti*- trypsin modulating oostatic peptide was cloned and expressed in the marine algae *Chlorella desiccata*, using two plasmids, pYES2 and pYDB4 (Borovsky et al., 2016). This was the first report of TMOF expression in stable recombinant algal cells. *Chlorella* cells (3×10^8 cells) having engineered pYES2-TMOF ($20 \pm 4 \mu\text{g}$ TMOF) or pYDB-TMOF ($17 \pm 3 \mu\text{g}$ TMOF) caused 75% and 60% mortality in *A. aegypti* larvae, respectively (Borovsky et al., 2016). These engineered *Chlorella* cells possess kanamycin and geneticin resistance, and green fluorescent protein (GFP) as marker. The Aea-TMOF was expressed in *Saccharomyces cerevisiae* within the plasmid pYDB2 having a strong galactose promoter using homologous recombination at *ura3-52* (Borovsky et al., 2018). Trypsin modulating oostatic factor was prepared as a fusion protein with GFP and expressed in *S. cerevisiae*. The engineered yeast cells with TMOF alone and GFP-TMOF led to a mortality of 66% and 83%, respectively, in *A. aegypti* larvae (Borovsky et al., 2018). The scientists thus report the possibility of using recombinant *Chlorella desiccata* and *S. cerevisiae* for field trials in order to control mosquito larval population.

The possibility of cloning and expressing multiple TMOF gene copies was reported in *Pichia pastoris* yeast cells (Borovsky et al., 2020). The plasmid vector pPICZB carrying AOX1 promoter was used for transforming KM71 and KM71H strains by homologous recombination. The genetically engineered cells possessed 10 copies of TMOF coding gene. The KM71 and KM71H strains produced 229 and 114 μM TMOF

respectively, and both of them caused 100 % mortality to *A. aegypti* larvae (Borovsky et al., 2020). Borovsky et al., 2020 reported larvicidal activity of engineered *P. pastoris* cells in *A. aegypti* and delayed growth in *C. quinquefasciatus* (Borovsky et al., 2020). The heat inactivation of engineered yeast cells expressing TMOF has increased potential as larvicides compared to non-heat inactivated (Borovsky et al., 2018; Borovsky et al., 2020).

1.3.11 The TMOF receptor

The earlier studies reported that TMOF peptide has specific binding sites at the gut membrane. The TMOF, when released into haemolymph, crosses the gut membrane by binding to the receptor and reaches the midgut, triggering inhibition of trypsin synthesis (Borovsky et al., 1990; Borovsky, Powell et al., 1994). The level of trypsin transcript remain unchanged after a blood meal, suggesting translational level regulation is made by the TMOF peptide (Borovsky et al., 1996; Borovsky et al., 2006). Several groups of receptors were studied to check its compatibility with TMOF peptide. Extracting the gut receptor from blood-fed *A. aegypti* females led to identifying the role of ATP-binding cassette (ABC) transporter in TMOF activity and the knockdown of ABC-TMOF receptor confirmed reduction of trypsin synthesis in the midgut (Borovsky et al., 2021). Borovsky et al., (2021) study also identified an Aea-TMOF (TMOF isolated from *Aedes aegypti*) binding site within the three-dimensional model of ABC transporter. In mosquitoes, Aea-TMOF binds at an exit tunnel in the A site of ribosomes, blocking the translation of trypsin mRNA, and hence ribosome is the ultimate target of Aea-TMOF (Borovsky et al., 2022).

1.4 MATERIALS AND METHODS

1.4.1 MATERIALS

pFN29AHis6Halo plasmid vector and DNA gel purification kit was purchased from Promega corporations, USA. AsiSI and PmeI restriction enzymes from New England Biolabs, UK. Agarose, imidazole and ampicillin sodium salt from Sigma, USA. DNA ladders, Luria Bertani broth, Luria Bertani agar, RNase A and Bovine serum albumin (BSA) were purchased from Himedia laboratories private limited, Mumbai, India. Isopropyl β -D-thiogalactopyranoside (IPTG), sodium dodecyl sulphate, sodium carbonate, sodium bicarbonate, sodium chloride, sodium hydroxide, Tris, boric acid, ethylene diamine tetra acetic acid (EDTA), sodium acetate, sodium chloride, sodium dihydrogen phosphate, disodium hydrogen phosphate, potassium acetate, glycerol and isopropanol were purchased from Sisco research laboratories limited, Mumbai, India. Protein marker from Genei laboratories private limited, Bangalore, India. Custom oligonucleotides were purchased from G Biosciences, USA. All other reagents used were of molecular biology grade.

1.4.2 METHODS

1.4.2.1 About *Aedes albopictus*

Experimental organism	: <i>Aedes albopictus</i>
Kingdom	: Animalia
Phylum	: Arthropoda
Class	: Insecta
Order	: Culicoides
Family	: Culicidae
Genus	: <i>Aedes</i>
Species	: <i>albopictus</i>

Aedes albopictus (Skuse), commonly referred to as the Asian tiger mosquito, is a native of Southeast Asia. Their geographic distribution at present extends over approximately 126 countries, including regions of Southeast Asia, Central Africa, Europe, America, and Australia. The widespread distribution of *A. albopictus* is facilitated by globalization and their ability to withstand varying climate conditions, and hence it has emerged as one of the most invasive species in the world.

Aedes albopictus is a biological vector of many infectious diseases including dengue, Zika, chikungunya etc. The adult mosquitoes are characterised by their small black and white body, with black and white striped legs.

1.4.2.2 Life cycle of *Aedes albopictus*

Aedes albopictus mosquitoes are holometabolous insects with four life stages. Their life cycle proceeds through morphologically and anatomically distinct forms called the egg, larva, pupa and adult (Fig. 1.4.1). The females lay eggs singly, which hatches into larvae. Larval form has four instars: first, second, third and fourth. Fourth instar larva pupates and emerges as adult. An egg takes about 7-10 days to emerge as an adult (Centers for Disease Control and Prevention, 2022).

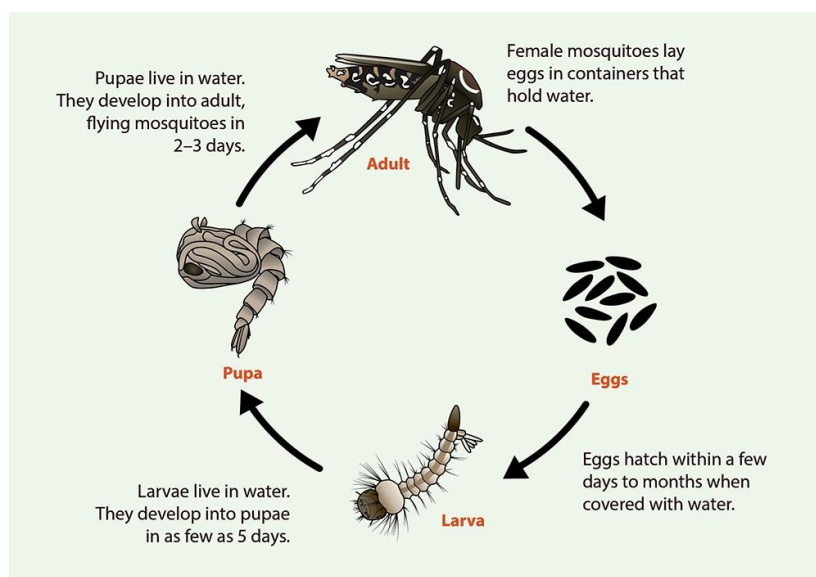


Fig. 1.4.1 Life cycle of *Aedes albopictus* (Reproduced from <https://www.cdc.gov/mosquitoes/about/life-cycle-of-aedes-mosquitoes.html>)

1.4.2.2.1 Eggs

Female *A. albopictus* lay 100-200 eggs after a blood meal, and they are the centrolecithal type. The number of eggs in a batch is dependent on the size of blood meal acquired (Das et al., 2018). Eggs are laid as single

in natural or artificial water reservoirs like tree holes, plastics etc. (Gunara et al., 2023) (Fig. 1.4.2). Sometimes eggs may be laid at damp surfaces at varying distances from the water level (Das et al., 2018). Cigar-shaped eggs are shiny jet black, having slight dorso-ventral curvature (Suman et al., 2011). Eggs can stay viable for long time and can hatch into larvae in a drop of water.



Fig. 1.4.2 Eggs of *A. albopictus* (Reproduced from <https://www.cdc.gov/mosquitoes/about/life-cycle-of-aedes-mosquitoes.html>)

1.4.2.2.2 Larvae

Eggs emerge into larvae and the larvae develops through four instars. They are actively swimming and feeding forms. The worm like body is divided into head, thorax and abdomen (Fig. 1.4.3). The head bears eyes, antennae and mouthparts. Larval development is temperature dependent (Das et al., 2018). The larvae have a posteriorly located siphon that helps in breathing by holding over the water level and keeping rest of the body vertical (Das et al., 2018). They feed on particulate matter in the water, including microbes, planktons etc. Usually, larvae pupate after 5-10 days.



Fig. 1.4.3 *Aedes albopictus* larva (Reproduced from <https://fmel.ifas.ufl.edu/mosquito-guide/mosquito-genera-and-species/genus-aedes/aedes-albopictus/>)

1.4.2.2.3 Pupae

Pupae of *A. albopictus* (Fig. 1.4.4) do not feed but move actively in the water. Being called as tumblers, the pupae will emerge into adult within three days (Das et al., 2018).



Fig. 1.4.4 *Aedes albopictus* pupa (Reproduced from <https://www.cdc.gov/mosquitoes/about/life-cycle-of-aedes-mosquitoes.html>)

1.4.2.2.4 Adult

Adults emerge from the pupae after breaking the pupal case by ingesting air to expand the abdomen (Das et al., 2018). The body is divided into head, thorax and abdomen. Adults have two pairs of legs and one pair

of wings. The body and legs of adult *A. albopictus* are striped in a black and white pattern (Fig. 1.4.5). *Aedes albopictus* is morphologically distinct from *A. aegypti* with the presence of a median silver scale line against black background in the thorax region (ECDC fact sheets, 2016).



Fig. 1.4.5 *Aedes albopictus* adult (Reproduced from <https://www.ecdc.europa.eu/en/disease-vectors/facts/mosquito-factsheets/aedes-albopictus>)

1.4.2.3 Laboratory maintenance of *Aedes albopictus*

Aedes albopictus larvae were collected from the field and allowed to grow to adults in the laboratory. The larvae were grown in distilled water and fed with yeast granules. They were identified with the help of Dr Raghu, Senior Scientist, Centre for Disease Control, Kozhikode.

1.4.2.4 Toxicity analysis on *Aedes albopictus* larvae

Toxicity analysis using *A. albopictus* larvae were done as per WHO protocol (WHO, 2005). For this, 25 larvae were taken in 100 mL distilled water. They were fed with yeast. Second instar larvae were used for toxicity experiments. Larvae were exposed to different concentrations of recombinant TMOF/mutant TMOF and mortality noted after 48 hours. The LC_{50} (48 hours) was calculated using probit regression analysis in SPSS version 23.

1.4.2.5 Conjugation of TMOF to BSA and its toxicity to *Aedes albopictus* and *Culex quinquefasciatus* larvae

The first four amino acids in TMOF (YDPA) are reported to retain 95% activity of full length TMOF decapeptide (Borovsky & Meola, 2004). This tetrapeptide with an additional lysine residue at N-terminus (KYDPA) was custom synthesised. The lysine act as a trypsin cleavage site. This pentapeptide was conjugated to BSA using glutaraldehyde. Steps for conjugation are described below.

- BSA solution (35 mg/mL) was prepared in phosphate buffer saline (PBS) (20 mM phosphate buffer, pH 7.4 with 150 mM NaCl)
- 25% glutaraldehyde was added slowly to the BSA solution while stirring to get a final concentration of 2%
- The mixture was allowed to incubate for 1 hour at room temperature with continuous stirring
- Peptide solution (250 µg) was added to the mixture with continuous stirring
- Reactive excess glutaraldehyde was removed by adding glycine at a final concentration of 10 mM to the mixture
- The peptide-conjugated solution was dialysed against PBS for 3 hours and used for toxicity studies

- Second instar *Aedes albopictus* and *Culex quinquefasciatus* larvae were treated with the BSA-conjugated pentapeptide at different concentrations

From the toxicity experiments lethal concentrations were calculated using probit analysis.

1.4.2.6 CLONING OF TMOF GENE

The gene coding for TMOF was designed and custom synthesised as single stranded oligos. They were annealed and cloned into the bacterial expression vector, pFN29AHis6Halo. The methods employed are discussed below.

1.4.2.6.1 Synthesis of gene coding for TMOF

The amino acid sequence of TMOF peptide was retrieved from the literature and is found to be YDPAPPPPPP. The triplet codon for corresponding amino acids in the peptide was codon optimised for expression in *Escherichia coli*. The codon optimisation table for *E. coli* is shown in table 1.4.1. Additionally, a trypsin cleavage site and stop codon were added at 5' and 3' ends, respectively.

Table 1.4.1 Codon optimisation table for *Escherichia coli*

TRIPLET	AMINO ACID	FRACTION	FREQUENCY/1000	OCCURENCE NO.
TAT	Y	0.59	17.5	63937
TAC	Y	0.41	12.2	44631
GAT	D	0.63	32.7	119939
GAC	D	0.37	19.2	70394
CCT	P	0.18	7.5	27340
CCC	P	0.13	5.4	19666
CCA	P	0.20	8.6	31534
CCG	P	0.49	20.9	76644
GCT	A	0.18	17.1	62479
GCC	A	0.26	24.2	88721
GCA	A	0.23	21.2	77547
GCG	A	0.33	30.1	110308

The gene construct was designed with half sites for restriction enzymes so that, no restriction digestion is required before ligation. The restriction enzymes in pFN29AHis6Halo vector chosen were AsiSI and PmeI. Half-sites for these enzymes were added at 5' and 3' ends respectively. An additional cytosine nucleotide was added between 5' restriction site and trypsin cleavage site, to make the TMOF gene in frame. The sequence designed is as follows.

5' CGCCAAATATGATCCGGCGCCGCCGCCGCCGCCGTAAGTTT 3'

5' AAAGTTTACGGCGGCGGGCGGGCGGGCGGGCGGATCATATTTGCGAT 3'

The restriction mapping of the construct was done using NEB cutter 2.0 to understand the sites where restriction enzymes cleave. The sequence confirmed was used to prepare single stranded oligos with separate antiparallel strands. They were custom synthesised from G biosciences, USA.

1.4.2.6.2 Preparation of vector

pFN29AHis6Halo vector (Fig. 1.4.6) was chosen for the cloning of TMOF gene. This bacterial expression vector consists of a poly-histidine tag of 6 histidine residues and a Halo tag for the purification of recombinant proteins. pFN29AHis6Halo vector works on the principle of suicidal cloning. Within the vector is a barnase gene, which encodes a bacterial toxin. Once an *E. coli* bacterium is transformed with this vector, the barnase gene also express in the bacterial host, producing the toxin, resulting in the death of the host. During cloning, the barnase gene in the vector is removed by restriction digestion, and an insert is placed at that position during ligation. Hence bacteria transformed with the recombinant vector will not have the gene for barnase toxin and remain alive, which assures positive cloning.

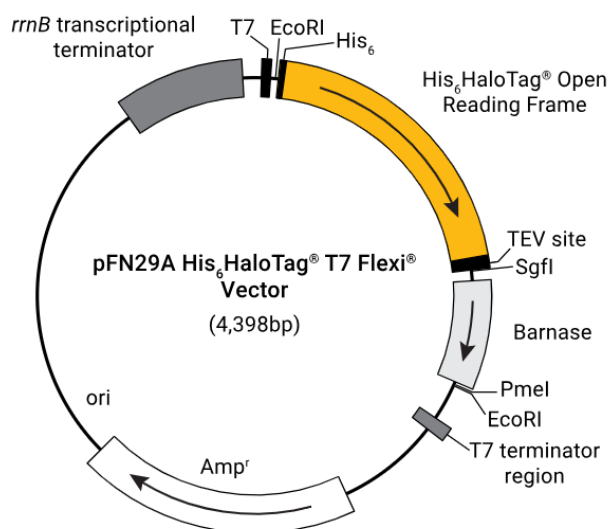


Fig 1.4.6 pFN29AHis6Halo vector. Vector map of the expression vector pFN29AHis6Halo showing cloning sites

1.4.2.6.2.1 Restriction digestion of the vector

pFN29AHis6Halo vector was digested using two restriction endonucleases, AsiSI and PmeI. The vector (1 µg) was digested with 1.5 units each of AsiSI and PmeI simultaneously at 37°C for one hour. After digestion, restriction mix was loaded on agarose gel and run the electrophoresis.

Restriction digestion mix (20 µL)

- pFN29AHis6Halo vector : 1 µg
- 10X restriction buffer : 1X buffer
- AsiSI enzyme : 1.5 units
- PmeI enzyme : 1.5 units
- Nuclease free water : up to 20 µL

1.4.2.6.2.2 Separation of restriction digested DNA by agarose gel electrophoresis

Reagents required

- Agarose
- TAE buffer, pH 8.3

Tris-acetate-EDTA buffer contains Tris, glacial acetic acid and EDTA at final concentrations of 40 mM, 20 mM and 1 mM, respectively. The pH of the solution was adjusted to 8.3 with concentrated HCl.

Casting of the gel

Agarose, 0.8 g was weighed and boiled in 100 mL TAE buffer, pH 8.3. Agarose was dissolved by heating to boiling. When the solution was cooled to about 60°C, 8-10µL safe DNA stain was added and mixed well. The solution was poured onto the casting tray without trapping air bubbles. Comb was inserted carefully. The poured solution was allowed to set at room temperature for about 20 minutes.

Loading the samples and running the gel

DNA samples were mixed with 5X DNA loading buffer with final buffer concentration of 1X. Tris-acetate-EDTA buffer, pH 8.3 was used for running the electrophoresis. After sample loading, electrodes were connected and a voltage of 100 V was applied.

1.4.2.6.2.3 Gel purification of digested vector DNA

After electrophoresis, DNA bands were visualized under a UV transilluminator, and the band corresponding to digested vector DNA was excised. Vector DNA from the gel slice was purified using gel elution kit. Gel purification was done by following steps

- The excised band was weighed and incubated in membrane-binding solution (100 mg DNA per 100 µL solution) at 50-65°C until the gel was dissolved
- Dissolved gel was transferred to spin column within a receptacle tube and centrifuged at 10000 x g for 3 minutes
- Solution in receptacle tube was discarded

- 700 μL wash buffer containing 95% alcohol was added to DNA bound to the spin column, centrifuged and the solution in the receptacle tube discarded
- Washing repeated with 500 μL wash buffer
- After washing, the spin column within receptacle tube incubated at room temperature for 1-2 minutes and centrifuged to remove residual alcohol
- The spin column was carefully placed in a fresh tube, and warm nuclease-free water was added to the spin column and incubated for 1-2 minutes
- Column within the new tube was centrifuged at 13000 x g for 2 minutes
- Eluted DNA kept at -20°C until use.

1.4.2.6.3 Preparation of Insert

Single stranded oligonucleotides (both upper and lower strands) were custom synthesised and annealed before ligation. Single stranded oligos (20 μM) were mixed and subjected to following conditions in a thermocycler (Table 1.4.2) to get 10 μM double-stranded DNA, which was used for ligation.

Table 1.4.2 Annealing conditions for insert gene

Temperature	Time
95°C	10 minutes
55 °C	30 minutes
4 °C	Temporary storage

1.4.2.6.4 Preparation of ligation mix and transformation

Ligation of double-digested vector with the TMOF insert was done using T4 DNA ligase at 22°C for three hours. Vector to insert molar ratios of 1:1, 1:4, 1:5, 1:10 and 1:20 were used for optimisation. Calculation of the mass of insert for a particular molar ratio and specific mass of vector was calculated using the formula,

Mass of insert in ng =

$$\frac{\text{mass in ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio of } \frac{\text{insert}}{\text{vector}}$$

After ligation, the ligation mix was used to transform DH5α competent cells.

1.4.2.6.4.1 Preparation of competent cells

Reagents required

Filter sterilized magnesium chloride (0.1M) and calcium chloride (0.1M), sterile glycerol (80%), LB broth and ampicillin solution

LB broth composition: 100 mL of LB broth contain 1 g tryptone, 1 g sodium chloride and 0.5 g yeast extract. The pH was adjusted to 7.0 and autoclaved.

Procedure

- DH5 α culture was revived from -80°C glycerol stock by inoculating a loop full of culture to 2mL LB broth and kept overnight at 37°C, vortexing at 200 rpm
- The culture obtained was expanded to 50 mL with fresh LB medium
- The culture was incubated in shaking incubator at 37°C, vortexing at 200 rpm until the OD₆₀₀ reaches between 0.35 and 0.4
- The culture was transferred to pre-cooled 15 mL centrifuge tubes and centrifuged at 3000 x g for 7 minutes at 4°C
- The supernatant was discarded and bacterial pellet was resuspended in 0.5 mL of ice cold, filter sterilized 0.1M MgCl₂
- The mixture was incubated on ice for 20 minutes and centrifuged at 3000 x g for 10 minutes at 4°C
- The supernatant was removed and the pellet was resuspended in 0.5 mL of ice cold 0.1M CaCl₂
- The mixture was incubated on ice for 30 minutes and centrifuged at 3000 x g for 10 minutes at 4°C

- The supernatant was removed and the pellet was resuspended in 0.125 mL of ice cold 0.1M CaCl_2 and 0.125 mL 80% sterile glycerol
- The mixture was made into 100 μL aliquots of competent cells and transferred immediately to -80°C

1.4.2.6.4.2 Transformation

- About 100 ng of plasmid DNA was added to 100 μL aliquot of competent cells and the tubes were tapped gently
- The plasmid- competent cells mix was incubated on ice for 30 minutes
- The cells were subjected to heat shock by placing the tubes at 42°C for 1-2 min
- The tubes were immediately transferred to ice and incubated for 5 minutes
- About 900 μL of LB broth was added to the mix and incubated at 37°C in a shaking incubator at 200 rpm for one and half hours
- The mix was centrifuged at $2500 \times g$ for 5 minutes at 4°C and the bacterial pellet with minimum volume of LB broth was collected
- The pellet was resuspended in the broth and used for spreading plates containing LB agar with ampicillin (100 $\mu\text{g}/\text{mL}$)

- The plates were incubated at 37°C overnight
- Single colonies obtained were picked on the next day and screened for colonies containing TMOF gene

1.4.2.6.5 Screening for positive colonies

Transformed colonies after ligation were screened to confirm the presence of vector containing insert. Plasmid containing insert was isolated from transformed colonies by modified alkaline lysis method.

1.4.2.6.5.1 Isolation of plasmid DNA

Materials required

Bacterial culture, RNase A solution, TE buffer (10mM Tris and 1 mM EDTA, pH 8.0), lysis buffer (NaOH (0.1N) and SDS (1%) solution), potassium acetate (3M, pH 4.7), isopropanol, 70% ethanol, nuclease free water, sterile tips and tubes.

Procedure

- Using a sterile loop, single colonies were picked from the transformed plate and transferred to 2 mL LB medium and incubated overnight at 37°C in a shaking incubator (200 rpm)
- About 2 mL bacterial culture centrifuged at 3000 x g for 5 minutes and supernatant removed
- Bacterial pellet obtained was resuspended in 300 µL RNase A solution in TE buffer and incubated for 5 minutes

- Incubated mixture was vortexed until the bacterial pellet resuspended completely
- Lysis buffer (300 μ L) was added to the pellet resuspended in RNase A, and incubated for 5 minutes
- Mixing was done immediately by inversion until the solution became clear
- Potassium acetate solution (300 μ L) was added and mixed by inverting tube and incubated for 5 minutes
- The mixture was centrifuged at 10000 x g for 10 minutes at 4°C and the supernatant was collected in a new tube
- Isopropanol (800 μ L) was added and incubated on ice for 10 minutes
- The mixture was centrifuged at 10000 x g for 15 minutes at 4°C and the supernatant was discarded
- The pellet was washed with 1 mL 70% cold ethanol
- The pellet was airdried by spinning in microcentrifuge with cap open for 5 minutes
- The pellet was kept at 70°C water bath until it became transparent
- Warm nuclease free water was added to the pellet and dissolved by tapping and kept frozen at -20°C.

The isolated plasmid was screened by restriction digestion. Confirmation of the sequence of the insert was done by PCR followed by Sanger sequencing.

1.4.2.6.5.2 Restriction digestion of recombinant vector

Restriction digestion of cloned recombinant DNA was done using PmeI enzyme, one of the enzymes used for cloning the TMOF gene. About 200 ng of recombinant vector was digested with 0.5 unit of PmeI at 37°C for 30 minutes. Digestion of original vector (untransformed) was also done in parallel. Digested DNA from cloned vector and original vector were run on agarose gel using Tris-borate-EDTA buffer and differences in size compared.

Tris-borate-EDTA (TBE) buffer: Tris-borate-EDTA buffer contains Tris, boric acid and EDTA at final concentrations of 90 mM, 90 mM and 2 mM, respectively. The pH of the solution was adjusted to 8.3 with concentrated HCl.

1.4.2.6.5.3 PCR

Polymerase chain reaction was run using recombinant vector (50 ng) as template. pFN29AHis6Halo vector-specific sequencing primers (flanking cloning site) were used for the amplification. The primers used were;

Forward Primer: 5' ACATCGGCCCGGGTCTGAATC 3'

Reverse Primer: 5' CTCCTTTCGGGCTTTGTTAG 3'

The composition of PCR mixture was prepared as given in table 1.4.3 and the conditions used for PCR are described in table 1.4.4. The amplicon size was used to confirm the presence of insert.

Table 1.4.3 Composition of PCR mixture

Components	Final concentration
Recombinant vector	1ng/ μ L
Forward primer	1 μ M
Reverse primer	1 μ M
10X Pfu buffer	1X buffer
Pfu polymerase	1 unit/ 10 μ L

Table 1.4.4 PCR conditions for amplification of the insert

Step	Temperature ($^{\circ}$ C)	Time
Initial denaturation	95	5 minutes
Denaturation*	95	30 seconds
Annealing*	54	30 seconds
Extension*	68	1 minute
Final extension	68	10 minutes
Temporary storage	4	

*Steps repeated in 34 cycles

The PCR products obtained were run on 1% agarose gel electrophoresis, and their sizes were compared with that amplified from the original vector.

1.4.2.6.5.4 Sanger sequencing

Sanger sequencing of the PCR product was done to confirm the presence and sequence of TMOF insert within pFN29AHis6Halo vector. Sequencing was done at Gene Spec, Ernakulam.

1.4.2.7 Cloning of mutant TMOF gene

The plasmid vector, pFN29AHis6Halo was used for cloning mutant TMOF gene. For this, a synthetic gene with random nucleotides in place of codons of TMOF coding region was synthesised as single stranded oligos and annealed. Half sites for restriction enzymes AsiSI and PmeI were incorporated in the 5' and 3' ends of the oligo, respectively. The sequence of oligo for mutant TMOF is given below.

5' CGCCAAANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNTAAGTTT 3'

5' AAACCTANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNTTTCGAT 3'

The methods adopted for cloning mutant TMOF was the same as those used for wild type TMOF and is described in section 1.4.2.6. Screening of the colonies were done by checking the activity of the TMOF in mosquito larval toxicity assay. Colonies with TMOF activity were selected, the plasmid was isolated, and the TMOF gene sequenced.

1.4.2.8 Expression of recombinant TMOF/mutant TMOF

Recombinant vector containing TMOF/mutant TMOF was expressed in *Escherichia coli* cells. BL21 (DE3) strain was used for expression of recombinant proteins. For this BL21 competent cells were transformed with recombinant vector with TMOF/mutant TMOF. Transformant colonies were inoculated in 5 mL LB medium overnight at 37°C and expanded on the following day using sterile LB with ampicillin. The culture was grown until the optical density at 600 nm reached 0.6. At this point, protein expression was induced by the addition of IPTG. The cells in each culture (BL21 with TMOF, BL21 with mutant TMOF and untransformed BL21) were harvested by centrifuging at 10000 x g for

10 minutes. Conditions for expression was optimised in terms of IPTG concentration (0.2, 0.5 or 1 mM final IPTG concentration), temperature (32°C, 37°C, or 40°C) and duration of induction (5 hours, 8 hours or 16 hours). The bacterial pellet from induced culture was kept at -20°C until use.

To check the level of expression, SDS-PAGE (Laemmli, 1970) was done. For this, the bacterial pellet was resuspended in 100 mM bicarbonate buffer, pH 9.0 and lysed by sonication in ice for 2 minutes. The cell lysate was centrifuged at 10000 x g for 10 minutes. The soluble fraction was collected and membrane fraction in the remaining pellet resuspended in 2% SDS. Protein concentration of these two fractions were estimated by modified Lowry's method (Markwell et al., 1978) using bovine serum albumin as the standard. Equal amount of protein from BL21 cells expressing TMOF, BL21 cells expressing mutant TMOF and untransformed BL21 cells were loaded onto 10% SDS-PAGE, and the size of the expressed recombinant protein was calculated. The soluble fraction of bacterial cell lysate from BL21 cells expressing TMOF, BL21 cells expressing mutant TMOF and untransformed BL21 cells were designated as crude TMOF, crude mutant TMOF and crude control, respectively.

1.4.2.8.1 SDS-PAGE

The level of expression of crude TMOF, crude mutant TMOF and crude control was checked using SDS-PAGE. This was done to determine the molecular weight of expressed recombinant TMOF. Samples were loaded onto 10% acrylamide gel. The molecular weight of the

recombinant TMOF/mutant TMOF was calculated by comparing the size with a standard protein (BSA) or protein marker.

Reagents for SDS-PAGE

1. Acrylamide solution (30%)

A stock solution of acrylamide (30%) was prepared by dissolving 15 grams of acrylamide and 0.4 grams of bis-acrylamide in distilled water and made up to 50 mL. The solution was filtered and stored at 4°C in an amber-coloured bottle.

2. Preparation of buffers

○ Buffer 1, pH 8.8

A Tris (0.614 M) solution was prepared in water by dissolving 7.43 g Tris and made up to 100 mL. The pH of the solution was adjusted to 8.8 with HCl, and 164 mg SDS was added to it.

○ Buffer 2, pH 6.8

A Tris (0.147 M) solution was prepared in water by dissolving 1.78 g Tris and made up to 100 mL. The pH of the solution was adjusted to 6.8 with HCl, and 108 mg SDS was added to it.

- Chamber buffer, pH 8.3

Chamber buffer was prepared in distilled water at a final concentration of 0.025 M Tris, 0.192 M glycine and 0.1 % SDS. The pH of the solution was adjusted to 8.3.

3. Ammonium per sulphate solution (APS)

This was prepared by dissolving 15 mg ammonium persulphate in 1 mL distilled water.

4. 6X loading dye

6X loading dye was prepared in distilled water at a final concentration of 375 mM Tris HCl (pH 6.8), 6% SDS, 48% glycerol, 9% β mercaptoethanol and 0.03% bromophenol blue.

5. Fixative

Fixative was prepared by mixing 75 μ L formaldehyde with 100 mL of 50% methanol.

6. Staining solution

Staining solution was prepared by dissolving 60 mg of Coomassie brilliant blue R-250 powder in a solution containing 44 mL methanol, 44 mL distilled water and 12 mL glacial acetic acid.

7. Destaining solution

Destaining solution was prepared by mixing 50 mL methanol, 75 mL glacial acetic acid and 875 mL distilled water.

Preparation of gel

A mini gel of 10% acrylamide was prepared, consisting of a separating gel and a spacer gel. They are prepared as follows

1. Separating gel (10%)

- Acrylamide (30%) : 3 mL
- Buffer 1, pH 8.8 : 5.5 mL
- TEMED : 10 μ L
- APS (15 mg/mL) : 450 μ L

2. Spacer gel (3%)

- Acrylamide (30%) : 500 μ L
- Buffer 2, pH 6.8 : 4.25 mL
- TEMED : 5 μ L
- APS (15 mg/mL) : 250 μ L

Sample loading and running the gel

The crude samples were mixed with 6X loading dye (final concentration 1X) and incubated for 5 minutes in a boiling water bath. They were then

loaded onto the gel after filling chamber buffer. Electrophoresis was run at 15 mA until the dye reached separating gel and then increased to 25 mA. Running was continued until the dye reached bottom line of the gel. Gel was then transferred to fixative and kept for 45 minutes. After that, the gel was transferred to staining solution and kept for 45 minutes and then transferred to destaining solution and left until destained. Expression of recombinant TMOF/mutant TMOF was confirmed by the presence of a protein band corresponding to the expected molecular weight.

1.4.2.9 Purification of recombinant TMOF/ mutant TMOF

The TMOF/mutant TMOF peptide is having a size of 1.046 kDa only. The expressed recombinant TMOF/mutant TMOF will have 6X histidine tag and Halo tag at its N-terminus, making it a 37 kDa protein. The poly-histidine tag serves as the affinity tag which can be used for purification using nickel affinity chromatography. In this work, purification of the recombinant TMOF/mutant TMOF was done by nickel affinity chromatography in batch method. The crude TMOF, crude mutant TMOF and crude control were subjected to nickel affinity chromatography. The procedure for the purification is described below.

1.4.2.9.1 Nickel affinity chromatography

Reagents required

- Tris buffer saline (TBS): 100 mM Tris buffer was prepared in distilled water with pH 8.0. NaCl was dissolved in the buffer with a final concentration of 150 mM.

- Imidazole: 2 M imidazole was prepared by dissolving imidazole in 100 mM tris buffer, 150 mM NaCl, pH 8.0. It was further diluted to 100 mM, 250 mM, 500 mM and 1 M imidazole solutions using Tris buffer saline.

Procedure

- Nickel IMAC resin (250 μ L) was equilibrated with 100 mM Tris buffer 150 mM NaCl, pH 8.0, in a 1.5 mL microcentrifuge tube
- 500 μ L crude sample (750 μ g protein) was mixed with the resin and incubated for 45 minutes
- Flowthrough was collected after centrifugation at 2000 x g for 5 minutes
- Unbound proteins were washed off with 100 mM Tris buffer containing 150 mM NaCl
- Elution was done using 250 mM imidazole in TBS
- Column was washed with 2 M imidazole to remove any protein left bound

For each sample, two elutes were collected and labelled as elute 1 and elute 2 (El 1 and El 2). The protein estimation of elutes were done using Bradford's dye binding method (Bradford, 1976) using Bovine serum albumin as the standard. To check the purity of eluted proteins, SDS-PAGE (Laemmli, 1970) was done.

1.4.2.10 Larvicidal activity of recombinant TMOF/mutant TMOF on *Aedes albopictus*

Larvicidal activity of crude TMOF/mutant TMOF and affinity purified TMOF/mutant TMOF was studied on *A. albopictus* larvae. For this, 25 numbers of second instar *A. albopictus* larvae were introduced into 100 mL distilled water. *Aedes albopictus* larvae were exposed to different concentrations of crude and affinity purified TMOF/mutant TMOF. Mortality was observed and the lethal concentrations were calculated using probit analysis.

1.4.2.10.1 Larvicidal activity of crude TMOF/mutant TMOF in terms of number of bacterial cells

The number of bacterial cells expressing TMOF/mutant TMOF required to cause mortality in *A. albopictus* larvae was calculated. For this, *E. coli* expressing TMOF and mutant TMOF were diluted using sterile 100 mM bicarbonate buffer, pH 9.0 and spread in ampicillin-agar plate. Single colonies obtained were counted, and each colony was considered as originated from one cell.

1.4.2.11 Effect of pH of storage buffer on stability of recombinant TMOF/mutant TMOF

To check the effect of the pH of storage buffer on stability of recombinant proteins, crude TMOF/mutant TMOF were resuspended in different pH solutions and stored for one week. Phosphate buffer with pH 6.0, 7.0, Tris buffer with pH 8.0 and bicarbonate buffer with 9.0 were used. *Aedes albopictus* larvae kept in distilled water were treated with crude samples prepared in buffers of different pH. The LC₅₀ (48 hours)

concentration of crude TMOF/mutant TMOF on *A. albopictus* was calculated.

1.4.2.12 Effect of pH of exposure medium on toxicity of recombinant TMOF/mutant TMOF

To check the effect of pH of the exposure medium on the toxicity of crude TMOF/mutant TMOF, the crude samples prepared (as mentioned in 1.4.2.8) and exposed to *A. albopictus* larvae kept in buffers of different pH (buffer solutions of pH 6.0, 7.0, 8.0 and 9.0). The LC₅₀ (48 hours) of crude TMOF/mutant TMOF on *A. albopictus* was used for the treatment.

1.4.2.13 Effect of storage temperature on stability of recombinant TMOF/mutant TMOF when stored as solution

Stability of recombinant TMOF at different temperatures in solution was studied. For this, crude TMOF/mutant TMOF were kept at two temperatures; -20°C and 4°C for one week. *Aedes albopictus* larvae were exposed to recombinant TMOF/mutant TMOF stored at -20°C and 4°C. The LC₅₀ (48 hours) concentration of crude TMOF/mutant TMOF on *A. albopictus* was used.

1.4.2.14 Effect of storage temperature on stability of dried recombinant TMOF/mutant TMOF

Crude TMOF/mutant TMOF were dried in an oven at 40°C for 24 hours. The dried powder was kept at -20°C, 4°C and 25°C for one week. Enough powder was resuspended in distilled water to get LC₅₀ (48 hours) of crude samples for *A. albopictus*. (For *Aedes albopictus*, the LC₅₀ (48 hours) for crude TMOF was 242.1 µg/mL and of crude mutant

TMOF, LC₅₀ (48 hours) was 236.54 µg/mL). *Aedes albopictus* larvae were treated with the dried recombinant TMOF/mutant TMOF and percentage mortality observed.

1.4.2.15 Effect of recombinant TMOF/mutant TMOF on *Culex quinquefasciatus* larvae

Larvicidal activity of crude TMOF/mutant TMOF was tested on *Culex quinquefasciatus*. For this, 25 numbers of second instar *C. quinquefasciatus* larvae were introduced into 100 mL distilled water. Different concentrations of the crude TMOF/mutant TMOF were used with appropriate controls. Lethal concentrations calculated using probit analysis.

1.4.2.16 Non-target toxicity of recombinant TMOF/mutant TMOF

Non-target toxicity of crude TMOF/mutant TMOF was studied. Organisms including vertebrate and invertebrates were selected for the study. The concentration used for the non-target toxicity study was the LC₅₀ (48 hours) for the *A. albopictus* larvae. The test organisms include *Drosophila melanogaster*, *Luprops tristis* and *Aplocheilus lineatus*.

Second instar larvae of *D. melanogaster* were used for the treatment. Recombinant TMOF was mixed with the medium (100 mL medium was composed of 1.5 g agar powder, 8 g jaggery, 8 g wheat powder and 7 mL vinegar) and fed to the larvae. Observations were made until adult emergence.

Adults of *L. tristis* were used in the study. Recombinant TMOF was coated on tender *Cassia* leaves and was fed to *L. tristis*. Treatment was continued for three days. Observations were made up to three weeks since the last treatment.

Adults of *A. lineatus* was cultured in pond water. Recombinant TMOF was added to the pond water, thrice with a gap of two days in the first week only. Observations were made up to three months.

1.4.2.17 Comparison of mosquito larvicidal efficacy of TMOF and mutant TMOF

Comparison of efficacy of the recombinant peptides were done by independent sample t test using their respective half lethal concentrations at 48 hours. Difference was considered significant at p value < 0.05.

1.4.2.18 Effect of TMOF/mutant TMOF on gut bacterial composition of *Aedes albopictus* larvae

Metagenomic analysis of gut bacteria was done using 16S rRNA gene sequencing of the mosquito larvae treated with recombinant TMOF/mutant TMOF.

1.4.2.18.1 Larval treatment and processing of larvae for NGS

Second instar *A. albopictus* larvae (50 numbers) were treated with LC₁₀ of crude TMOF and crude mutant TMOF. A control was done by exposing the larvae to crude control (soluble fraction of untransformed BL21 cell lysate). They were grown until fourth instar stage and then processed for metagenomic analysis. Surface sterilization was done sequentially with 0.5% tween 20, 0.5% sodium hypochlorite and 80% ethanol treatment for 30 seconds each. Then washed in 1M PBS and the larvae were subjected to metagenomic analysis at Oneomics, Thiruchirappally.

1.4.2.18.2 DNA extraction

DNA was extracted from the larval tissue using Oneomics DNA extraction kit.

1.4.2.18.3 PCR Amplification and Sequencing:

Genomic DNA obtained was used as template for PCR amplification of the V3-V4 region of the 16S rRNA gene using gene specific primer 341F -CCTAYGGGRBGCASCAG and 806R -GGACTACNNGGGTATCTAAT. The PCR conditions were;

- Initial denaturation at 98°C for 1 minute
- 30 cycles of denaturation at 98°C (10 seconds), annealing at 57°C (30 seconds) and extension at 72°C (1 minute).
- A final extension at 72°C for 5 minutes

The amplicons thus obtained from the PCR reaction were purified and checked with Bioanalyser 2100 (Agilent Technologies), and libraries were prepared using TruSeq DNA PCR-Free Library Preparation Kit (Illumina, USA). The prepared libraries were quantified using Qubit 4 Fluorometer (Thermo Fisher Scientific, USA) and QuantStudio 5 Real Time PCR (Applied Biosystems, USA). Qualified libraries were sequenced using paired end chemistry in NovaSeq 6000 platform (Illumina, USA) with read length of 250 bp.

1.4.2.18.4 Metagenomic sequence analysis

The sequence data were quality-filtered at various levels: first, allowing 3' trimming (trim back valley filter). After splitting the sequences according to their bar code, chimeras were detected with UCHIME. The feature table and feature table summary were generated which provides

the information of sequence associated with the samples and feature. Representative sequence for each OTU was screened for further annotation. The pre-trained naive classifier was used to classify each read or amplicon sequence variant (ASV). To get the phylogenetic relationship of all OTUs, the representative sequences were subjected to alignment using MAFFT. The site which are not phylogenetically informative were masked and the phylogenetic tree was generated. Subsequent analysis of alpha diversity and beta diversity were all performed based on this output normalized data. The table, taxonomy and tree were exported for further analysis in R. Alpha diversity is applied in analysing complexity of species diversity for sample through indices, including Observed - species, evenness, Shannon, faith-pd. All these indices in the samples were calculated with QIIME2. Beta diversity analysis was used to evaluate differences of samples in species complexity. To compare microbial communities between every pair of community samples, a square matrix of "distance" or dissimilarity was calculated to reflecting the dissimilarity between certain samples, such as Unweighted Unifrac and Weighted Unifrac distance. Cluster analysis was preceded by principal component analysis (PCA).

1.4.2.19 Statistical analysis

Independent sample t test was done to compare the mean activity of TMOF and mutant TMOF. The p values < 0.05 was considered to be significant. Statistical analysis was done using SPSS version 23 and Microsoft Excel version 2021.

1.5 RESULTS

1.5.1 CONJUGATION OF TMOF TO BSA AND ITS TOXICITY TO *AEDES ALBOPICTUS* AND *CULEX QUINQUEFASCIATUS* LARVAE

The tetrapeptide from the first four amino acids of the TMOF peptide (YDPA) reported to have 95% TMOF activity of the full-length peptide. This tetrapeptide with an additional lysine (K) residue at N-terminus was synthesised. This TMOF pentapeptide (KYDPA) was conjugated to BSA using glutaraldehyde. The BSA-conjugated peptide makes the peptide bit longer and accessible for trypsin enzyme. Trypsin can cleave at the C-terminus of lysine residue of the pentapeptide, leaving active peptide inside the gut. *Aedes albopictus*, *Culex quinquefasciatus* and *Drosophila melanogaster* larvae were treated with the BSA-conjugated TMOF pentapeptide. Mortality and delayed development were observed in *A. albopictus* and *C. quinquefasciatus* larvae, while *D. melanogaster* larvae remain unaffected. The LC₅₀ (48 hours) for *A. albopictus* larvae was found to be 2.12 ± 0.02 µg/mL and that of *C. quinquefasciatus* larvae was found to be 3.05 ± 0.02 µg/mL. Figure 1.5.1 represents the toxicity analysis of conjugated peptide.

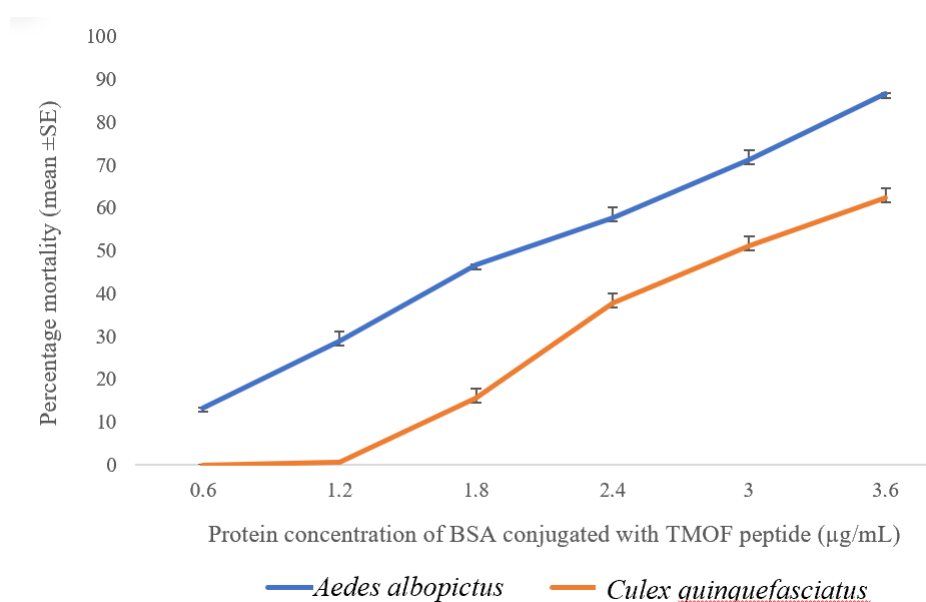


Fig. 1.5.1 Toxicity of BSA-conjugated TMOF peptide to *A. albopictus* and *C. quinquefasciatus* larvae

1.5.2 CLONING TMOF/MUTANT TMOF GENE

1.5.2.1 Synthesis of gene coding for TMOF/mutant TMOF

The TMOF/mutant TMOF genes were designed and custom synthesised. The amino acid sequence of the TMOF decapeptide was retrieved from the literature. They were codon optimised for expression in *Escherichia coli* (marked in brown colour in the sequence given below). Trypsin cleavage site (AAA) was added at the N-terminus of the peptide, and at C-terminus a stop codon (TAA) was added. Restriction enzyme sites added as half-sites so that no restriction digestion was required while preparing the TMOF insert for cloning. The restriction enzymes chosen for the cloning were AsiSI and PmeI. So, half sites for these enzymes added, AsiSI (CGC) at N-terminus and PmeI (GTTT) at

5' CGCCAAATATGATCCGGCGCCGCCGCCGCCGCCGTAAGTTT 3'
5' AAAGTTACGGCGGGCGGGCGGGCGGGCGCCGGATCATATTTGCGAT 3'

5' CGCCAAANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNTAAGTTT 3'
5' AAAGTTANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNTTTCGCGAT 3'

The designed construct was subjected to restriction mapping to identify unwanted recognition sites, if any, using NEB cutter 2.0. Restriction mapping of designed construct is represented in figure 1.5.2.

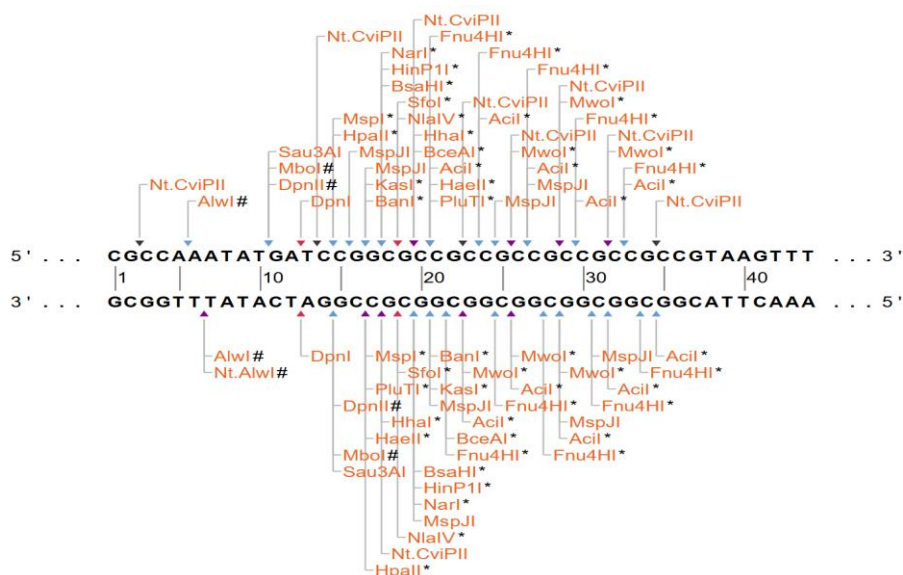


Fig. 1.5.2 Restriction mapping of TMOF construct. Restriction mapping of TMOF gene insert using NEB cutter showing no other sites for AsiSI and PmeI enzymes

1.5.2.2 Restriction digestion of pFN29AHis6Halo and agarose gel electrophoresis

pFN29AHis6Halo vector was double digested with AsiSI and PmeI enzymes. Digested DNA was run in an agarose gel to separate the fragments. The size of vector after double digestion was reduced from 4398 bp to 4030 bp by the removal of 368 bp long barnase gene. The double digested vector after electrophoresis is shown in figure 1.5.3 A.

1.5.2.3 Gel purification of double digested pFN29AHis6Halo

Separated vector DNA after double digestion was sliced from the gel under UV. The sliced gel piece dissolved and DNA extracted. The extracted DNA gel quantified. Figure 1.5.3 B represents electrophoresis using gel purified DNA.

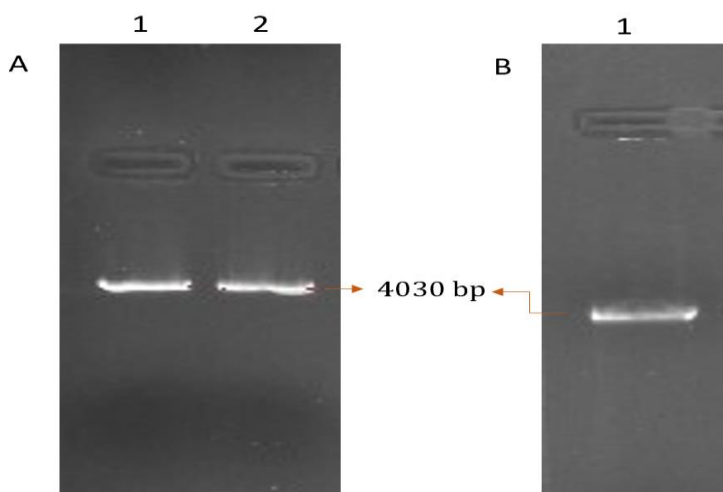


Fig 1.5.3 Double digestion and gel purification of pFN29AHis6Halo vector. A. Lane 1 and 2. Double digested pFN29AHis6Halo vector (4030 bp) using AsiSI and PmeI enzymes, B. Lane1. Double digested vector after gel purification

1.5.2.4 Preparation of insert, ligation and transformation

Gel-purified double digested vector and double stranded insert (TMOF/mutant TMOF gene) were ligated using T4 DNA ligase. The ligation mix was used to transform competent DH5 α cells. Transformed colonies obtained only in the plate ligated in 1:5 molar ratio of vector to insert. There were about 35 colonies obtained after the vector ligated with the TMOF gene. Only 4 colonies obtained in vector ligated with the mutant TMOF gene. Images of transformed colonies are represented in figure 1.5.4.

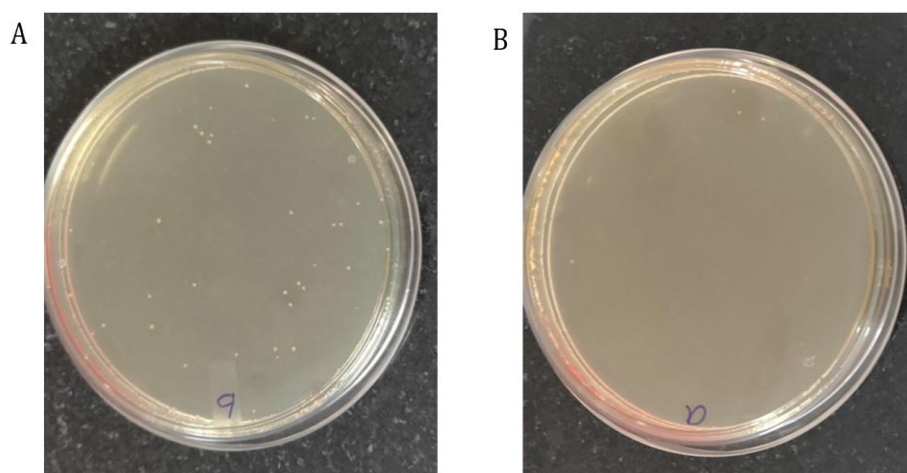


Fig. 1.5.4 Transformation of DH5 α cells with TMOF/ mutant TMOF gene. A. Bacterial cells transformed with pFN29AHis6Halo vector containing TMOF gene. B. Bacterial cells transformed with pFN29AHis6Halo vector containing mutant TMOF gene.

1.5.2.5 Screening for transformed colonies

Recombinant vector was isolated from transformed colonies and screened for the presence of insert within vector. For this, restriction digestion/ PCR was used. Sequence was confirmed by DNA sequencing.

1.5.2.5.1 Restriction digestion

Isolated recombinant vector was digested with PmeI, one of the enzymes used for cloning. The original vector was also digested with the same enzyme and both of them were run on 0.8% agarose gel. A 1 kb DNA ladder was run along to compare size differences. The original vector after digestion had a size of 4398 bp whereas in recombinant vector (pFN29A-TMOF/mutant TMOF) the size was 4075 bp. A difference in 323 bp was clear from the gel and is represented in figure 1.5.5. A vector map representing TMOF/mutant TMOF insert within pFN29AHis6Halo is shown in figure 1.5.6.

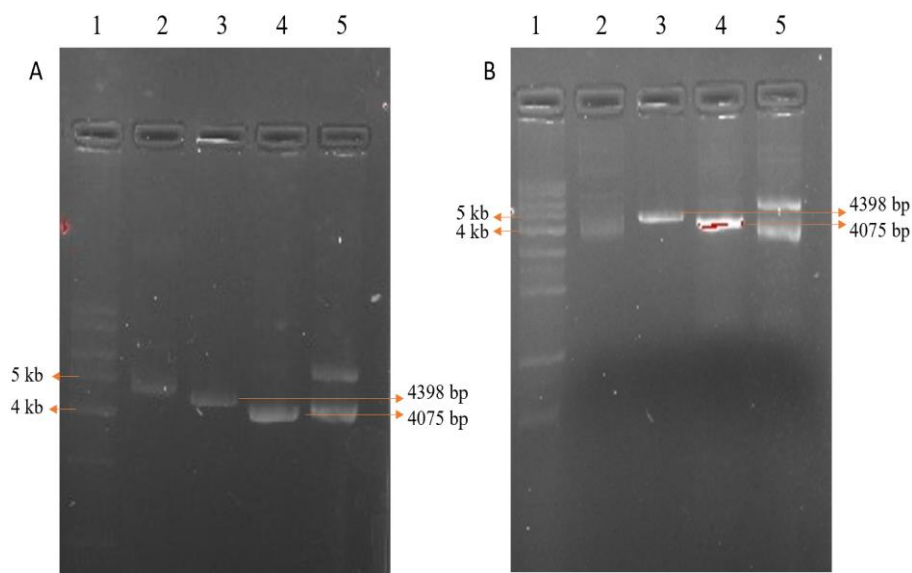


Fig. 1.5.5 Restriction digestion of vector containing TMOF/ mutant TMOF. **A.** Restriction digestion and electrophoresis of pFN29AHis6Halo vector containing TMOF gene, Lane 1. 1Kb DNA ladder, lane 2. Undigested pFN29AHis6Halo vector, lane 3. pFN29AHis6Halo digested with PmeI enzyme (4398 bp), lane 4. pFN29AHis6Halo with TMOF digested with PmeI enzyme (4075 bp), lane 5. Undigested pFN29AHis6Halo with TMOF. **B.** Restriction digestion and electrophoresis of pFN29AHis6Halo vector containing mutant TMOF gene, Lane 1. 1Kb DNA ladder, lane 2. Undigested pFN29AHis6Halo vector, lane 3. pFN29AHis6Halo digested with PmeI enzyme (4398 bp), lane 4. pFN29AHis6Halo with mutant TMOF digested with PmeI enzyme (4075 bp), lane 5. Undigested pFN29AHis6Halo with mutant TMOF.

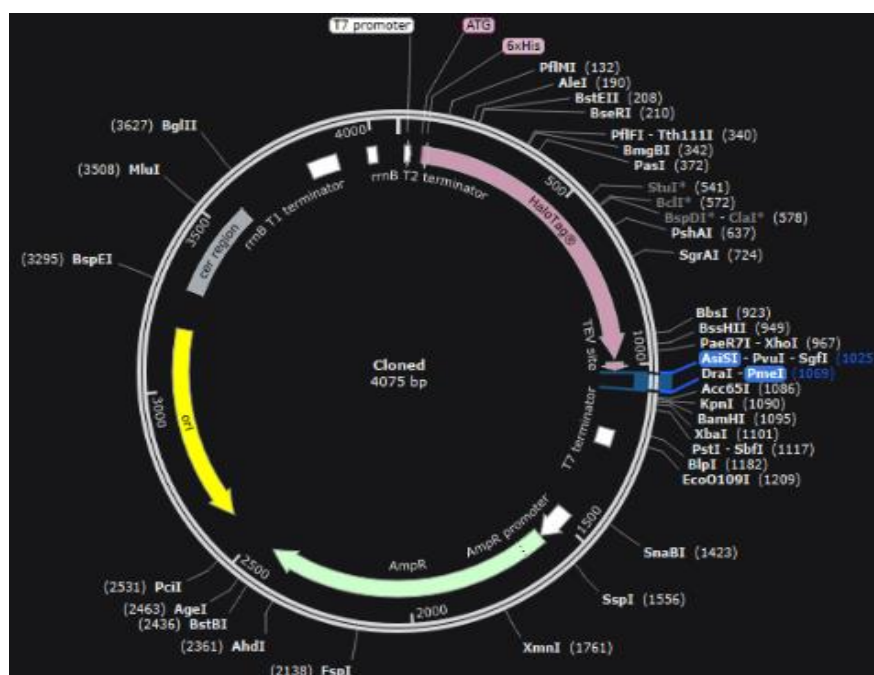


Fig. 1.5.6 Vector map. pFN29AHis6Halo vector map containing TMOF/ mutant TMOF gene

1.5.2.5.2 PCR amplification

PCR amplification of the vector with TMOF/mutant TMOF were done. Vector-specific primers flanking the insert were used. The original vector was also amplified. Amplification of the original vector produced a fragment of 596 bp, while the vector with TMOF/mutant TMOF produced a fragment of 277 bp. The amplified PCR products were run on 1% agarose gel with 100 bp DNA ladder (Fig 1.5.7).

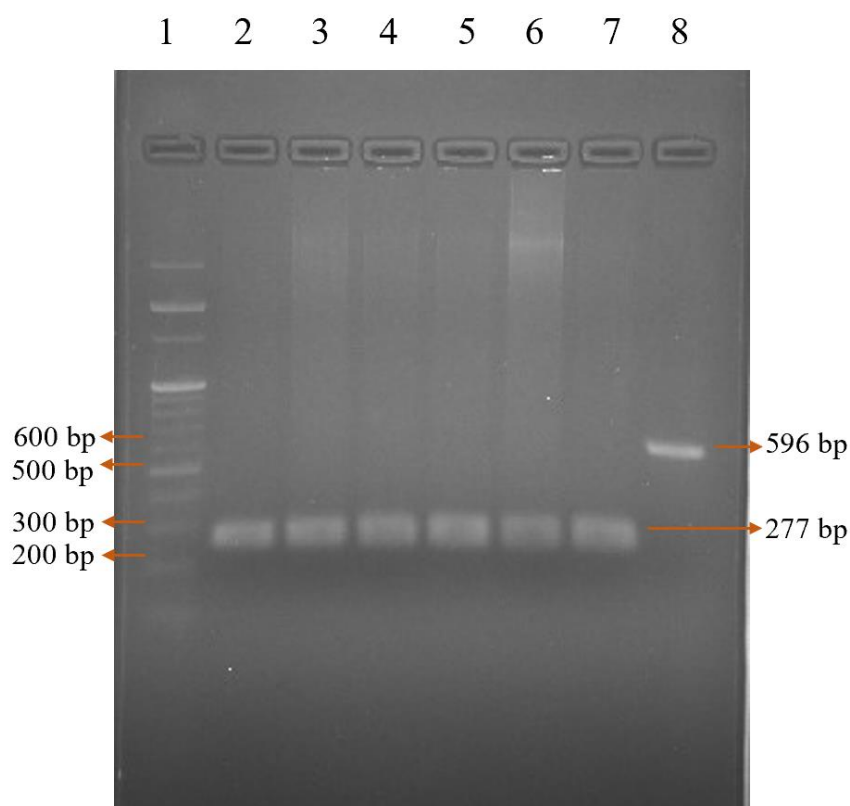


Fig. 1.5.7 PCR amplification of vector with TMOF/mutant TMOF. 0.8% Agarose gel of PCR amplified TMOF/ mutant TMOF. Lane 1. 100 bp DNA ladder, lane 2 to 5. TMOF amplified with sequencing primers, lane 6-7. Mutant TMOF amplified with sequencing primers, lane 8. pFN29AHis6Halo vector amplified with sequencing primers

1.5.2.5.6 Sanger sequencing

Amplified PCR products containing TMOF/mutant TMOF gene were sequenced by Sanger sequencing, and the chromatogram is represented in figure 1.5.8.

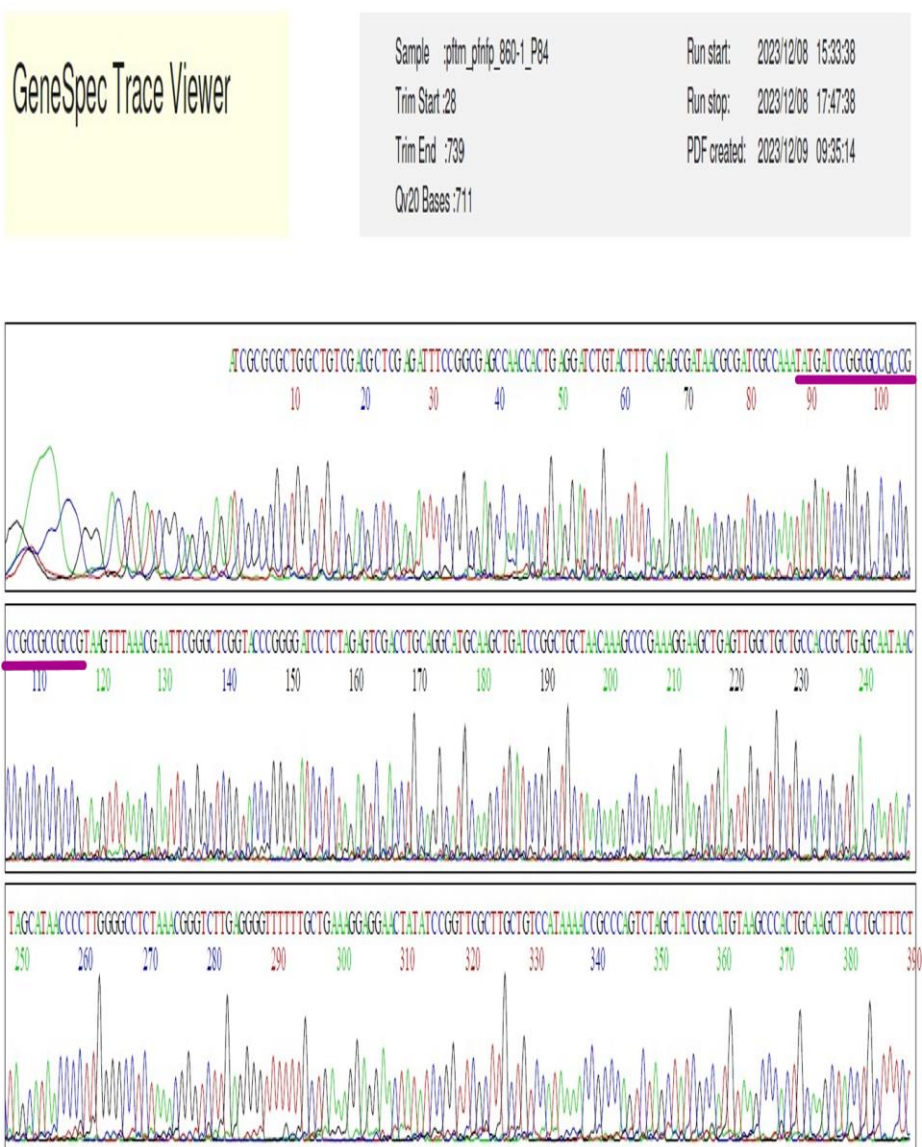


Fig. 1.5.8 Chromatogram of PCR product amplifying the TMOF gene. Sanger sequencing chromatogram of PCR product amplifying the region of TMOF gene (81-123) (marked using underline) in pFN29AHis6Halo vector

The nucleotide sequence of the recombinant protein containing TMOF gene is given below (The TMOF gene is marked in red colour).

```
ATGAAACATCATCACCATCACCACGCAGAAATCGGTACTGGCTTTCC
ATTCGACCCCCATTATGTGGAAGTCCTGGGCGAGCGCATGCACTACG
TCGATGTTGGTCCGCGCGATGGCACCCCTGTGCTGTTCTGACGGT
AACCCGACCTCCTCCTACGTGTGGCGCAACATCATCCCGCATGTTGC
ACCGACCCATCGCTGCATTGCTCCAGACCTGATCGGTATGGGCAAAT
CCGACAAACCAGACCTGGGTTATTTCTTCGACGACCACGTCCGCTTC
ATGGATGCCTTCATCGAAGCCCTGGGTCTGGAAGAGGTCGTCCTGGT
CATTACGACTGGGGCTCCGCTCTGGGTTTCCACTGGGCCAAGCGCA
ATCCAGAGCGCGTCAAAGGTATTGCATTTATGGAGTTCATCCGCCCT
ATCCCGACCTGGGACGAATGGCCAGAATTTGCCCGCGAGACCTTCCA
GGCCTTCCGCACCACCGACGTCGGCCGCAAGCTGATCATCGATCAGA
ACGTTTTTATCGAGGGTACGCTGCCGATGGGTGTCGTCCGCCCGCTG
ACTGAAGTCGAGATGGACCATTACCGCGAGCCGTTCTGAATCCTGT
TGACCGCGAGCCACTGTGGCGCTTCCCAAACGAGCTGCCAATCGCCG
GTGAGCCAGCGAACATCGTCGCGCTGGTCTGAAGAATACATGGACTGG
CTGCACCAGTCCCCTGTCCCGAAGCTGCTGTTCTGGGGCACCCAGG
CGTTCTGATCCCACCGGCCGAAGCCGCTCGCCTGGCCAAAAGCCTGC
CTAACTGCAAGGCTGTGGACATCGGCCCGGGTCTGAATCTGCTGCAA
GAAGACAACCCGGACCTGATCGGCAGCGAGATCGCGCGCTGGCTGTC
GACGCTCGAGATTTCCGGCGAGCCAACCACTGAGGATCTGTACTTTC
AGAGCGATAACGCGATCGCCAAATATGATCCGGCGCCGCCGCCG
CCGCCGTAA
```

Translated amino acid sequence of the recombinant protein containing the TMOF peptide is given below (Recombinant TMOF peptide is marked in red colour).

```
MKHHHHHHAEIGTGFPFDPHYVEVLGERMHYVDVGPRDGTPVLFLHG
NPTSSYVWRNIIPHVAPTHRCIAPDLIGMGKSDKPDLGYFFDDHVRF
MDAFIEALGLEEVVLVIHDWGSALGFHWAKRNPERSVKIAFMFIRP
IPTWDEWPEFARETFAQFRITDVGRKLIIDQNVFIEGTLPMGVVRPL
TEVEMDHYREPFLNPVDREPLWRFPNELPIAGEPANIVALVEEYMDW
LHQSPVPKLLFWGTPGVLIIPPAEAAARLAKSLPNCKAVDIGPGLNLLQ
EDNPDIGSEIARWLSTLEISGEPTTEDLYFQSDNAIAKYDPAPPPP
PP
```

- Number of amino acids: **331**
- Molecular weight: **37513.79 Daltons**
- Theoretical pI: **5.08**

The chromatogram of pFN29A-mutant TMOF is shown in figure 1.5.9.

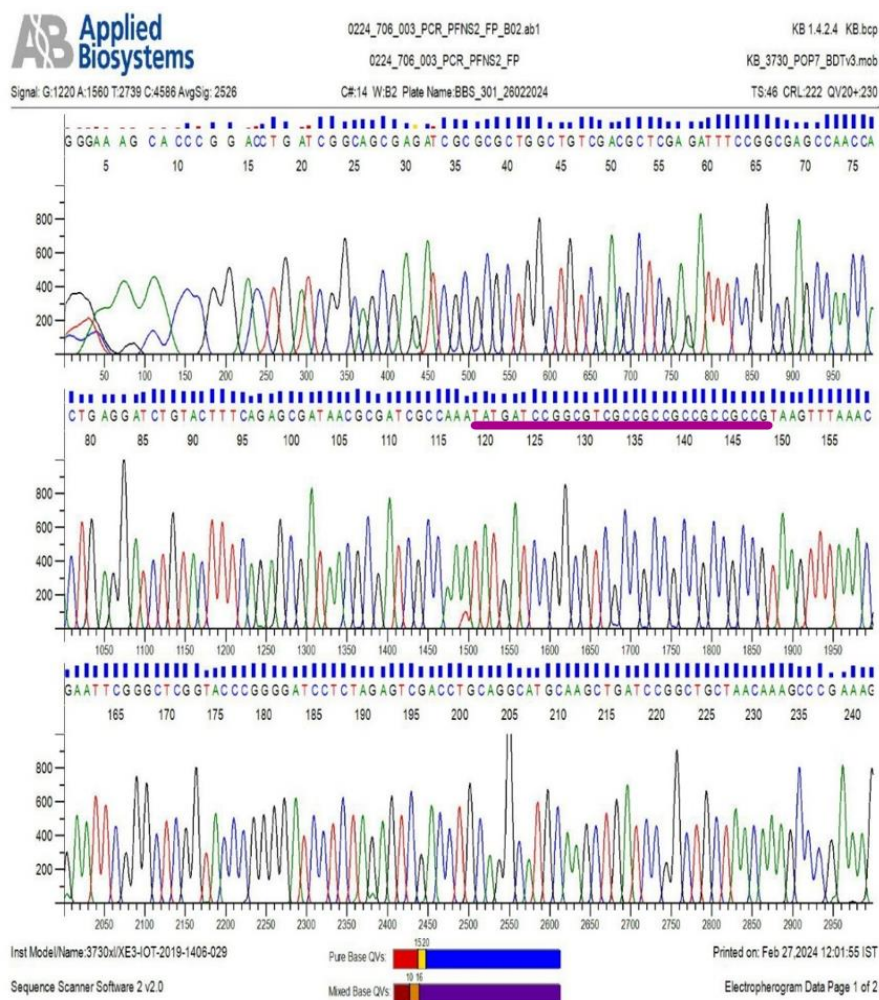


Fig. 1.5.9 Chromatogram of PCR product amplifying the mutant TMOF gene. Sanger sequencing chromatogram of PCR product amplifying the region of mutant TMOF gene (112- 159) (marked using underline) in pFN29AHis6Halo vector

The nucleotide sequence of the recombinant mutant TMOF protein is given below (the mutant TMOF gene is marked in red colour).

```
ATGAAACATCATCACCATCACCACGCAGAAATCGGTACTGGCTTTCC
ATTCGACCCCCATTATGTGGAAGTCCTGGGCGAGCGCATGCACTACG
TCGATGTTGGTCCGCGCGATGGCACCCCTGTGCTGTTCCCTGCACGGT
AACCCGACCTCCTCCTACGTGTGGCGCAACATCATCCCGCATGTTGC
ACCGACCCATCGCTGCATTGCTCCAGACCTGATCGGTATGGGCAAAT
CCGACAAACCAGACCTGGGTTATTTCTTCGACGACCACGTCCGCTTC
ATGGATGCCTTCATCGAAGCCCTGGGTCTGGAAGAGGTCGTCCTGGT
CATTACGACTGGGGCTCCGCTCTGGGTTTCCACTGGGCCAAGCGCA
ATCCAGAGCGCGTCAAAGGTATTGCATTTATGGAGTTCATCCGCCCT
ATCCCGACCTGGGACGAATGGCCAGAATTTGCCCGCGAGACCTTCCA
GGCCTTCCGCACCACCGACGTCGGCCGCAAGCTGATCATCGATCAGA
ACGTTTTTATCGAGGGTACGCTGCCGATGGGTGTGTCGCCCGCGCTG
ACTGAAGTCGAGATGGACCATTAACGCGAGCCGTTCTGAATCCTGT
TGACCGCGAGCCACTGTGGCGCTTCCCAAACGAGCTGCCAATCGCCG
GTGAGCCAGCGAACATCGTCGCGCTGGTCTGAAGAATACATGGAAGTGG
CTGCACCAGTCCCCTGTCCCGAAGCTGCTGTTCTGGGGCACCCAGG
CGTTCTGATCCCACCGGCCGAAGCCGCTCGCCTGGCCAAAAGCCTGC
CTAACTGCAAGGCTGTGGACATCGGCCCGGGTCTGAATCTGCTGCAA
GAAGACAACCCGGACCTGATCGGCAGCGAGATCGCGCGCTGGCTGTC
GACGCTCGAGATTTCCGGCGAGCCAACCACTGAGGATCTGTACTTTC
AGAGCGATAACGCGATCGCCAAATATGATCCGGCGTCGCCGCCGCCG
CCGCCGTAA
```

Translated amino acid sequence of the recombinant mutant TMOF protein is given below (Recombinant mutant TMOF peptide is marked in red colour and the mutated amino acid residue is marked in blue colour).

```
MKHHHHHHAEIGTGFPDPHYVEVLGERMHYVDVGPRDGPVLFHLHG
NPTSSYVWRNIIPHVAPTHRCIAPDLIGMGKSDKPDLGYFFDDHVRF
MDAFIEALGLEEVVLVIHDWGSALGFHWAKRNPervKGIAFMefIRP
IPTWDEWPEFARETFQAFRTTDVGRKLIIDQNVFIEGTLPMGVVRPL
TEVEMDHYREPFLNPVDREPLWRFPNELPIAGEPANIVALVEEYMDW
LHQSPVPKLLFWGTPGVLIIPPAEAARLAKSLPNCKAVDIGPGLNLLQ
EDNPDIGSEIARWLSTLEISGEPTTEDLYFQSDNAIAKYDPASPPP
PP
```

- Number of amino acids: **331**
- Molecular weight: **37503.75 DALTONS**
- Theoretical pI: **5.08**

1.5.3 Expression of recombinant TMOF/mutant TMOF

The recombinant vector containing TMOF/mutant TMOF were expressed in *E. coli* cells. Expression of recombinant proteins as induced by IPTG. BL21 competent cells were transformed with vector containing TMOF/mutant TMOF gene. Single colony of the transformed bacteria cultured and induced for protein expression. The optimum temperature, IPTG concentration and duration of induction was optimised for maximum expression of protein. Optimum expression of recombinant TMOF/mutant TMOF was obtained at 37°C with 0.5 mM IPTG concentration for a duration of 5 hours. The bacterial pellet after expression was lysed and its supernatant was used as crude TMOF, crude mutant TMOF and crude control as described in methods. SDS-PAGE was run with crude samples to check level of expression. Protein concentration of the crude samples is represented in table 1.5.1. The figure 1.5.10 represent SDS-PAGE image showing expression of recombinant protein in the crude samples.

Table 1.5.1 Protein concentration of crude samples

Sample	Protein concentration (($\mu\text{g}/\mu\text{L}$))
Control	9.12
Crude TMOF	9.64
Crude mutant TMOF	9.04

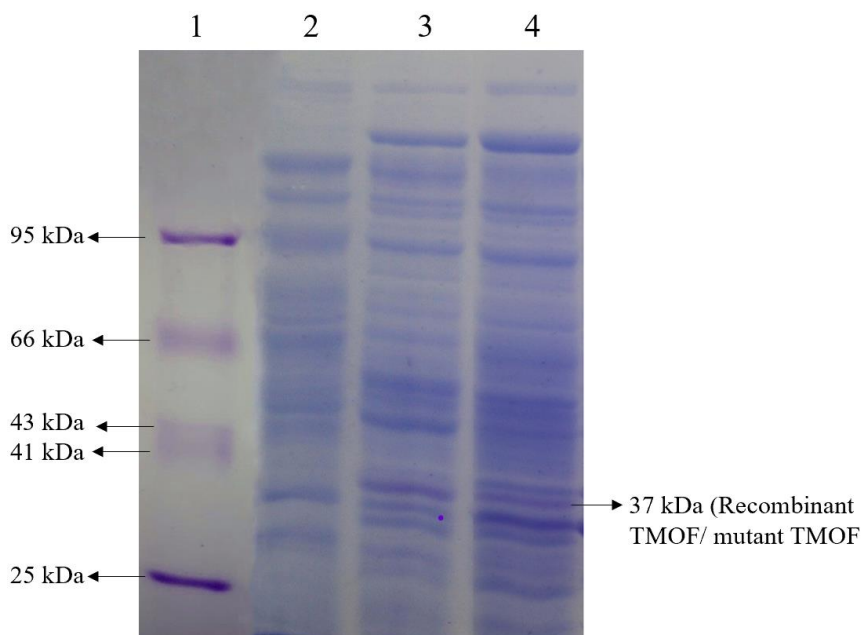


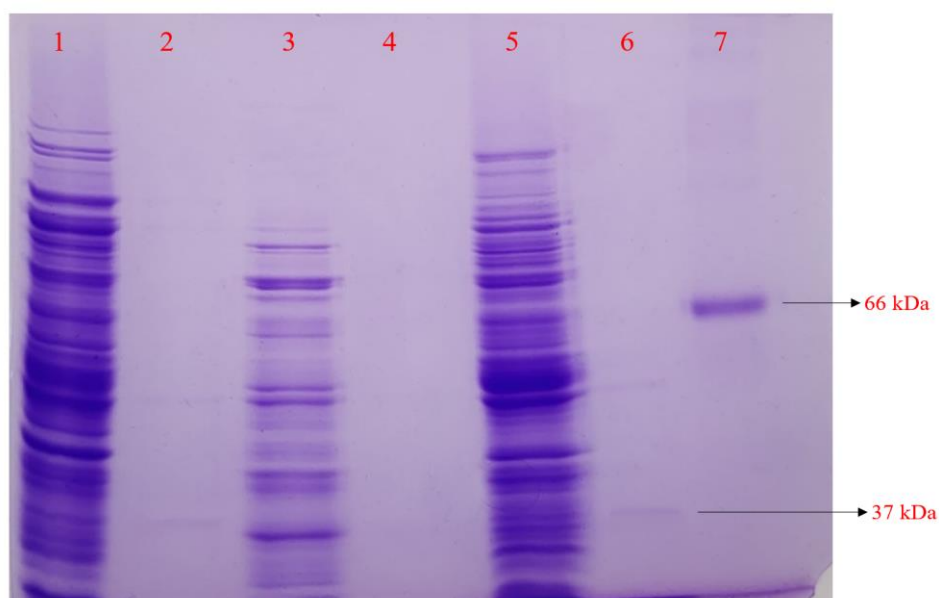
Fig. 1.5.10 SDS-PAGE showing expression of recombinant TMOF/ mutant TMOF. Expression of recombinant proteins. Lane 1. Protein marker, Lane 2. Crude control, Lane 3. Crude TMOF, Lane 4. Crude mutant TMOF

1.5.4 Purification of recombinant TMOF/mutant TMOF

Crude TMOF and crude mutant TMOF were purified using nickel affinity chromatography. Elution was done using 250 mM imidazole. Crude control was also run through a nickel affinity column for comparing the elutes obtained. Eluted proteins were loaded onto 10% SDS-PAGE to check the purity (Fig. 1.5.11). Protein concentration of eluted samples is represented in table 1.5.2. The recombinant TMOF/mutant TMOF was found to have a size of 37.5 kDa.

Table 1.5.2 Protein concentration of Ni-IMAC elutes containing recombinant TMOF

Sample	Protein concentration (($\mu\text{g}/\mu\text{L}$))
Control elute 1	1.49
Control elute 2	1.38
TMOF elute 1	1.56
TMOF elute 2	1.45
Mutant TMOF elute 1	1.49
Mutant TMOF elute 2	1.4

**Fig. 1.5.11** SDS-PAGE showing purification of TMOF/mutant TMOF. Lane 1. Crude TMOF, Lane 2. Purified TMOF, Lane 3. Crude control, Lane 4. Purified control, Lane 5. Crude mutant TMOF, Lane 6. Purified mutant TMOF, Lane 7. BSA

1.5.5 Larvicidal activity of recombinant TMOF/mutant TMOF on *Aedes albopictus*

Larvicidal activity of crude TMOF/mutant TMOF and affinity purified TMOF/mutant TMOF were checked. For this, 25 numbers of *A. albopictus* larvae were treated and observed until adult emerged. Mortality after 48 hours was used for LC₅₀ calculation.

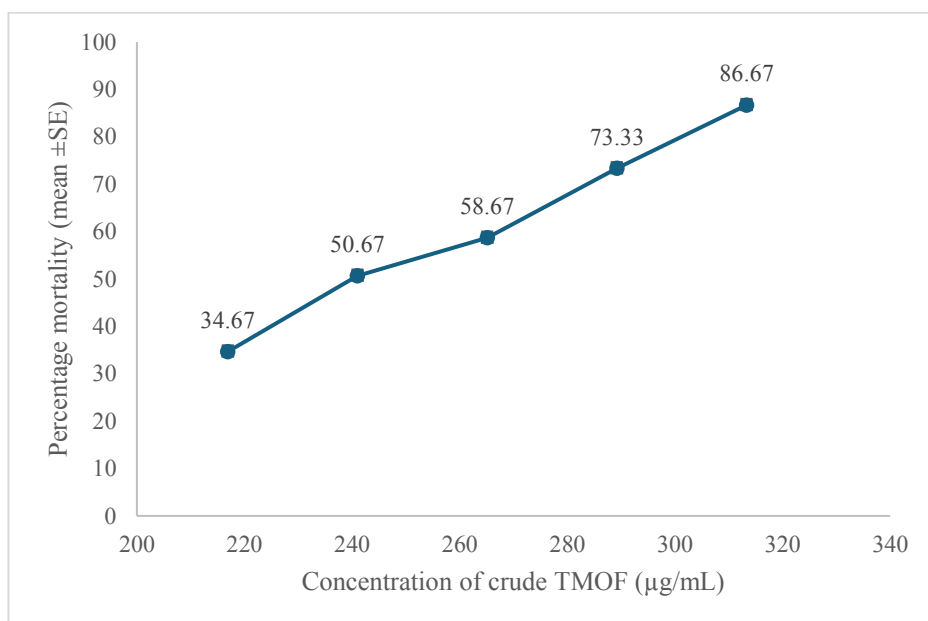
1.5.5.1 Toxicity of crude TMOF/mutant TMOF

Aedes albopictus larvae were treated with different concentrations of crude TMOF/mutant TMOF. Lethal concentrations were calculated using probit analysis. Larval mortality shows a direct relation with concentration of crude TMOF/mutant TMOF. For crude TMOF, larval mortality increases from $34.67 \pm 1.33\%$ to $86.67 \pm 1.33\%$ at concentrations 216.9 and 313.3 $\mu\text{g/mL}$ respectively. The LC₅₀ (48 hours) was found to be $242.10 \pm 6.04 \mu\text{g/ml}$. In case of crude mutant TMOF, $36.00 \pm 0.00\%$ mortality was obtained at 216 $\mu\text{g/mL}$ concentration and it was increased to $92.00 \pm 2.33\%$ at 306 $\mu\text{g/mL}$. The LC₅₀ (48 hours) for mutant TMOF was found to be $236.54 \pm 6.49 \mu\text{g/mL}$.

Results of toxicity analysis using crude TMOF is shown in table 1.5.3 and of crude mutant TMOF in table 1.5.4. Toxicity results using crude TMOF in terms of protein concentration is represented in figure 1.5.12 and that of crude mutant TMOF is represented in figure 1.5.13. It is noted that the protein concentration and the larval mortality is in direct proportion. As the protein concentration increases, mortality of treated larvae also increases.

Table 1.5.3 Toxicity analysis using crude TMOF

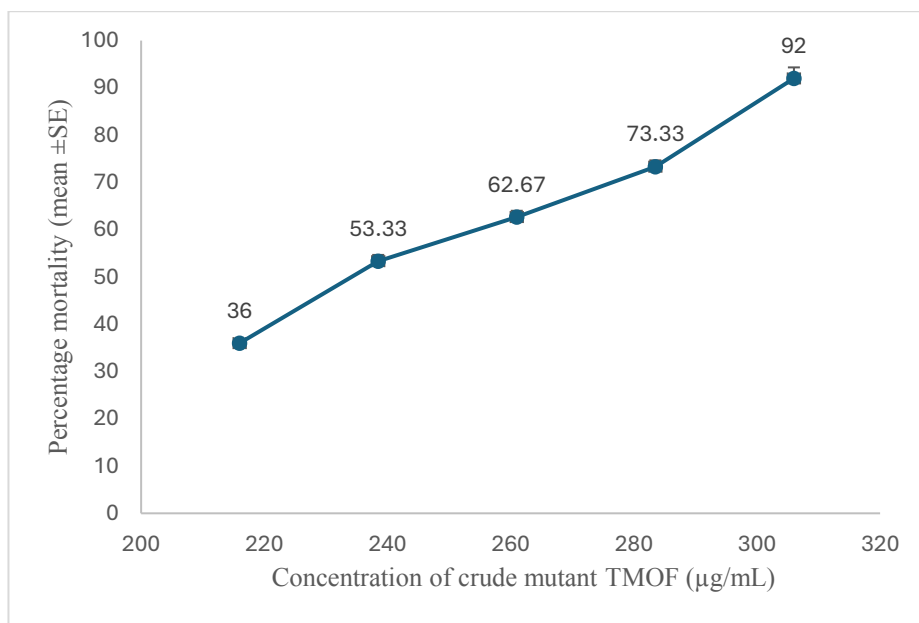
Protein concentration (µg/mL) of supernatant from bacterial lysate (TMOF)	Number of bacterial cells	Percentage mortality (mean ± SE) (after 48 hours)
216.9	$5.61 \times 10^6 \pm 2.89 \times 10^4$	34.67 ± 1.33
241.0	$6.26 \times 10^6 \pm 3.21 \times 10^4$	50.67 ± 1.33
265.1	$6.85 \times 10^6 \pm 3.53 \times 10^4$	58.67 ± 1.33
289.2	$7.48 \times 10^6 \pm 3.85 \times 10^4$	73.33 ± 1.33
313.3	$8.10 \times 10^6 \pm 8.22 \times 10^4$	86.67 ± 1.33

**Fig. 1.5.12** Larvicidal activity of crude TMOF on *Aedes albopictus*

LC₅₀ (48 hours) of crude TMOF on *A. albopictus* was found to be 242.10 ± 6.04 µg/mL.

Table 1.5.4 Toxicity analysis using crude mutant TMOF

Protein concentration (µg/mL) of supernatant from bacterial lysate (mutant TMOF)	Number of bacterial cells	Percentage mortality (mean ± SE) (after 48 hours)
216.0	$5.25 \times 10^6 \pm 6.39 \times 10^4$	36.00 ± 0.00
238.5	$5.80 \times 10^6 \pm 7.06 \times 10^4$	53.33 ± 1.33
261.0	$6.35 \times 10^6 \pm 7.73 \times 10^4$	62.67 ± 1.33
283.5	$6.89 \times 10^6 \pm 8.39 \times 10^4$	73.33 ± 1.33
306.0	$7.44 \times 10^6 \pm 9.06 \times 10^4$	92.00 ± 2.33

**Fig. 1.5.13** Larvicidal activity of crude mutant TMOF on *Aedes albopictus*

LC₅₀ (48 hours) of crude mutant TMOF on *A. albopictus* was found to be 236.54 ± 6.49 µg/mL.

1.5.5.2 Larvicidal activity of crude TMOF/mutant TMOF in terms of number of bacterial cells

Larvicidal activity of crude TMOF/mutant TMOF was also expressed in terms of number of cells expressing respective recombinant proteins. Number of cells required to produce definite amount of TMOF/mutant TMOF were calculated. Lethal concentrations were also expressed based on the number of cells. In the case of crude TMOF, $6.34 \times 10^6 \pm 5.38 \times 10^4$ cells expressing recombinant TMOF was required to cause 50% mortality in the larvae (Fig. 1.5.14). In the case of crude mutant TMOF, $5.76 \times 10^6 \pm 4.53 \times 10^4$ cells expressing mutant TMOF was required to cause 50% death in the larvae (Fig. 1.5.15).

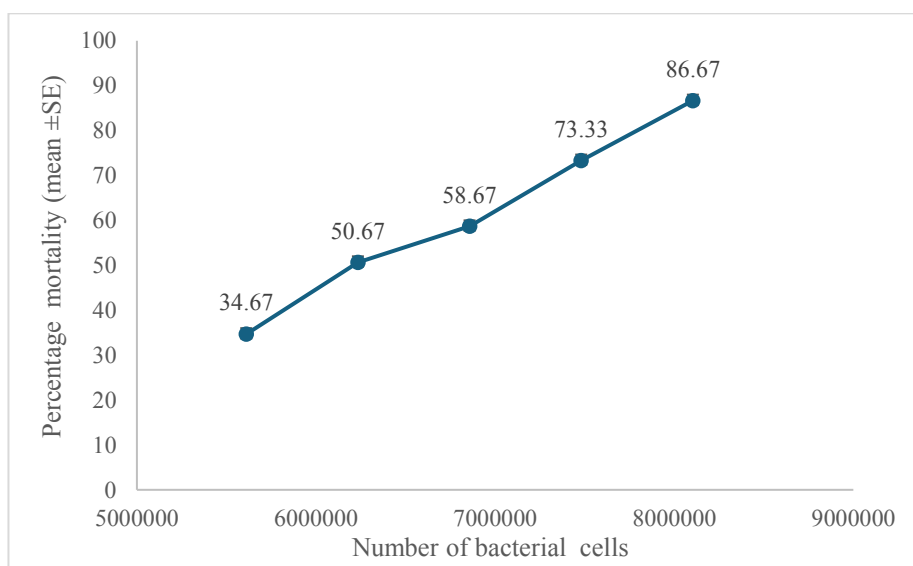


Fig 1.5.14 Larvicidal activity of crude TMOF on *Aedes albopictus* in terms of number of bacterial cells

LC₅₀ (48 hours) of crude TMOF on *A. albopictus* was found to be $6.34 \times 10^6 \pm 5.38 \times 10^4$ cells.

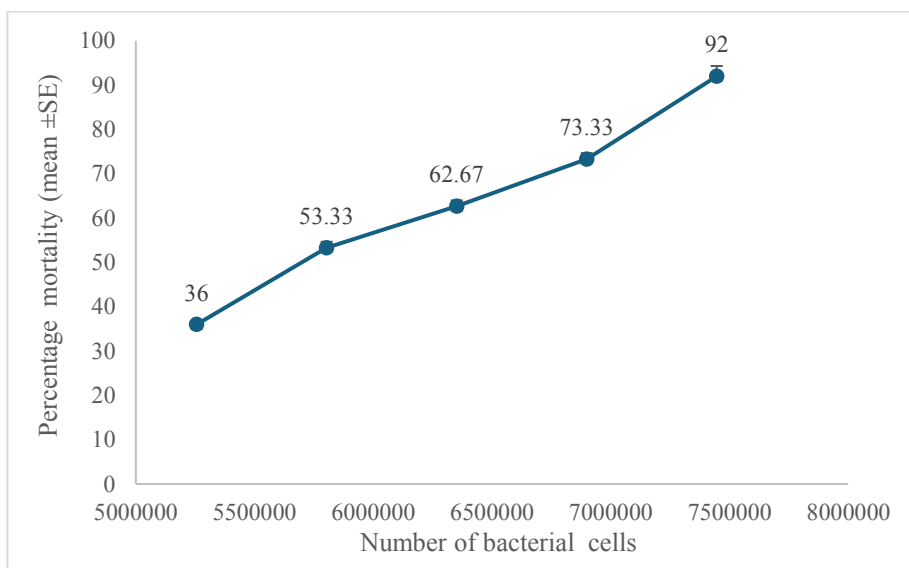


Fig. 1.5.15 Larvicidal activity of crude mutant TMOF on *A. albopictus* in terms of number of bacterial cells

LC₅₀ (48 hours) of crude mutant TMOF on *A. albopictus* was found to be $5.76 \times 10^6 \pm 4.53 \times 10^4$ cells.

1.5.5.3 Toxicity of affinity purified TMOF/mutant TMOF

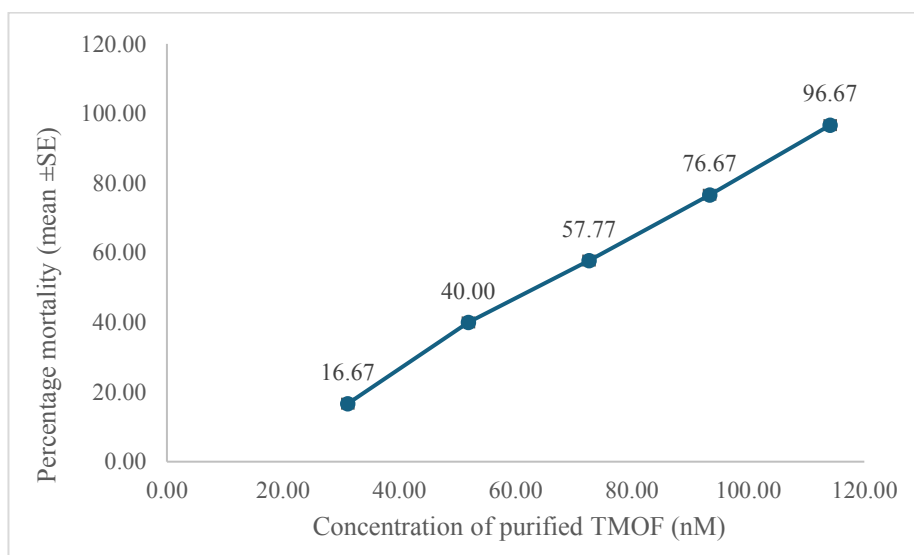
Aedes albopictus larvae were treated with different concentrations of affinity purified TMOF and mutant TMOF. As the concentration of purified TMOF/mutant TMOF increases, percentage mortality also increased. The highest mortality ($96.67 \pm 1.14\%$) for purified TMOF was obtained at a concentration of 114.13 nM and for purified mutant TMOF, the highest mortality (100.00 ± 1.53) was obtained at 109.01 nM. The results are given in table 1.5.5 and 1.5.6 for TMOF and mutant TMOF, respectively. Lethal concentrations were calculated using probit analysis and the results are represented in figure 1.5.16 and 1.5.17.

Table 1.5.5 Toxicity of affinity purified TMOF

Concentration (nM) of affinity purified TMOF	Percentage mortality (mean $\pm$ SE) (after 48 hours)
31.13	16.67 $\pm$ 1.14
51.88	40.00 $\pm$ 1.14
72.63	57.77 $\pm$ 1.14
93.38	76.67 $\pm$ 1.14
114.13	96.67 $\pm$ 1.14

Table 1.5.6 Toxicity of affinity purified mutant TMOF

Concentration (nM) of affinity purified mutant TMOF	Percentage mortality (mean $\pm$ SE) (after 48 hours)
29.73	16.67 $\pm$ 1.53
49.55	38.89 $\pm$ 1.53
69.37	68.89 $\pm$ 1.53
89.19	85.56 $\pm$ 1.53
109.01	100.00 $\pm$ 1.53

**Fig. 1.5.16** Larvicidal activity of affinity purified TMOF on *Aedes albopictus* larvae

LC₅₀ (48 hours) for affinity purified TMOF on *A. albopictus* was found to be 56.89 ± 1.42 nM (2.13 ± 0.02 µg/mL).

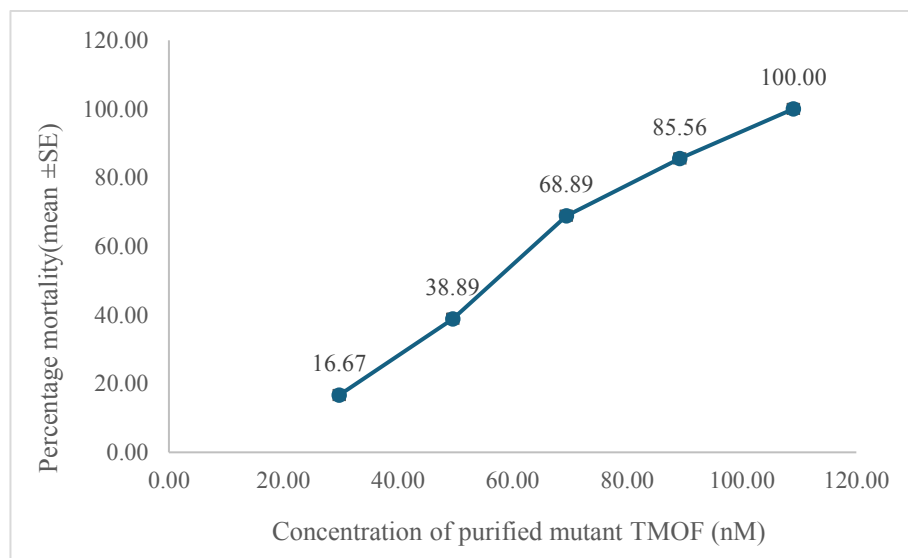


Fig. 1.5.17 Larvicidal activity of affinity purified mutant TMOF on *Aedes albopictus* larvae

LC₅₀ (48 hours) for affinity purified mutant TMOF on *A. albopictus* was found to be 50.93 ± 1.50 nM (1.91 ± 0.02 µg/mL).

1.5.6 Effect of pH of storage buffer on stability of recombinant TMOF/mutant TMOF

The crude TMOF and mutant TMOF samples resuspended in four different buffers of pH, 6.0, 7.0, 8.0 and 9.0 and stored for one week at -20°C. *Aedes albopictus* larvae were treated with the LC₅₀ (48 hours) for the crude samples and the stability of samples in different pH were analysed. The results are represented in figure 1.5.18. For crude TMOF, toxicity was the highest when stored in buffer with pH 9.0 (53.33 ± 1.33 %) and toxicity observed in buffer pH 8.0 was 48.00 ± 0.00 %. But

TMOF toxicity was lower when stored in buffer with pH 7.0 (33.33 ± 1.33 %) and the least in pH 6.0 (14.67 ± 1.33 %). In case of mutant TMOF, toxicity was the highest when stored in buffer with pH 9.0 (53.33 ± 1.33 %) followed by buffer pH 8.0 (49.33 ± 1.33 %), pH 7.0 (34.67 ± 1.33 %) and the least in pH 6.0 (16.00 ± 0.00 %). Crude TMOF and crude mutant TMOF were most stable when stored at pH 9.0.

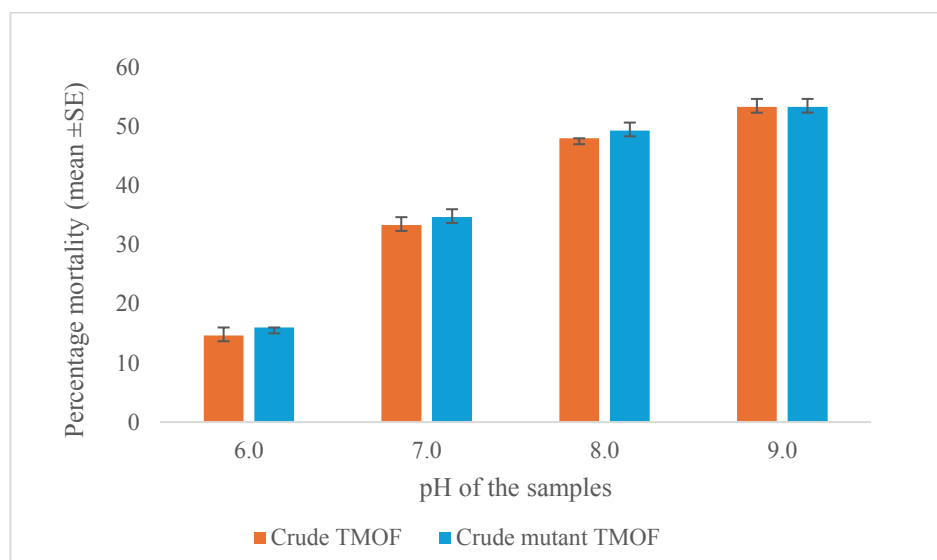


Fig. 1.5.18 Stability of crude TMOF/mutant TMOF in different pH

1.5.7 Effect of pH of exposure medium on toxicity of recombinant TMOF/ mutant TMOF

The toxicity of crude samples was checked by treating larvae in different buffer at pH 6.0, 7.0, 8.0 and 9.0 at LC_{50} (48 hours) concentrations. The results are shown in figure 1.5.19. Toxicity of crude TMOF was the highest in pH 9.0 (53.33 ± 1.33 %), followed by pH 8.0 (49.33 ± 1.33 %), pH 7.0 (37.33 ± 1.33 %) and the least in pH 6.0 (21.33 ± 1.33 %). Similar pattern observed in crude mutant TMOF also with the highest

mortality in pH 9.0 (53.33 ± 1.33 %), followed by pH 8.0 (49.33 ± 1.33 %), pH 7.0 (33.33 ± 1.33 %) and pH 6.0 (21 ± 33 %).

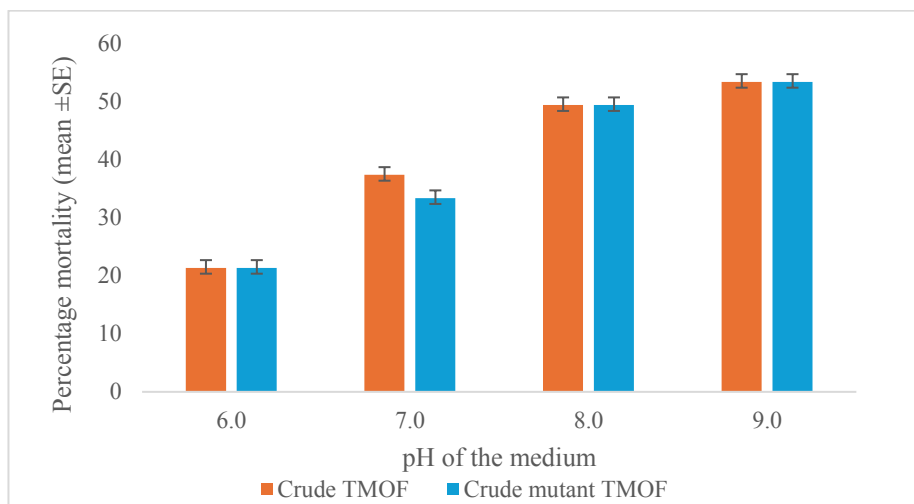


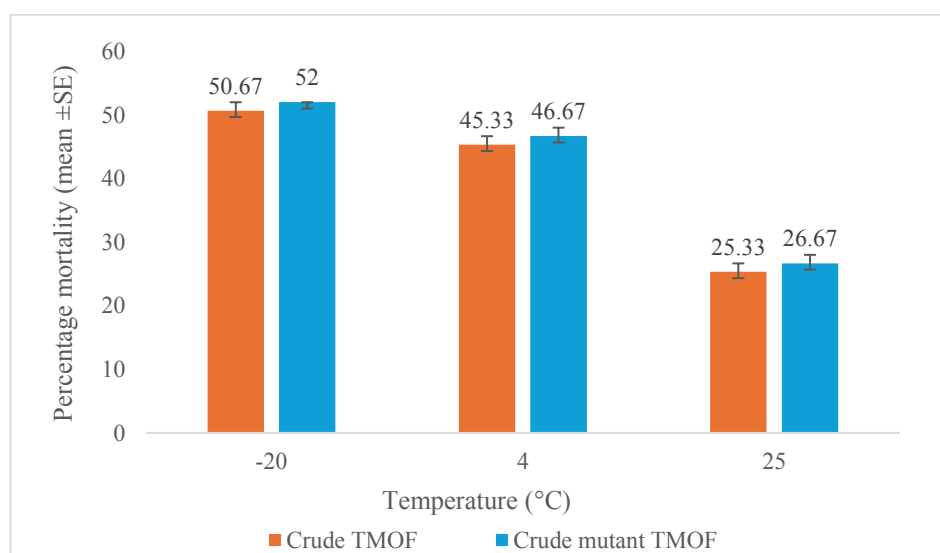
Fig. 1.5.19 Toxicity of crude TMOF/mutant TMOF in medium with different pH

1.5.8 Effect of storage temperature on stability of recombinant TMOF/mutant TMOF when stored as solution

Stability of crude TMOF and mutant TMOF were studied in different temperature conditions. For this, *A. albopictus* larvae were treated with LC₅₀ (48 hours) concentration of crude TMOF/mutant TMOF after storing at -20°C, 4°C and 25°C for one week. Observations were made after 48 hours and is shown in table 1.5.7 and figure 1.5.20. Toxicity of crude TMOF was unchanged for frozen sample (50.67 ± 1.33 %) and reduced for that kept at 4°C (45.33 ± 1.33 %) and the least at 25°C (25.33 ± 1.33 %). For crude mutant TMOF, highest mortality was for sample at -20°C (52.00 ± 0.00 %), followed by 4°C (46.67 ± 1.33 %) and 25°C (26.67 ± 1.33 %). Larvicidal activity was more stable when crude TMOF and mutant TMOF kept at lower temperatures (-20°C, 4°C).

Table 1.5.7 Stability of crude TMOF/mutant TMOF in different temperatures

Temperature	Percentage mortality (mean $\pm$ SE)	
	Crude TMOF	Crude mutant TMOF
-20°C	50.67 $\pm$ 1.33	52.00 $\pm$ 0.00
4°C	45.33 $\pm$ 1.33	46.67 $\pm$ 1.33
25°C	25.33 $\pm$ 1.33	26.67 $\pm$ 1.33

**Fig. 1.5.20** Effect of temperature on stability of crude TMOF/mutant TMOF when stored as solution

1.5.9 Effect of storage temperature on stability of dried recombinant TMOF/mutant TMOF

Stability of the dried crude TMOF at different temperatures was tested by keeping at different temperatures (-20°C, 4°C and 25°C) for one week followed by exposing *A. albopictus* larvae to LC₅₀ (48 hours) concentrations. The highest mortality was observed in sample kept at -20°C (34.67 $\pm$ 1.33%) followed by 4°C (29.33 $\pm$ 1.33%) and 25°C

($18.67 \pm 1.33\%$). The toxicity of crude mutant TMOF powder was the highest when kept at -20°C followed by 4°C and 25°C with percentage mortality 37.33 ± 1.33 , 32.00 ± 0.00 and 18.67 ± 1.33 respectively. The results are shown in table 1.5.8 and figure 1.5.21.

Table 1.5.8 Stability of dried crude TMOF/mutant TMOF in different temperatures

Temperature	Percentage mortality (mean $\pm$ SE)	
	Crude TMOF	Crude mutant TMOF
-20°C	34.67 ± 1.33	37.33 ± 1.33
4°C	29.33 ± 1.33	32.00 ± 0.00
25°C	18.67 ± 1.33	18.67 ± 1.33

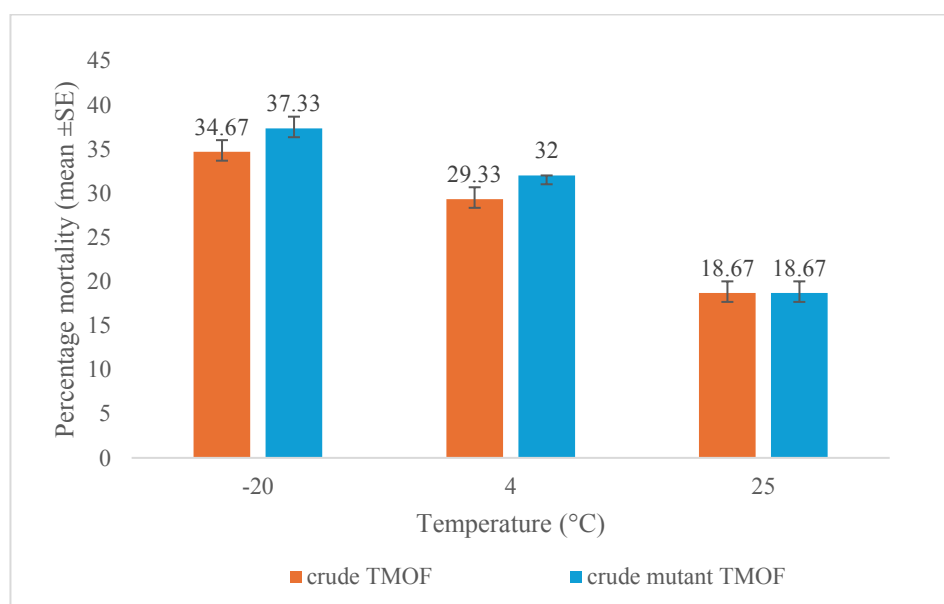


Fig. 1.5.21 Stability of dried crude TMOF/mutant TMOF

1.5.10 Effect of recombinant TMOF/mutant TMOF on *Culex quinquefasciatus* larvae

The larvicidal activity of crude TMOF and mutant TMOF was studied in *Culex quinquefasciatus* larvae. When *C. quinquefasciatus* larvae were treated with crude recombinant TMOF/mutant TMOF, the LC₅₀ (48 hours) was found to be 285.10 ± 6.16 and was 290.17 ± 1.92 $\mu\text{g/mL}$ respectively. Crude TMOF/mutant TMOF concentration has a direct effect on mortality of mosquito larvae. For crude TMOF, 216.9 $\mu\text{g/mL}$ protein concentration gave a mortality of 34.67 ± 1.33 % and the mortality became 86.67 ± 1.33 % at 313.3 $\mu\text{g/mL}$ crude TMOF concentration. In case of crude mutant TMOF, 216.0 $\mu\text{g/mL}$ gave a mortality of 36.00 ± 0.00 % and was increased to 92.00 ± 2.33 % at a crude mutant TMOF concentration 306.0 $\mu\text{g/mL}$. Results of toxicity analysis using crude TMOF is shown in table 1.5.9 and figure 1.5.22 and that of crude mutant TMOF in table 1.5.10 and figure 1.5.23.

Table 1.5.9 Toxicity of crude TMOF to *Culex quinquefasciatus* larvae

Protein concentration of crude TMOF ($\mu\text{g/mL}$)	Percentage mortality (mean $\pm$ SE) (after 48 hours)
216.9	34.67 ± 1.33
241.0	50.67 ± 1.33
265.1	58.67 ± 1.33
289.2	73.33 ± 1.33
313.3	86.67 ± 1.33

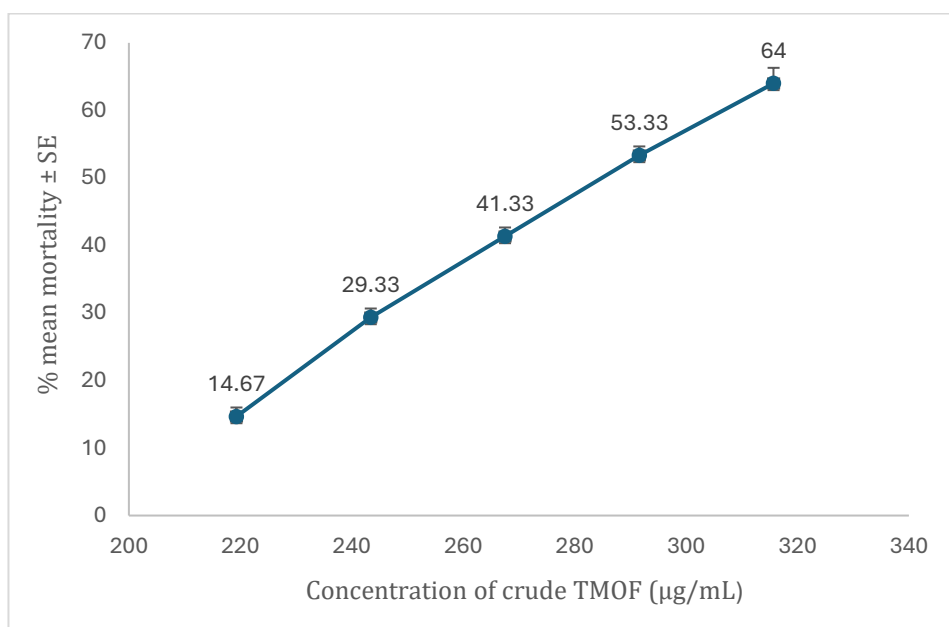


Fig. 1.5.22 Larvicidal activity of crude TMOF on *C. quinquefasciatus*

LC₅₀ (48 hours) of crude TMOF on *C. quinquefasciatus* larvae was found to be 285.10 ± 6.16 µg/mL.

Table 1.5.10 Toxicity of crude mutant TMOF to *Culex quinquefasciatus* larvae

Protein concentration of crude mutant TMOF (µg/mL)	Percentage mortality (mean± SE) (after 48 hours)
216.0	36.00 ± 0.00
238.5	53.33 ± 1.33
261.0	62.67 ± 1.33
283.5	73.33 ± 1.33
306.0	92.00 ± 2.33

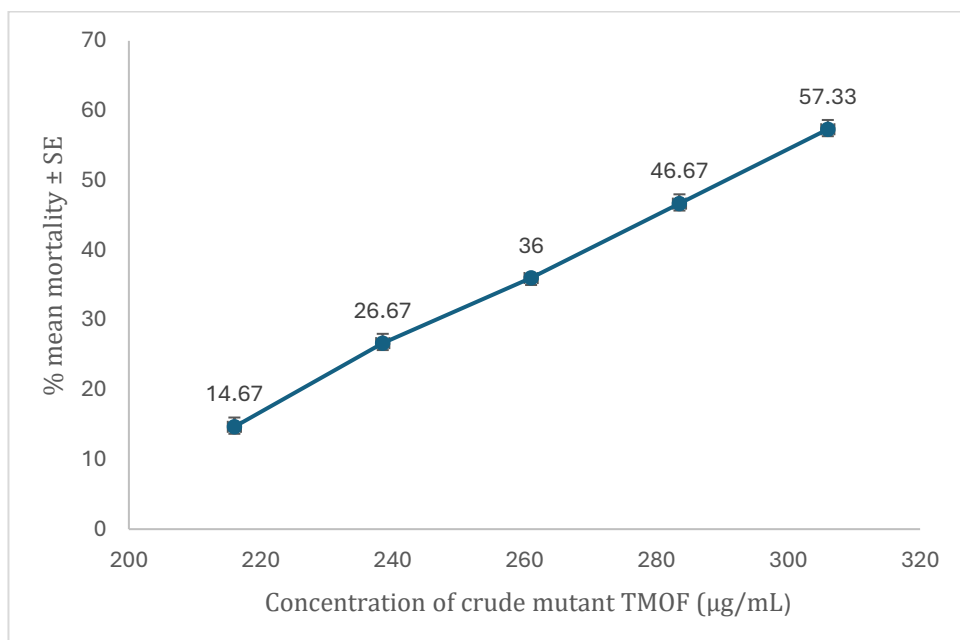


Fig. 1.5.23 Larvicidal activity of crude mutant TMOF on *C. quinquefasciatus*

LC₅₀ (48 hours) of crude TMOF on *C. quinquefasciatus* larvae was found to be 290.33 ± 1.33 µg/mL.

1.5.11 Non-target toxicity of recombinant TMOF/mutant TMOF

Non-target toxicity of crude TMOF and mutant TMOF was tested on *Drosophila melanogaster*, *Luprops tristis* and *Aplocheilus lineatus*. The LC₅₀ (48 hours) for *A. albopictus* was the concentration tested. There was no significant difference noted in the treated organisms compared to control.

In *D. melanogaster*, treated and untreated larvae emerged simultaneously, no death or anomaly noted in larvae, pupae and adult. The adults emerged from treated and untreated larvae were similar in appearance and in general behaviour.

In *L. tristis*, leaves coated with crude TMOF/mutant TMOF were used for treatment. Adult *L. tristis* fed the leaves, but there were no observable changes in behaviour of treated adults compared to control. No mortality was observed in the treated adults even after three weeks of treatment.

Aplocheilus lineatus was exposed to crude TMOF/mutant TMOF three times in the first week only. There were no behavioural changes compared to control and no mortality observed.

1.5.12 Comparison of mosquito larvicidal efficacy of TMOF and mutant TMOF

Crude TMOF as well as crude mutant TMOF shows a mosquito larvicidal effect on *A. albopictus* larvae. From the toxicity experiments on second instar *A. albopictus* larvae, the LC₅₀ (48 hours) for crude TMOF was 242.10 ± 6.04 µg/mL and that of crude mutant TMOF was 236.54 ± 6.49 µg/mL. The efficacy of wild type and mutant TMOF was compared statistically using independent sample t test (table 1.5.11). Mutant TMOF has a slight increase in toxicity (1.02 times) compared to the wild type and the increase was significant ($p < 0.05$). In case of affinity purified fractions, LC₅₀ (48 hours) for affinity purified recombinant TMOF to *A. albopictus* larvae was found to be 56.89 ± 1.42 and for mutant TMOF was 50.93 ± 1.50 nM respectively. The purified mutant TMOF is 1.12 times more efficient than the purified TMOF ($p < 0.05$). The sole difference between the two peptides is the presence of serine instead of proline at fifth position in TMOF mutant peptide. Table 1.5.11 list the differences between efficacy of recombinant TMOF with that of recombinant mutant TMOF.

Table 1.5.11 Comparison of efficacy of TMOF with mutant TMOF

Sample	LC ₅₀ (48 hours) (µg/mL)	p value
Crude TMOF	242.10± 6.04	<0.05
Crude mutant TMOF	236. 54 ± 6.49	
Affinity purified TMOF	2.13 ± 0.02	<0.05
Affinity purified mutant TMOF	1.91 ± 0.02	

1.5.13 Effect of crude TMOF/mutant TMOF on gut bacterial composition of *Aedes albopictus* larvae

Metagenomic analysis using 16S rRNA gene sequencing was done to check alteration in microbial population on exposure to recombinant TMOF/mutant TMOF treatment. Second instar *A. albopictus* larvae were treated with LC₁₀ (48 hours) concentrations of crude TMOF and crude mutant TMOF and the gut bacterial composition compared with that of control. The analysis gave insights into the changes in diversity as well as relative abundance of bacterial population in the larval gut.

1.5.13.1 Gut bacterial diversity measurement – Rarefaction curve

The rarefaction curve reflects the overall bacterial diversity measured in the larvae. A total of 1717 OTUs were described in untreated control. In crude TMOF treated larvae, only 950 OTUs were obtained and shows a 1.8-fold decrease in gut bacterial population compared to control (crude from untransformed BL21 cells). In the case of crude mutant TMOF treated larvae, 1117 OTUs were obtained and shows 1.54-fold decrease in gut bacterial population compared to the control. The figure 1.5.24 represents rarefaction curve of control and crude TMOF, and figure 1.5.25 control with crude mutant TMOF.

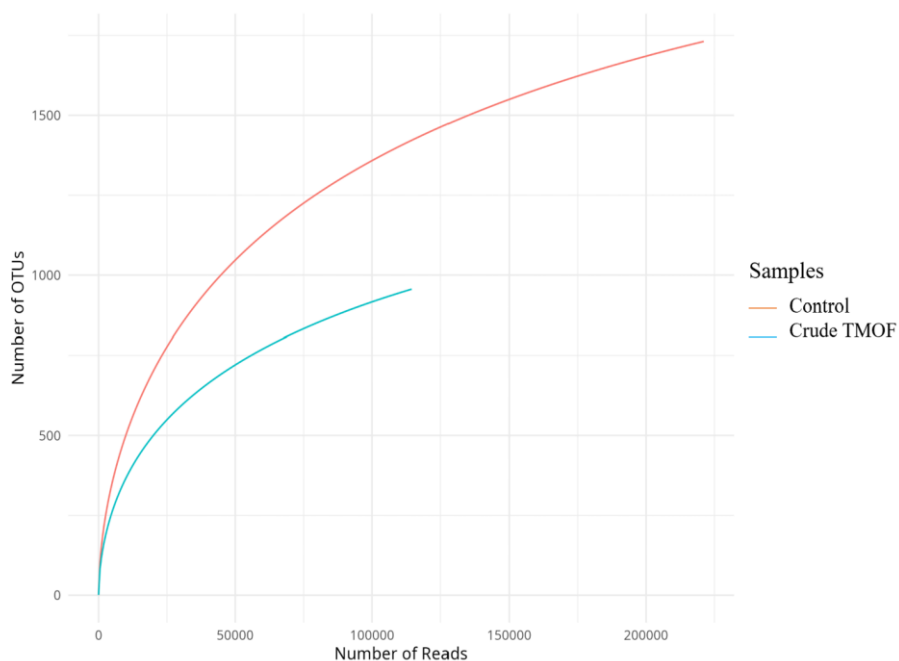


Fig. 1.5.24 Rarefaction curve- control Vs crude TMOF treated larvae

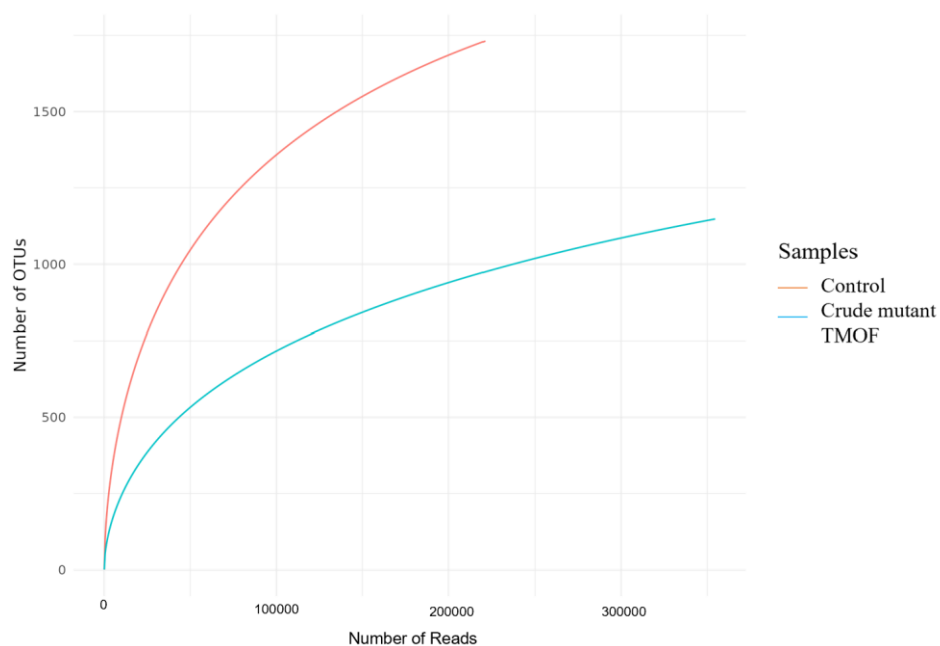


Fig. 1.5.25 Rarefaction curve- control Vs crude mutant TMOF treated

1.5.13.2 Alpha diversity indices

Boxplots were plotted to analyse the difference in alpha diversity indices between TMOF and mutant TMOF treated larvae. Indices Chao1 and ACE represents species richness. Species richness and abundance are represented by Shannon, Simpson, InvSimpson and Fisher indices. The differences in alpha diversity indices of gut bacteria in control and that of TMOF and mutant TMOF treated larvae are shown in figure 1.5.26 and 1.5.27, respectively. These indices shows that the species diversity of gut bacteria is decreased in larvae treated with crude TMOF and crude mutant TMOF compared to control.

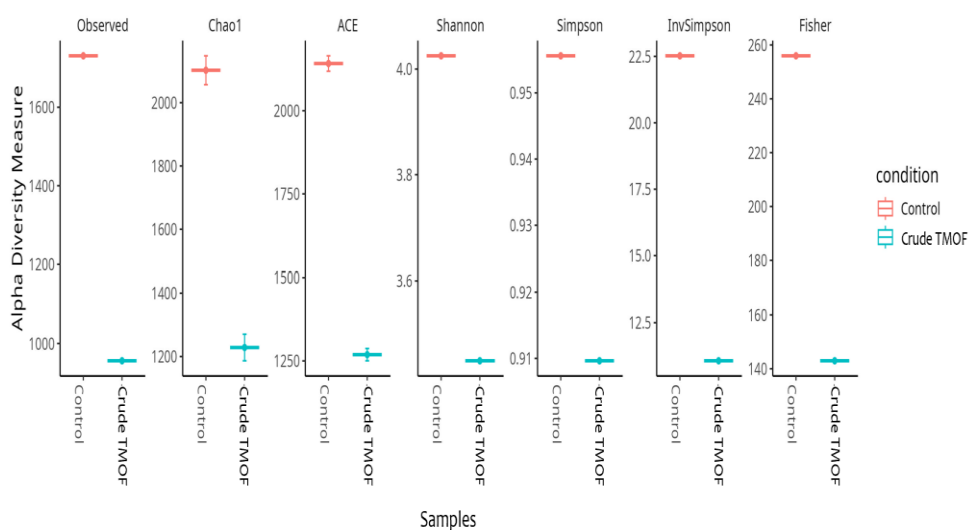


Fig. 1.5.26 Alpha diversity measurements of control Vs crude TMOF treated larvae

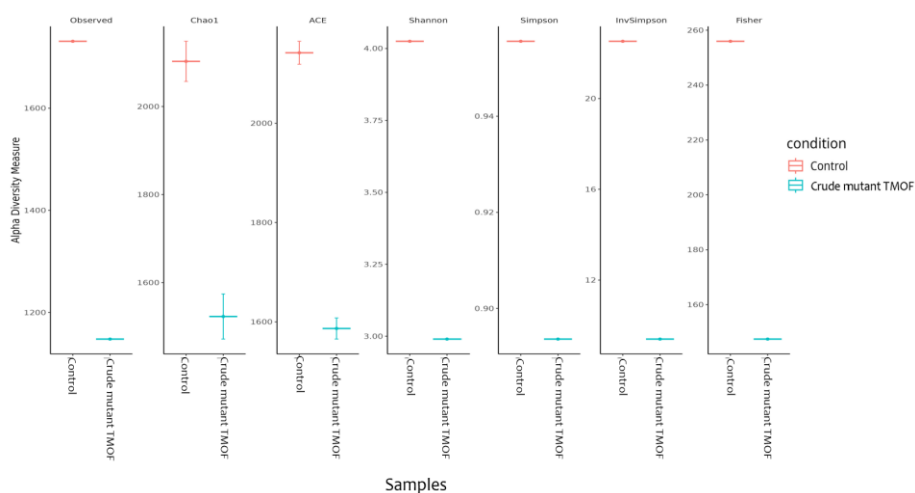


Fig. 1.5.27 Alpha diversity measurements for control Vs crude mutant TMOF treated larvae

1.5.13.3 Beta diversity analysis

Beta diversity represents the explicit comparison of microbial communities based on their composition. Beta-diversity metrics thus assess the differences between microbial communities. To compare microbial communities between every pair of community samples, a square matrix of "distance" or dissimilarity was calculated to reflect the dissimilarity between certain samples. Beta diversity measurements between control and crude TMOF is shown in figure 1.5.28 and that of control and crude mutant TMOF in figure 1.5.29. The PCoA plot shows decrease in bacterial communities in TMOF/ mutant TMOF treated sample compared to control.

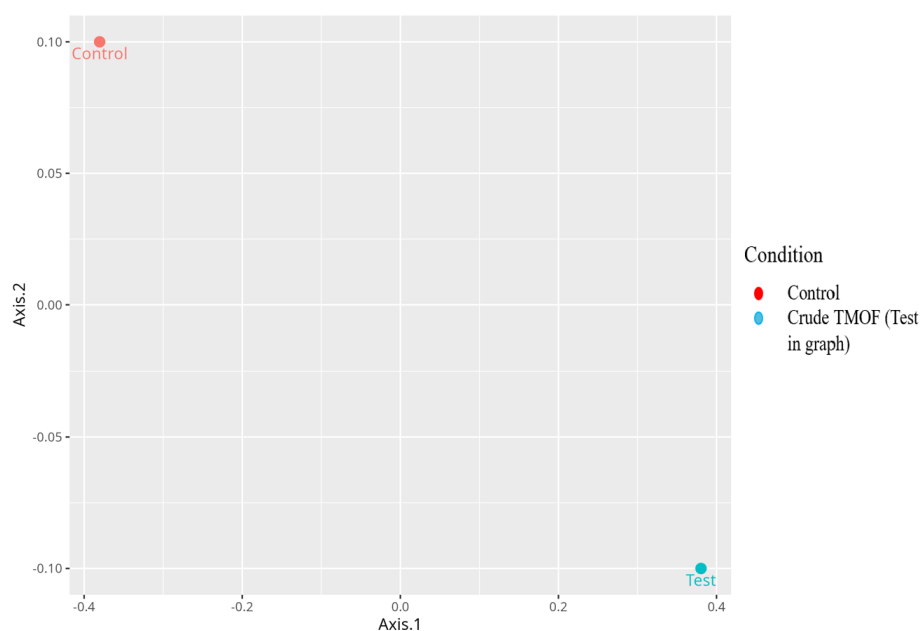


Fig. 1.5.28 Beta diversity measurements of control Vs crude TMOF treated larvae

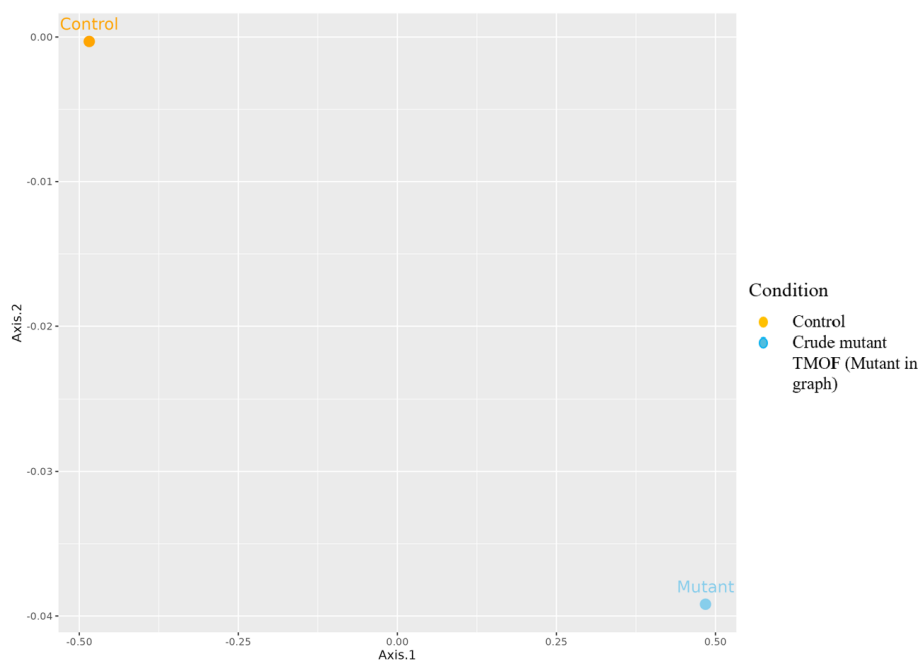


Fig. 1.5.29 Beta diversity measurements of control Vs crude mutant TMOF treated larvae

1.5.13.4 Abundance

Abundance describes the number of individuals in a specific area. Species abundance in *A. albopictus* larvae treated with recombinant TMOF/mutant TMOF were checked using metagenomic analysis with 16S rRNA gene. Relative abundance of gut bacterial population between the larvae treated with crude TMOF/mutant TMOF with control was analysed. Analysis was done at phylum, genus and species level. It had shown differences in proportion and distribution of various taxa. A total of 1717 OTUs were obtained in control whereas in crude mutant TMOF, it is only 1117 and in crude TMOF treated, it is only 950. Distribution of taxonomic levels also varies like number of phyla or genera present and number of individuals in a particular taxon. The changes may be the contributed by shortage of nutrient availability due to inhibition of trypsin synthesis and raising the competition levels among existing species. The relative abundance of bacterial populations at different taxa is described below.

1.5.13.4.1 Relative abundance at phylum level

A total of 31 phyla were identified in control and mutant TMOF treated sample while in TMOF treated sample, only 22 phyla could be identified. The most abundant phyla in control and crude TMOF treated sample is Actinomycetota with 34% and 57%, respectively. In crude mutant TMOF treated sample, Bacillota is the topmost with 44% abundance. In all three samples the second most abundant phylum is Pseudomonadota with 32%, 20% and 43% in control, TMOF treated and mutant TMOF, respectively. The top four phyla in control, TMOF and mutant TMOF treated larvae are represented in table 1.5.12. Relative abundance between the samples is shown in figure 1.5.30 and 1.5.31.

Table 1.5.12 Phylum level relative abundance – crude control Vs crude TMOF Vs Crude mutant TMOF

Control	Crude TMOF	Crude mutant TMOF
Actinomycetota (34%)	Actinomycetota (57%)	Bacillota (44%)
Pseudomonadota (32%)	Pseudomonadota (20%)	Pseudomonadota (43%)
Bacillota (23%)	Bacillota (16%)	Bacteroidota (11%)
Mycoplasmata, Myxococota, Planctomycetota (1%)	Candidatus Saccharibacteria (4%)	Actinomycetota (0.04%)

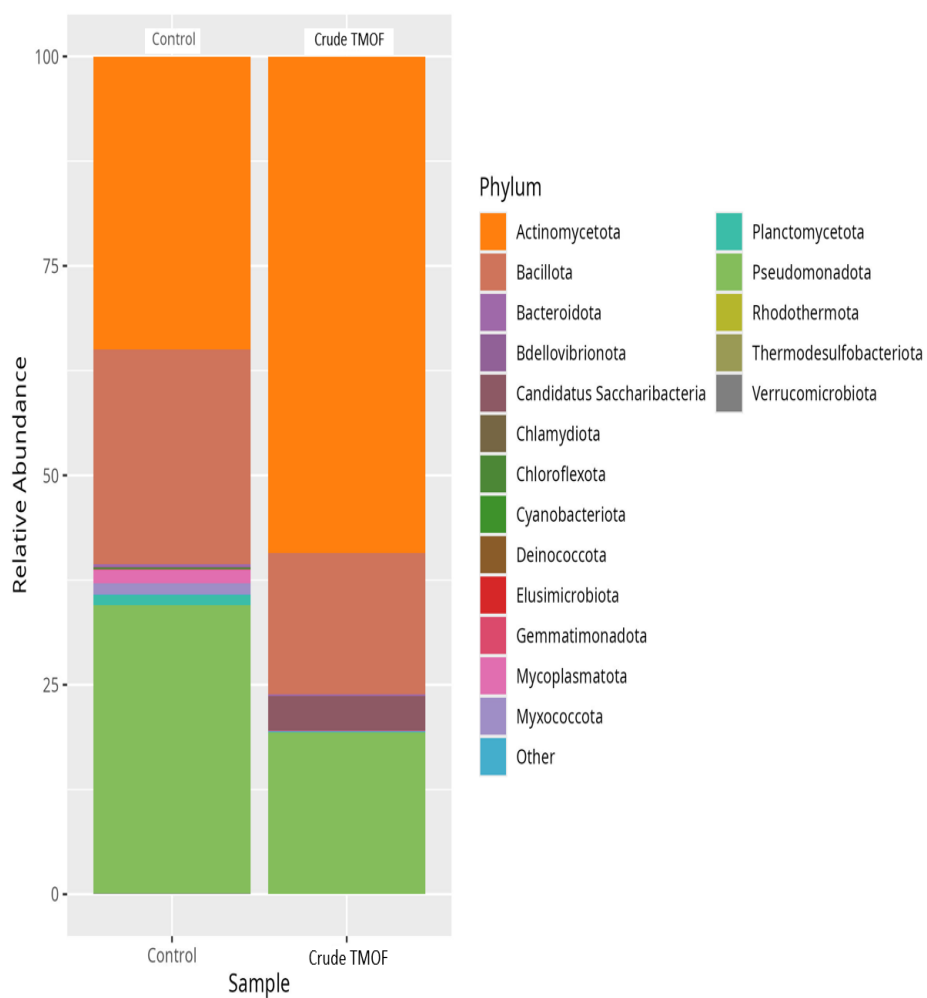


Fig. 1.5.30 Phylum level relative abundance – control Vs crude TMOF

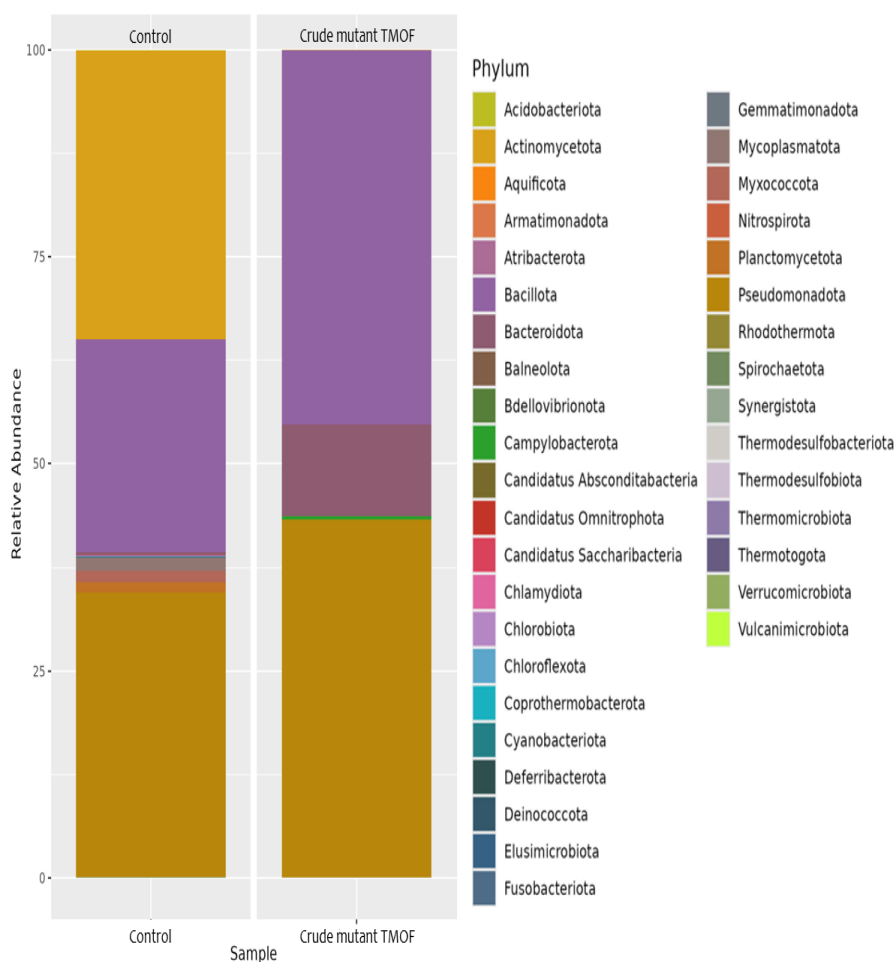


Fig. 1.5.31 Phylum level relative abundance – control Vs crude mutant TMOF

1.5.13.4.2 Relative abundance at genus level

At genus level, 795 genera were identified in control sample, 466 in crude TMOF treated and 548 in crude mutant TMOF treated. Top four genera in either group are listed in table 1.5.13 and in figures in 1.5.32 and 1.5.33. *Streptococcus* is the most abundant in control, while *Microbacterium* and *Cellulomonas* shares highest abundance in TMOF

treated sample and in crude TMOF treated, *Clostridium* shows the highest abundance (26%).

Table 1.5.13 Genus level relative abundance- control Vs crude TMOF Vs crude mutant TMOF

Control	Crude TMOF	Crude mutant TMOF
<i>Streptococcus</i> (9%)	<i>Microbacterium</i> , <i>Cellulomonas</i> (7%)	<i>Clostridium</i> (26%)
<i>Cellulosimicrobium</i> (7%)	<i>Lysinibacillus</i> (6%)	<i>Morganella</i> (20%)
<i>Kaistia</i> (6%)	<i>Rathayibacter</i> (4%)	<i>Lactococcus</i> (12%)
<i>Pseudoxanthomonas</i> , <i>Sediminicoccus</i> (5%)	<i>Candidatus</i> <i>Sachharimonas</i> , <i>Kaistia</i> (3%)	<i>Paraclostridium</i> (4%)

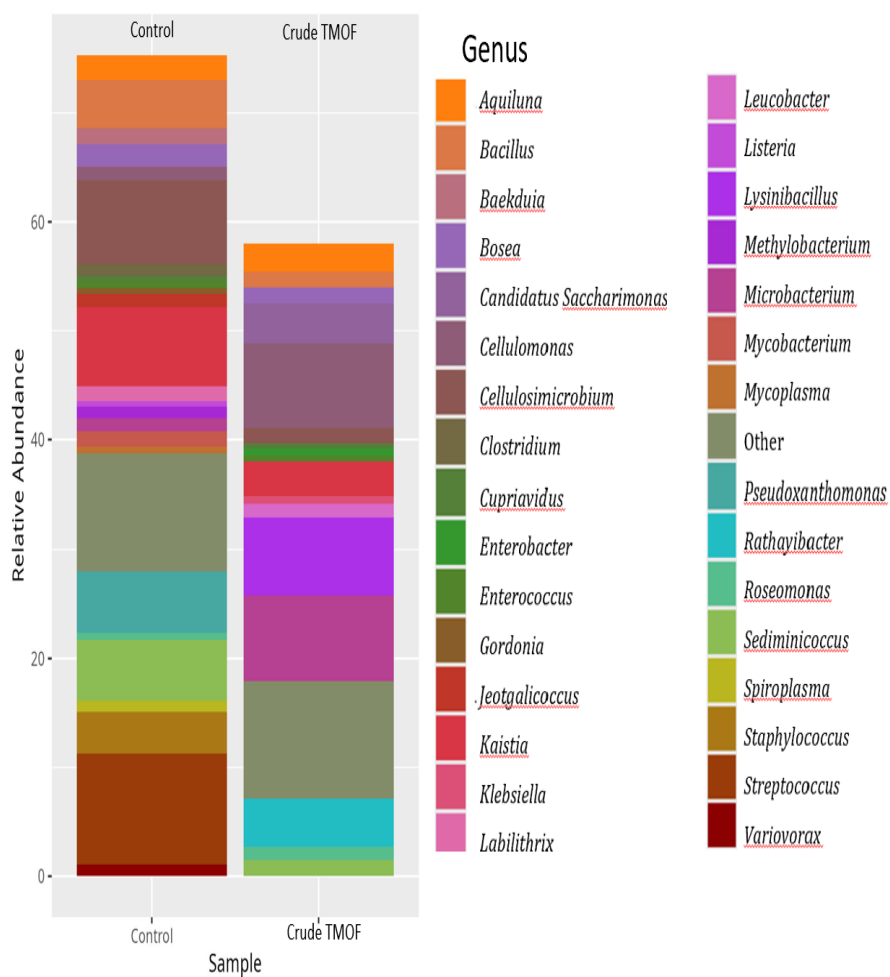


Fig. 1.5.32 Genus level relative abundance – control Vs crude TMOF

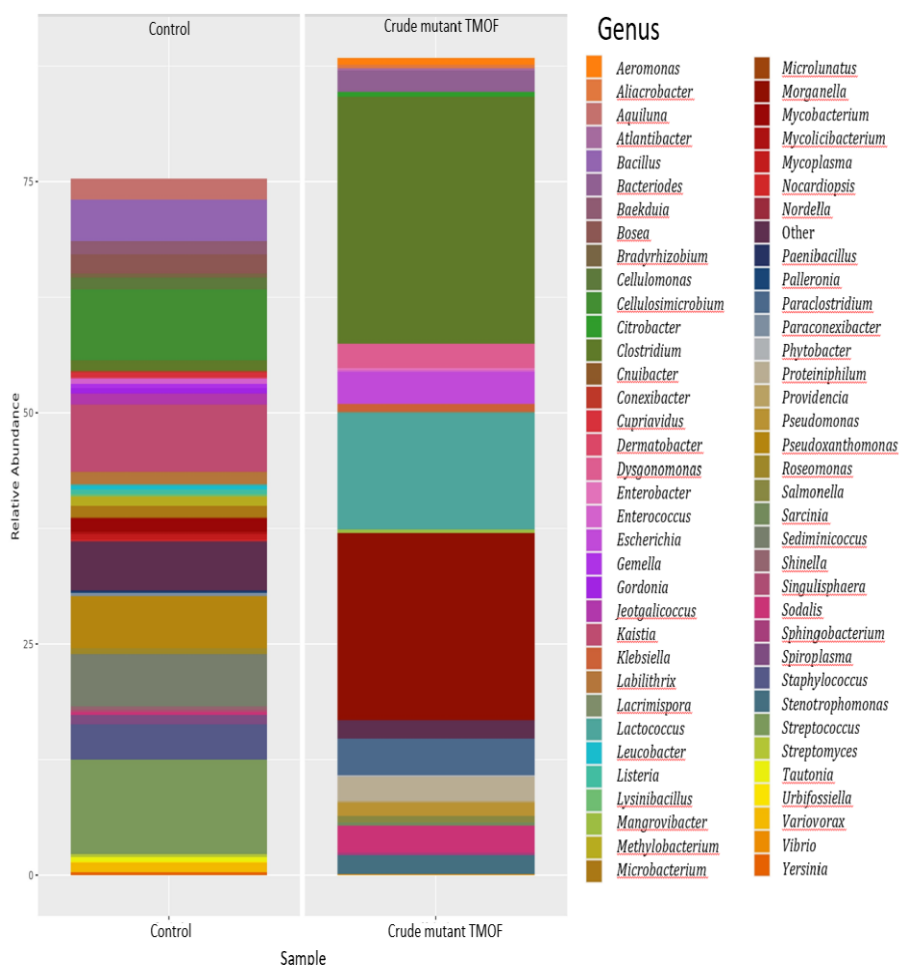


Fig 1.5.33 Genus level relative abundance – control Vs crude mutant TMOF

1.5.13.4.3 Relative abundance at species level

A total of 1349 species were identified in control sample, 757 in crude TMOF treated sample and 864 in crude mutant TMOF treated. The most abundant four species in each sample is listed in table 1.5.14 and figures representing relative abundance in 1.5.34 and 1.5.35. *Kaistia* species representing 100% of *Kaistia* genus is the topmost in control sample. In

TMOF treated, *Cellulomonas gilvus* representing 100% of *Cellulomonas* genus tops first. In crude mutant TMOF treated sample, *Morganella morganii* is the most abundant species representing 100% of *Morganella* genus.

Table 1.5.14 Species level relative abundance- control Vs crude TMOF Vs crude mutant TMOF

Control	Crude TMOF	Crude mutant TMOF
<i>Kaistia</i> species (6%)	<i>Cellulomonas gilvus</i> (7%)	<i>Morganella morganii</i> (20%)
<i>Listeria monocytogenes</i> (4%)	<i>Microbacterium sufflavum</i> (6%)	<i>Paraclostridium bifermentans</i> (4%)
<i>Staphylococcus aureus</i> (3%), <i>Pseudoxanthomonas suwonensis</i> (3%), <i>Bacillus velezensis</i> (3%)	<i>Candidatus Saccharimonas aalborgensis</i> (3%), <i>Kaistia</i> species (3%)	<i>Eschericia coli</i> , <i>Sodalis ligni</i> , <i>Proteiniphilum saccharomentans</i> (3%)
<i>Bosea beijingensis</i> (2%), <i>Aquilina borgonia</i> (2%)	<i>Aquilina borgonia</i> (2%)	<i>Clostridium novyi</i> , <i>C. baratii</i> , <i>Dysgonomonas</i> species (2%)

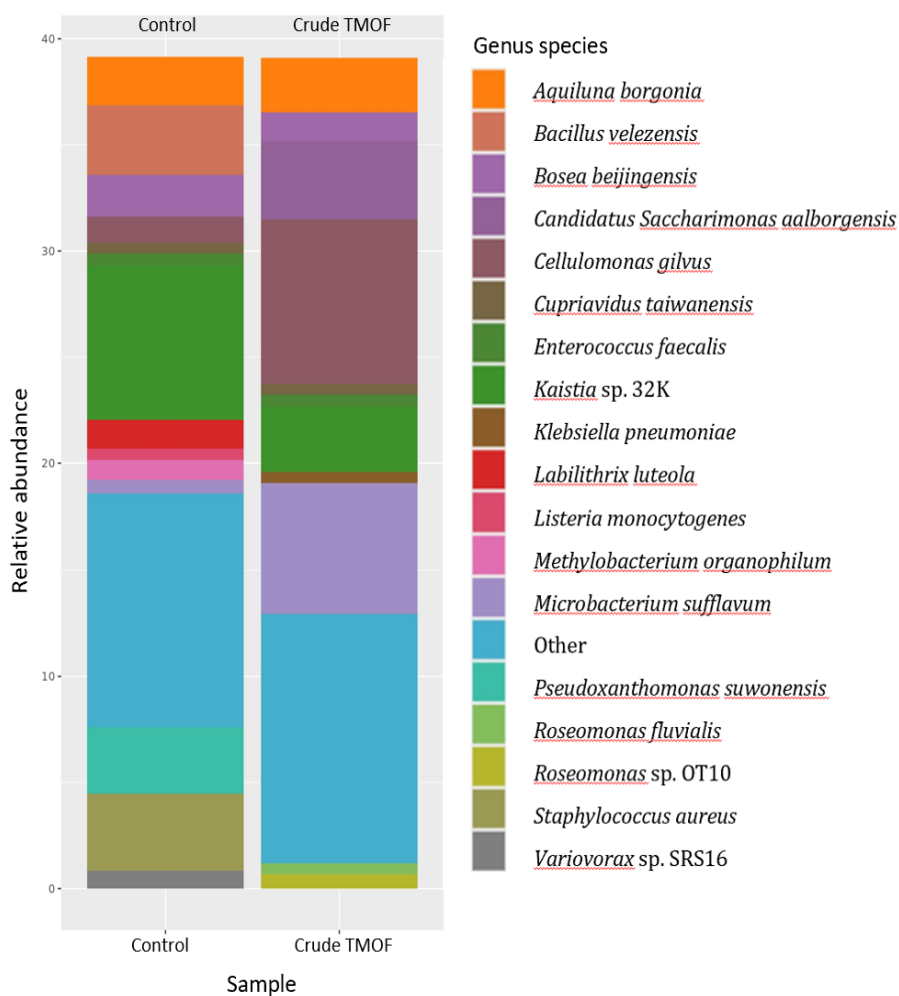


Fig. 1.5.34 Species level relative abundance – control Vs crude TMOF

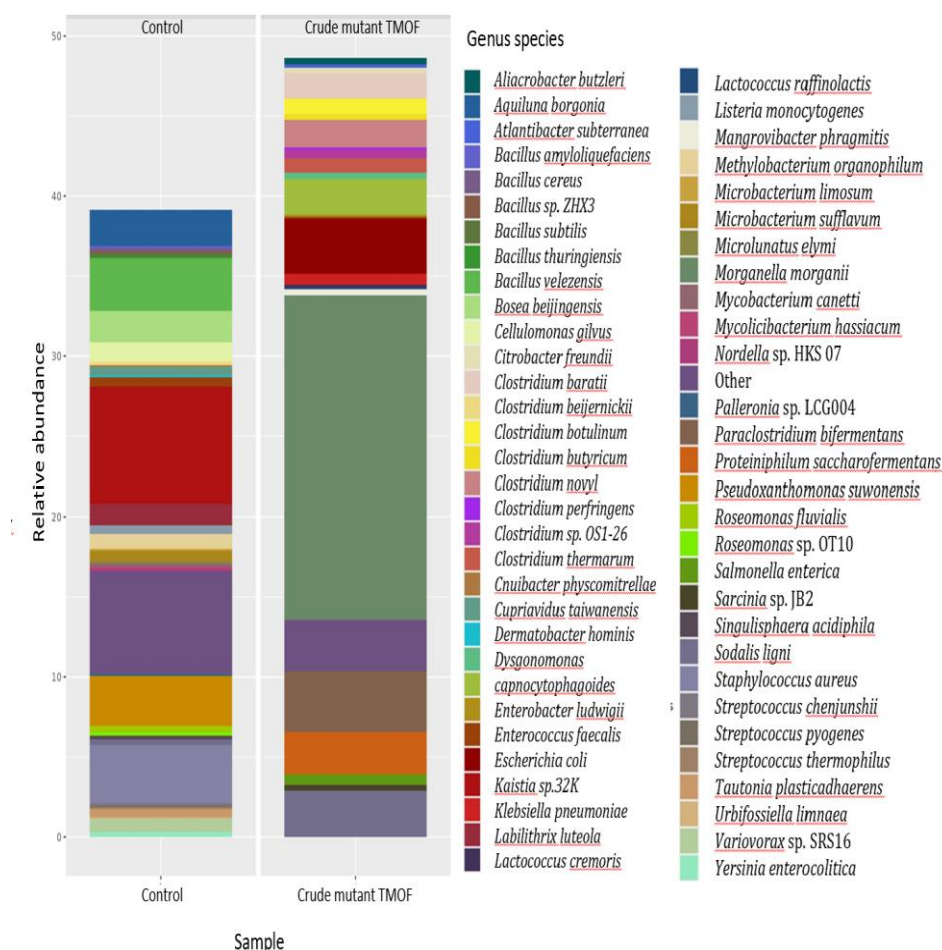


Fig. 1.5.35 Species level relative abundance – control Vs crude mutant TMOF

1.5.13.5 Species annotations using Krona maps

Species annotations of gut bacteria from larvae treated with control, crude TMOF and crude mutant TMOF are represented in Krona maps for a comparative analysis of each. The arrangement of circles from inner to outer layers represents different taxonomic levels from higher to lower categories, and the area of each sector indicates the relative

1.6 DISCUSSION

The trypsin modulating oostatic factor, a decapeptide interfering with trypsin biosynthesis in the midgut of *Aedes aegypti*, has been isolated. It is also known that the first four amino acids of the peptide can cause 95% larval mortality of the full length decapeptide. In the first part of the chapter 1, the potential of TMOF tetrapeptide conjugated with BSA against *Aedes albopictus* and *Culex quinquefasciatus* was studied. The possibility for cloning and expressing the TMOF gene in bacteria was studied in thereafter, and its toxicity against *A. albopictus* was analysed. An active mutant of the TMOF peptide also identified in this study. The toxicity of TMOF/mutant TMOF expressed in bacteria to *A. albopictus* larvae and its effect on the gut bacterial profile of larvae were also examined. This study reports that the mutant peptide is more toxic than the wild type and will be useful in mosquito control.

1.6.1 Conjugation studies

Earlier studies with TMOF peptide and its analogues had shown that the first four amino acids of the decapeptide (YDPA) has 95% activity of the full-length decapeptide (Borovsky & Meola, 2004). It is also reported that the addition of arginine at the C- terminus of YDPA tetrapeptide is active (Borovsky & Meola, 2004). A TMOF analogue with first five amino acids of the decapeptide (YDPAP) had lost its biological activity when lysine or arginine is added at their C-terminus (Borovsky & Meola, 2004). So, based on this study, we custom synthesised TMOF pentapeptide with lysine residue at the N-terminus of YDPA tetrapeptide (KYDPA). The lysine residue act as a trypsin

cleavage site such that to release active YDPA inside the mosquito gut. Trypsin will cleave at the C-terminus of lysine or arginine residue. Trypsin cleavage occurs only when lysine or arginine is not followed by proline, and the rate of cleavage will be lower if lysine or arginine is attached to an acidic amino acid (Simpson, 2006). In the synthesised pentapeptide (KYDPA), lysine was at the N-terminus of the peptide, attached to a neutral amino acid, tyrosine, so that the trypsin cleavage will not be affected. But no toxicity was observed on treatment with this pentapeptide. It may be due to, lack of cleavage of lysine at the N-terminus, as it is the terminal residue. Thus, we conjugated TMOF pentapeptide (KYDPA) to BSA by glutaraldehyde coupling. This led to regaining the toxicity of peptide due to cleavage of lysine by trypsin in the larval gut. It was toxic to *Aedes albopictus* and *Culex quinquefasciatus* larvae but not to *Drosophila melanogaster* larvae confirming the specificity to mosquito larvae. The LC₅₀ (48 hours) for the peptide for *A. albopictus* larvae was found to be 2.12 ± 0.02 µg/mL and that of *C. quinquefasciatus* larvae was found to be 3.05 ± 0.02 µg/mL. The TMOF decapeptide has been conjugated to poly-ethylene glycol and developed enhanced toxicity to *Aedes aegypti* larvae (Jeffers et al., 2011). This preliminary experiment prompted us to clone and express the TMOF gene in bacteria so as to produce large quantities of the peptide economically.

1.6.2 Cloning TMOF/ mutant TMOF gene

In this study, the Trypsin Modulating Oostatic Factor (TMOF) gene was cloned and expressed in *Escherichia coli* and studied its impact on *Aedes albopictus* larvae and the gut bacterial profile of the larvae. The

oligonucleotide coding the TMOF gene was synthesised and annealed with its complementary strand and used for cloning. The pFN29AHis6Halo flexi vector was chosen for cloning and expression of TMOF gene in bacteria. This vector is a bacterial expression vector that satisfies requirements for cloning and expression in bacteria (Gaastra & Bergmans, 1987; Walker et al., 2008).

The pFN29AHis6Halo flexi vector makes use of two rare cutting enzymes, SgfI and PmeI. The AsiSI is an isoschizomer of SgfI that recognises the GCGGTCGC sequence and cuts between the T and C sites, making staggered ends the same as the SgfI. Their efficacy is validated, and hence AsiSI is used in this work. PmeI recognises GTTTAAAC sequence and cuts between T and A, making blunt ends.

The oligonucleotide containing the TMOF gene construct consists of the TMOF coding gene, restriction enzyme sites, trypsin cleavage site, stop codon and an additional base to make the gene in frame. In the gene construct, AsiSI half site is added at the 5' end and PmeI site at the 3' end making the construct with a 5' staggered end and 3' blunt end. An additional base 'C' was added just after the 5' restriction site to make the TMOF gene in frame. The TMOF coding sequence was codon optimised for expression in *E. coli*. A trypsin cleavage site which is a lysine residue (AAA) was added at the 5' end (N-terminus of the peptide) whereas the 3' (C-terminus of the peptide) ends in a stop codon (TAA). The restriction enzyme sites for cloning were added as half-sites. This is because conventional cloning methods, including restriction digestion followed by ligation will not be suitable for inserts below 150 bp and in such cases inserts can be directly synthesised (Tao et al.,

2020). Mutant TMOF gene constructs were prepared in similar way with nucleotide changes in TMOF coding sequence. Site directed mutagenesis had already been carried out to generate mutant TMOF (Borovsky & Meola, 2004). To explore the maximum output of all combinations, we went for random mutagenesis. Hence, for cloning mutant gene, oligos containing random nucleotides were synthesised, cloned in the same vector and screened by bioassay using mosquito larvae for identifying active mutant gene.

The custom synthesised oligos were annealed in a thermocycler, and the differences in single-stranded and double-stranded oligos were checked in agarose gel and using ethidium bromide (EtBr) staining. In 2% agarose gel, the double-stranded oligos appeared with more intense band and occupied higher position compared to the single-stranded oligos. In the case of EtBr staining, fluorescence appeared in both samples, but with varying intensity (more intense in double-stranded oligo). So, based on these, annealing of single-stranded oligos was confirmed and used directly for ligation with the double digested vector using T4 DNA ligase. The transformants were grown in LB medium and screened.

The cloning using pFN29AHis6Halo flexi vector offers a system of suicidal cloning as described in some gram negative bacteria by Quandt & Hynes, 1993. The vector possesses a barnase gene which encodes a toxin that kill *E. coli* cells. So, when *E. coli* transformed with pFN29AHis6Halo vector, the bacteria cannot survive due to the expression of barnase toxin. This flexi vector system works on the principle that, during cloning using AsiSI and PmeI, the barnase gene is removed, and during ligation, the insert is added at the place of barnase

gene. So, the transformants grown will have the insert gene in place of barnase, confirming the successful cloning of insert.

Further screening was done by restriction digestion (Sambrook et al., 1989; Peterson, 1998), PCR and sequencing (Gubili & Borthakur, 1996). Restriction digestion with one of the enzymes used for cloning was done for both the original and recombinant vectors. The original vector with 368 bp barnase gene had a size of 4398 bp. The size was reduced to 4075 bp in the recombinant vector due to the replacement of barnase gene by the 45 bp long TMOF/mutant TMOF gene construct. The difference of 323 bp between the original and recombinant vector was distinguished from the agarose gel. Using PCR, the original vector with barnase gene and recombinant with TMOF/mutant TMOF were amplified by vector-specific primers. The original vector yields an amplicon of size 596 bp whereas in recombinant vector, the amplicon is 277 bp long. Here also, size difference was noted from the agarose gel. A final confirmation of cloning TMOF/mutant TMOF in the pFN29AHis6Halo vector was obtained by Sanger sequencing. The sequencing using vector-specific sequencing primers, specifically amplify regions, 142 bp upstream of AsiSI recognition site and 87 bp downstream the PmeI recognition site. In addition, sequencing confirmed the actual difference between TMOF and mutant TMOF sequence. It was confirmed that at the gene level, there was a single base change, that is, thymine instead of cytosine at the first position of the codon coding for proline residue in the TMOF gene. This is the result of transition (T/C) of pyrimidines in the gene sequence ligated to the vector. This led to the replacement of fifth proline in the TMOF peptide

to serine in mutant TMOF peptide at the fifth position. This change in amino acid sequence may lead to altered expression of mutant gene. Mutations can alter the protein expression of a gene depending upon their relative position (Robert & Pelletier, 2018). The presence of substitution mutation other than the third base of a codon within the coding sequence of a gene will lead to amino acid change which will result in the modification of a protein's activity. If the mutation does not change the nature of the amino acid, then translation rates or mRNA half-life may be affected or if the mutation causes a premature stop codon, production of a truncated protein product or a near-null phenotype may occur (Mendell & Dietz, 2001; Nicholson et al., 2010). So, the change in single amino acid can cause alteration in physical and functional aspects of the protein.

1.6.3 Expression of recombinant TMOF/ mutant TMOF

The vector pFN29AHis6Halo consists of T7 RNA polymerase promoter which can trigger in vivo expression in *E. coli* cells containing T7 RNA polymerase. The BL21 (DE3) strains of *E. coli* has T7 RNA polymerase under the control of lacUV₅ promoter which can be induced by Isopropyl-β-D-1 thiogalactopyransoside (IPTG) (Jeong et al., 2009; Du et al., 2021). In this work, the cloned TMOF/mutant TMOF gene was expressed in BL21 cells using IPTG as an inducer. The recombinant vector was used to transform BL21 competent cells. The transformants grown and expanded in LB medium with ampicillin. The growth of BL21 cells involves the exponential increase of cell biomass during uninduced condition and the production of proteins during induced condition (Du et al., 2021). The BL21 cells were grown until they

reached an OD₆₀₀ of 0.60 when, IPTG was added. Different concentrations of IPTG, temperature and duration of induction were used to optimise maximum expression of recombinant protein. The IPTG concentration used were 0.2, 0.5 and 1 mM. Temperature conditions used were 32°C, 37°C and 40°C and the post induction time used were 5 hours, 8 hours and 16 hours. Based on the SDS-PAGE analysis, the maximum expression was obtained at 37°C with 0.5 mM final IPTG concentration for 5 hours. The Jembrana trans-activator transcription protein (JTAT) was expressed in *E. coli* at 37°C for three hours using 0.2 – 0.6 mM IPTG (Margawati et al., 2017). A duration of induction of 3-5 hour with 0.1-1 mM IPTG concentration was used for the expression of IL-23p19 in *E. coli* (Gutiérrez-González et al., 2019). The expression of single chain variable fragment (ScFv) antibodies against epithelial cell adhesion molecule extracellular domain in BL21 (DE3) cells report maximum expression at 37°C for 7 hours at an IPTG concentration of 0.5 mM (Soulari et al., 2020).

The recombinant TMOF/mutant TMOF was expressed as a fusion protein with poly-histidine and Halo tag at the N-terminus as the vector contains sequences for coding these tags. So that, the size was not too small as it is expected for the decapeptide, but has 37.5 kDa in both TMOF and mutant TMOF. The bacterial cells expressing recombinant TMOF/ mutant TMOF were collected and lysed. The soluble and membrane fractions obtained were loaded onto SDS-PAGE and compared the protein profile. Also, *A. albopictus* larvae were treated with soluble and membrane fractions of the lysate. From this, it was confirmed that the soluble fraction contains the recombinant

TMOF/mutant TMOF. Recombinant biotinylated fusion proteins containing nerve growth factor and semaphorin 3A were purified from the soluble fraction of *E. coli* cell lysate (McCormick et al., 2014).

1.6.4 Purification of recombinant TMOF/ mutant TMOF

Purification of the protein can be achieved by chromatographic techniques which aid in high resolution purifications. The immobilized nickel affinity chromatography (IMAC) is an affinity chromatography with metal ion fixed to the adsorbing column. The technique is based on the interaction between transition metal ions of intermediate polarizability such as Cu^{2+} , Ni^{2+} , Zn^{2+} , and Co^{2+} which are particularly used since they interact only with the nitrogen, oxygen and sulphur in amino- and imino-groups (Porath et al., 1975). Thus, it is possible to capture and purify proteins having these functional groups, which are in fact present in histidine and cysteine residues. The poly-histidine tag has affinity to nickel ions and the recombinant protein having this tag can be purified in Ni^{2+} ion loaded in nitrilotriacetic acid-agarose resins (Porath & Olin, 1983; Bornhorst & Falke, 2000).

The recombinant TMOF/mutant TMOF has a 6x histidine tag at its N-terminus which allowing purification by IMAC. The soluble fraction of lysate was used for purifying recombinant TMOF/mutant TMOF by nickel affinity chromatography. The soluble fraction of the bacterial lysate expressing TMOF/mutant TMOF is referred as crude TMOF and crude mutant TMOF, respectively. Crude TMOF/mutant TMOF were incubated with nickel affinity resin for purification by batch method. Mild competing agents like glycine, histidine, or imidazole, or an

organic modifier is frequently used for the elution of proteins (Freitag & Horváth, 1996). After the removal of unbound proteins, elution was done using imidazole in Tris buffer saline, pH 8.0. The concentration of imidazole was optimised by eluting at 100 mM, 250 mM, 500 mM and 1 M concentrations. Elutes obtained were loaded onto SDS-PAGE and analysed. Maximum elution of the recombinant TMOF/mutant TMOF was obtained at 250 mM imidazole concentration. Purification of protease inhibitor from *Spatholobus parviflorous* was done by Ni-IMAC using 250 mM imidazole as eluent (Patinhara Parambil & Vadakkadath Meethal, 2022). The bead concentration and protein elution have a direct relationship, if bead concentration is above 4 mg/ ml, contaminating proteins also may adsorb into resin, which may not elute even after 2M imidazole wash. This can be reduced by lowering bead concentration and elution is possible with 250 mM imidazole (Nejadmoghaddam et al., 2011). Accordingly, proportion of bead and protein adjusted and recombinant proteins were purified at 250 mM imidazole concentration. Even after optimisations, a few contaminating proteins were present in the elute fraction. This suggest the possibility of presence of endogenous *E. coli* proteins having affinity for metal ions as reported by Bolanos-Garcia & Davies, 2006 co-purified after IMAC. The yield of purified TMOF was 3.12 µg per mg of bacterial pellet and the yield of mutant TMOF was 2.98 µg per mg of bacterial pellet.

1.6.5 Toxicity of recombinant TMOF/mutant TMOF to *Aedes albopictus* mosquito larvae

In this work, *A. albopictus* larvae were treated with crude and affinity purified recombinant TMOF /mutant TMOF. Different concentrations of crude and affinity purified TMOF/mutant TMOF were used for feeding experiments. Control samples also kept, derived from untransformed BL21 cells. Feeding experiments were done using 25 second instar *A. albopictus* larvae in 100 mL distilled water (WHO, 2005). There were no significant changes observed in control larvae during treatments, but in recombinant protein treated larvae, delayed development and mortality were observed. As the concentration of the recombinant TMOF/mutant TMOF increased, significant increase in larval mortality was observed within 48 hours. The lethal concentration values were calculated from probit analysis. The LC_{50} (48 hours) for crude TMOF was $242.10 \pm 6.04 \mu\text{g/mL}$ and for crude mutant TMOF was $236.54 \pm 6.49 \mu\text{g/mL}$. The level of expression of mutant TMOF was 1.05 times less compared to TMOF which may tend to lower toxicity than the actual toxicity. Thus, the actual LC_{50} (48 hours) after taking into account the lower level of expression will be $224.5 \mu\text{g/mL}$. Based on this, crude mutant TMOF is 1.08 times more efficient than crude TMOF ($p \text{ value} < 0.05$). Mutations have the potential to cause alteration in all stages of gene expression be it in regulatory sequences or in coding region (Robert & Pelletier, 2018). Mutational incorporation of alanine, serine, proline, lysine, cysteine or threonine at the second position enhanced the expression of Ig α (Bivona et al., 2010). The α mating factor secretion signal mutations affect the recombinant protein

expression in *Pichia pastoris* (Lin-Cereghino et al., 2013). For *Culex quinquefasciatus* larvae, an LC_{50} (48 hours) of 285.10 ± 6.16 and 290.17 ± 1.92 $\mu\text{g/mL}$, respectively, was obtained for crude TMOF and crude mutant TMOF. The lethal concentration obtained for *C. quinquefasciatus* is significantly higher than that of *A. albopictus*, indicating that the efficiency of recombinant TMOF/mutant TMOF varies with organism. There are reports indicating *Aedes* and *Anopheles* mosquitoes showing variations in susceptibility to pyrethroids (Pusawang et al., 2022).

The toxicity was also expressed in terms of number of bacterial cells required. The LC_{50} (48 hours) for crude TMOF expressing bacterial cells was $6.34 \times 10^6 \pm 5.38 \times 10^4$ cells and that of crude mutant TMOF was $5.76 \times 10^6 \pm 4.53 \times 10^4$ cells. In terms of number of bacterial cells also, the efficiency of crude mutant TMOF is significantly high compared to those of crude TMOF ($p < 0.05$).

The LC_{50} (48 hours) for affinity purified TMOF and mutant TMOF were 56.89 ± 1.42 and 50.93 ± 1.50 nM (2.13 and 1.91 $\mu\text{g/mL}$), respectively. The affinity purified mutant TMOF was 1.12-fold more toxic compared to affinity purified wild type TMOF ($p < 0.05$). The yield of wild type TMOF was 3.12 μg per milligram of bacterial pellet. In the case of mutant TMOF, the yield was 2.98 μg per milligram of bacterial pellet. The difference between the wild type and mutant TMOF was in the fifth position where proline in the wild type was replaced by serine in mutant TMOF. At the codon level, the 'C' in the triplet 'CCG' (proline of wild type) was replaced by 'T', leading to change in codon to 'TCG' (serine).

The trypsin modulating oostatic factor peptide isolated from *Aedes aegypti* was found to have larvicidal effect on *Aedes taeniorhynchus*, *Culex quinquefasciatus*, *C. nigripalpus*, *Anopheles quadrimaculatus*, *Heliothis virescens*, *Plutella xylostella* and *Helicoverpa zea* (Borovsky & Meola, 2004; Borovsky & Nauen, 2006; Jeffers et al., 2011). Several analogues of TMOF were also studied and observed to have toxicity in the larvae. *Aedes aegypti* larvae were fed with *Chlorella desiccata* cells expressing TMOF, resulted in mortality up to 75% (Borovsky et al., 2016). Similarly, TMOF expressed in *Saccharomyces cerevisiae* caused 83% larval mortality in *A. aegypti* (Borovsky et al., 2018). The yield of TMOF from 3×10^8 *Chlorella* cells is 20 µg and from same number of *Saccharomyces* cells, it is 2 µg. The same number of bacterial cells (3×10^8) in this study contain 24.19 µg TMOF and 24.53 µg mutant TMOF. Trypsin modulating oostatic factor expressed in $6.34 \times 10^6 \pm 5.38 \times 10^4$ bacterial cells cause 50% mortality in *A. albopictus* larvae whereas $5.76 \times 10^6 \pm 4.53 \times 10^4$ cells expressing mutant TMOF is enough to produce 50% larval mortality. Thus, it is clear that the bacterial expression of TMOF/mutant TMOF in this study is more effective and economical compared to previous expression systems.

1.6.6 Effect of pH of storage buffer on stability of recombinant TMOF/mutant TMOF

Effect of pH of the storage buffer on the stability of recombinant TMOF/mutant TMOF were studied. This was done by resuspending the crude recombinant proteins in different pH conditions. Buffers with pH 6.0, 7.0, 8.0 and 9.0 were used. For this, the bacterial pellet collected after expression was resuspended in buffers of different pH, and stored

for one week at -20°C. The second instar *A. albopictus* larvae (kept in distilled water) were treated with the soluble fraction with protein concentration equated to actual LC₅₀ (48 hours) obtained in earlier bioassays and toxicity analysed. Recombinant TMOF in pH 9.0 had shown highest mortality followed by pH 8.0, 7.0 and 6.0. Similar result was obtained in recombinant mutant TMOF also. The difference in larval mortality obtained by recombinant TMOF when stored in buffer with pH 6.0 and 7.0 was significant ($p < 0.05$). Mortality obtained at pH 8.0 was nearly similar to that of pH 9.0 and the change is not significant ($p > 0.05$). The decrease in larval mortality may be due to variation in solubility or loss of conformation of the recombinant TMOF/ mutant TMOF at lower pH. The role of pH in determining the stability and conformation of a recombinant protein is already reported (Swietnicki et al., 1997). There are also reports of effect of pH on stability of emulsion (Östbring et al., 2021). So, pH 9.0 will be better for the storage of recombinant TMOF/mutant TMOF.

1.6.7 Effect of pH of the exposure medium on toxicity of recombinant TMOF/mutant TMOF

The effect of pH of the exposure medium on toxicity of recombinant TMOF/ mutant TMOF was checked. *Aedes albopictus* larvae were treated with respective LC₅₀ (48 hours) concentration of crude TMOF ($242.10 \pm 6.04 \mu\text{g/mL}$) and crude mutant TMOF ($236.54 \pm 6.49 \mu\text{g/mL}$) at buffers of different pH (6.0, 7.0, 8.0 and 9.0). This was done to check whether the recombinant peptides will be active in fields with different pH. Toxicity of crude TMOF was the highest in pH 9.0, followed by pH 8.0, pH 7.0 and 6.0. Similar trend in mortality was observed in crude

mutant TMOF. In either case, significant change in mortality was observed in pH 6.0 and 7.0 ($p < 0.05$). So, it is found that pH 9.0 is found to be the optimum medium pH for the toxicity of crude TMOF/mutant TMOF. The gut pH of mosquito larvae is mostly alkaline ranging from 8.5 to 9.5. The peptide may be better stable in the alkaline pH as it is designed to work under alkaline conditions. The toxicity of an endotoxin from *Bacillus thuringiensis kurstaki* was reported to be insecticidal only after dissolution at pH > 9 (Venugopal et al., 1992). The recombinant enzyme alginate lyase expressed in *E. coli* cells shows a similar optimum activity at pH 9.0 compared to its commercial form favoured at pH 8.0 (Grobler et al., 2024).

1.6.8 Effect of storage temperature on stability of recombinant TMOF/mutant TMOF when stored as solution

The expression of recombinant TMOF/mutant TMOF was optimised at a temperature of 37°C. But, the storage of recombinant proteins needs to be considered to maintain its biological activity. To find the optimum storage temperature, the crude TMOF/mutant TMOF solutions were kept at three storage temperatures viz. -20°C, 4°C and 25°C for one week. After incubation, *A. albopictus* larvae were treated with LC₅₀ (48 hours) concentration. Toxicity of crude TMOF was highest when stored at -20°C followed by at 4°C and the least at 25°C. Similar results was obtained for crude mutant TMOF also. There is significant loss in toxicity of TMOF/mutant TMOF when stored at 25°C compared to the LC₅₀ (48 hours) for *A. albopictus* ($p < 0.05$). Storage of biological products at lower temperature is ruled by the inverse relation of protein stability with temperature (Correia et al., 2020). It is observed that at

25°C, proteins in the crude settled down. Increased temperature may be the reason for this aggregation. It is reported that incubating protein solutions at high temperatures results in physical degradation by irreversible aggregation of proteins (Chi et al., 2003). It is possible that the storage temperature may affect the stability and structure of recombinant TMOF/mutant TMOF in solution. Reduced toxicity when kept at higher temperature may be due to the denaturation and aggregation of recombinant TMOF/mutant TMOF. It is reported that the variation in the structure contribute to changes in biological responses (Scoble & Martin, 1990). Even though there are reports of cold induced degradation of proteins, but some proteins may retain its biological activity even after freezing. For example, protease inhibitor from *Fortunella margarita* can retain biological activity even after freezing (H. Yang et al., 2023).

1.6.9 Effect of storage temperature on stability of dried recombinant TMOF/mutant TMOF

The stability of crude TMOF/mutant TMOF in powder form was also studied. The crude solutions were dried at 40°C overnight and was kept at three temperature conditions; -20°, 4° and 25° C for one week. *Aedes albopictus* larvae were exposed to dried TMOF/mutant TMOF resuspended in water. The weight of dried TMOF/mutant TMOF was equated to LC₅₀ (48 hours) concentration and used for treatment. However, reduced toxicity was observed in dried form compared to TMOF/mutant TMOF in solution. Storage temperature also influences the stability since with increase in the temperature, toxicity is reduced. Highest toxicity of dried TMOF/mutant TMOF was observed when kept

at -20°C followed by 4°C and 25°C. Toxicity of crude mutant TMOF powder follows the same order. Significant loss in larvicidal potential of TMOF/mutant TMOF was observed when stored at room temperature ($p < 0.05$). Dehydration of proteins may result in measurable and significant changes in conformation (Prestrelski et al., 1993) and may account for the reduced toxicity. It is usually observed during heating that aggregation starts at temperatures well below the equilibrium melting temperature of the protein and it appears that partially unfolded protein molecules are the reactive species that form aggregates (Dong et al., 1995; Chi et al., 2003). In addition to the aggregation of protein, it's also possible that incomplete dissolution of dried TMOF/mutant TMOF in water make them unavailable for feeding by the larvae. Since the larvae are filter feeders, size of the particle is a major factor to be considered here. So, it will be better if TMOF is conjugated to a particle size ingestible by mosquito larvae, it may help in better ingestion.

1.6.10 Toxicity of recombinant TMOF/mutant TMOF to non-target organisms

In this study, we have checked the toxicity of recombinant TMOF/mutant TMOF to *Drosophila melanogaster*, *Luprops tristis* and *Aplocheilus lineatus*. Behavioural changes and mortality were observed after the treatment. Second instar larvae of *D. melanogaster* were treated with crude TMOF/mutant TMOF. The larvae fed the media mixed with crude TMOF/mutant TMOF and no notable changes in behaviour was observed compared to control. The life span was same for both control and treated larvae and all larvae emerged as adults. The adults reproduced similar to that of control and confirms that TMOF/mutant

TMOF is not toxic to *D. melanogaster*. In case of *L. tristis*, adult organisms were used for treatment. The tender leaves of *Hevea brasiliensis*/*Cassia fistula* were coated with crude TMOF/ mutant TMOF and used to feed *L. tristis*. They fed the leaves and there was no observable behavioural change as well as mortality compared to control. In case of *A. lineatus* also, adults were fed with crude TMOF/ mutant TMOF in water. Neither behavioural changes nor mortality was observed until three months after treatment compared to control. This experiment confirms that no toxicity was reported in non-target organisms tested. There are reports of non-target toxicity of chemicals commonly used in pest control. Malathion, one of the common organophosphates used in target pest control, including mosquitoes, inhibits acetylcholine esterase enzyme and reported to have its toxicity in fishes as well (Ahammad Sahib et al., 1980; Aker et al., 2008; Jebali et al., 2006; Silva de Souza et al., 2020). Pyrethroids used in agricultural pest control measures acts on the sodium potassium channels and reported to have carcinogenic, neurotoxic and immunosuppressive effects in mammals (Aznar-Aleman & Eljarrat, 2020).

Earlier studies reported that in insects like *Stomoxys calcitrans*, *Culicoides variipenis*, *Musca domestica* etc. TMOF peptide inhibits biosynthesis of trypsin like enzymes (Borovsky et al., 1990; Borovsky et al., 1993). These studies report there may be some TMOF like peptides in other dipterans for the regulation of trypsin like enzyme synthesis. But the peptide concentration required for the inhibition of enzyme synthesis in different organisms may vary. In addition, the TMOF peptide is transported across the gut by ABC transporter

(Borovsky et al., 2021). After entering the gut epithelial cells, TMOF binds to ribosome and inhibits trypsin mRNA translation (Borovsky et al., 2022). The TMOF peptide is non-toxic to organisms like mice, rabbit, mallard duck and *Daphnia* (D. M. Thompson et al., 2004). So, it is concluded that the lethal concentrations obtained in this study is not toxic to other non-target organisms, tested.

1.6.11 Effect of TMOF/mutant TMOF on gut bacterial composition

The gut microbiota has a significant role in pathogen transmission and mosquito biology (Douglas, 2011). To check whether treatment with recombinant TMOF/ mutant TMOF alters the gut microbiota, the second instar larvae of *A. albopictus* was exposed to LC₁₀ (48 hours) concentration of crude TMOF/mutant TMOF and allowed to grow until fourth instar stage. Bacterial composition of the treated larvae was assessed using 16S rRNA gene sequencing and compared it with that of control. A change in bacterial profile at the phylum, genus, and species levels was observed in TMOF treated larvae compared to control. A similar change in bacterial profile was also observed in mutant TMOF treated larvae compared to control. A total of 31 phyla, 795 genera and 1349 species were identified in control. In TMOF treated larvae, only 22 phyla with 466 genera with 757 species of gut bacteria were identified. In the case of mutant TMOF treated larvae, 31 phyla were present but, only 548 genera and 864 species were identified. Even though the same number of phyla was observed in mutant TMOF treated larvae and the control, taxonomic groups coming under each phylum were not the same.

There are alterations in the gut bacterial diversity of larvae treated with TMOF/mutant TMOF compared to control. Also, differences in bacterial profile of larvae treated with TMOF compared to larvae treated with mutant TMOF was observed. In general, there is a decrease in number of genus and species in the TMOF/mutant TMOF treated larvae compared to control. This may be due to several factors including non-availability of nutrients. It is reported that malnutrition can alter the composition of gut microbiota in humans (Biesalski, 2016). In TMOF/mutant TMOF treated larvae, inhibition of trypsin synthesis leads to indigestion resulting an unavailability of nutrients which may affect the gut bacterial community. The composition of mosquito gut bacteria at any life stages is influenced by the diet they taken (Merritt et al., 1992; Coon et al., 2014).

Predominant phyla in the *A. albopictus* gut microbiome include Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes (Baltar et al., 2023). The bacterial phyla Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes are renamed as Pseudomonadota, Actinomycetota, Bacillota and Bacteroidota respectively (Oren, 2024) and is used in this study. Pseudomonadota represents 20% gut bacteria of TMOF treated larvae, while it constitutes 43% bacterial population in mutant TMOF treated larvae. Actinomycetota (57%), the topmost phyla in TMOF treated larvae is represented by only 0.04% bacteria in mutant TMOF treated larvae. Bacillota comprising 16% bacteria in TMOF treated larvae constitutes 44% of gut bacteria in mutant TMOF treated larvae. Bacteroidota is represented by 0.1% bacteria in TMOF treated larvae whereas in mutant TMOF treated larvae, Bacteroidota constitutes

11% of bacterial population. Comparing the abundance of these phyla with respect to bacterial abundance in control larvae, the TMOF treated larvae and the control shows a similar pattern viz. Actinomycetota> Pseudomonadota> Bacillota> Bacteroidota. In the case of mutant TMOF treated larvae, the gut bacterial phyla are in the order Bacillota> Pseudomonadota> Bacteroidota> Actinomycetota.

At genus level, *Microbacterium* and *Cellulomonas* (7% each) are the major groups present in TMOF treated larvae, which are less than 0.01% in mutant TMOF treated larvae. In mutant TMOF treated larvae, *Clostridium* (26%) and *Morganella* (20%) are the major genera present. Comparing the abundance of these genera in that of TMOF treated larvae, *Clostridium* represents 0.07% bacteria and, *Morganella* has lower than 0.01% abundance.

At species level, *Cellulomonas gilvus* (7%) is the most abundant species in TMOF treated larvae followed by *Microbacterium sufflavum* (6%). Both of these species are entirely absent in mutant TMOF treated larvae. Instead, mutant TMOF treated larvae consists *Morganella morganii* (20%) and *Paraclostridium bifermentans* (4%) with highest abundance. *M. morganii* represents negligible percent of abundance in TMOF treated larvae, while *P. bifermentans* is completely absent.

Different microbes present in larval gut has found to have mosquitocidal activity. Insecticidal crystals from *Bacillus thuringiensis* and *Lysinibacillus sphaericus* have been used for the control of mosquito larvae (Lacey, 2007; Silva-Filha et al., 2021). *Morganella morganii* is the only gram-negative bacteria among the species described above and

is reported to have insecticidal activity (Nishiwaki et al., 2007). Copper nanoparticles bound to secondary metabolites of *M. morganii* found to have toxicity to the larvae of *Culex quinquefasciatus*, *Anopheles stephensi* and *Aedes aegypti* (Lalitha et al., 2020). A toxin isolated from *Paraclostridium bifermentans* targets nervous system of Anopheline mosquitoes (Contreras et al., 2019). Influence of diet on gut bacterial composition can complement or detract specific bacterial populations. The role of gut bacteria in the toxicity of the insecticide needs to be investigated.

In mammals, the gut microbiota play an important role in host metabolism by complementing various enzymes including digestion of polyphenols, synthesis of vitamins etc. thus contributing to maintain intestinal health (J. K. Nicholson et al., 2012; Rowland et al., 2018). The fish gut microbiome is influenced by diet and water nutrients (Talwar et al., 2018). The observed shift in microbial composition of larval gut microbiota may be the result of TMOF inhibition of trypsin synthesis which affects the nutrient availability to the microorganisms. This can lead to delayed development or mortality of the mosquito larvae. Whether the alteration in the gut microbiota also contributes towards toxicity of TMOF needs to be investigated.

1.7 SUMMARY

The gene coding for trypsin modulating oostatic factor (TMOF) was cloned and expressed in *Escherichia coli* cells after codon optimisation for bacterial expression. Mutant TMOF was also expressed in *E. coli*. Crude extracts of recombinant TMOF/mutant TMOF were prepared from bacterial cell lysate. Second instar *A. albopictus* larvae were treated with the crude TMOF/mutant TMOF and significant mortality was observed within 48 hours of treatment. On treatment with crude TMOF, an LC_{50} (48 hours) of $242.1 \pm 6.04 \mu\text{g/mL}$ was obtained and 100 % mortality was observed at a concentration of $435.00 \mu\text{g/mL}$ after 48 hours. For crude mutant TMOF, an LC_{50} (48 hours) of $236.54 \pm 6.49 \mu\text{g/mL}$ was obtained. The crude TMOF and crude mutant TMOF were toxic to *C. quinquefasciatus* larvae with an LC_{50} (48 hours) 285.10 ± 6.16 and $290.17 \pm 1.92 \mu\text{g/mL}$, respectively. The recombinant TMOF/mutant TMOF were purified by nickel affinity chromatography. The purified TMOF found to have an LC_{50} (48 hrs) of $2.13 \pm 0.02 \mu\text{g/mL}$ ($56.89 \pm 1.42 \text{ nM}$) and that of mutant TMOF was $1.91 \pm 0.02 \mu\text{g/mL}$ ($50.93 \pm 1.50 \text{ nM}$). The yield of purified TMOF was $3.12 \mu\text{g}$ per milligram of bacterial pellet and the yield of purified mutant TMOF was $2.98 \mu\text{g}$ per milligram of bacterial pellet. The affinity purified mutant TMOF is 1.12 times better than the purified TMOF as a larvicide in *A. albopictus*.

No toxicity on non-target organisms like *Drosophila melanogaster*, *Luprops tristis*, *Aplocheilus lineatus* observed indicating its specificity to mosquito larvae. Analysis of the gut microbiota of *A. albopictus* larvae on treatment with recombinant TMOF/mutant TMOF revealed

that there is a reduction in the gut bacterial population compared to untreated larvae. The alteration in the gut microbiota may also be contributing towards the toxicity of recombinant TMOF/mutant TMOF. As the mechanism of action of TMOF/mutant TMOF is receptor mediated and the receptor is only reported from mosquitoes, using recombinant TMOF/mutant TMOF as a larvicide is an ecofriendly method of mosquito control.

CHAPTER 2

**Isolation of a new trypsin inhibitor from
Calopogonium mucunoides and its toxicity to the
larvae of the Dengue vector, *Aedes albopictus***

2.1 INTRODUCTION

Proteases are hydrolytic enzymes involved in the cleavage of peptide bonds. Their role has contributed to the catalytic functions of many physiological processes like growth, nutrient mobilization, immune responses and reproduction. The protease action may not be similar in all organisms but does vary according to the requirement or extent to which the enzymes act upon it. Based on the catalytic site, proteases are categorised as serine, cysteine, aspartyl and metalloproteases. There are certain molecules that work antagonistically to proteases, which primarily aim to regulate protease activities. Such molecules are protease inhibitors, which are mostly proteins. Like the proteases, protease inhibitors are also categorised as serine protease inhibitors, cysteine protease inhibitors, aspartyl protease inhibitors and metalloprotease inhibitors.

Generally, the protease inhibitors are involved in regulating metabolic reactions catalysed by proteases by suppressing the ability of proteases to act. These molecules are distributed in a wide range of organisms, including microbes, fungi, plants and animals. The importance of proteases in physiological processes has contributed to the potential of protease inhibitors in industrial applications, especially therapeutics, food and beverages and pest control. The proteinaceous nature of inhibitors makes them easy to produce and apply by genetic engineering techniques. Protease inhibitors are specific in their action, as in the case with proteases.

Protease inhibitors in plants are defence mechanisms against herbivory and have found a potential role in the control of phytophagous pests. They are even reported to have antimicrobial, antioxidant, anti-

inflammatory and anticancer properties. Protease inhibitors are reported from many plants, including *Glycine max*, *Sinapis alba*, *Brassica hirta*, *Oryza sativa*, *Helianthus annuus*, *Solanum tuberosum*, *Zea mays*, *Momordica cochinchinensis*, *Triticum aestivum*, *Acacia confuse*, etc. (Habib & Fazili, 2007). Cell line experiments, animal studies and cytotoxicity experiments had shown the efficiency of protease inhibitors in treating various human diseases including cancer.

Plant protease inhibitors (PPIs) are found to have insecticidal toxicity, thus interfering with their growth and development, which can be utilised for the control of such insect pests. There are many reports of PPIs with potential pest control applications, like trypsin inhibitors from soybean, barley, cow pea etc. Proteinaceous inhibitors can be produced as recombinant proteins, and developing transgenic plants will be useful in crop protection and for enhancing the yield. A combination of inhibitors as recombinant fusion proteins will be useful in overcoming the adaptations developed by insects against a single inhibitor.

Identification and purification of protease inhibitors with potential mosquitocidal activities will be useful as an eco-friendly method of mosquito control. Plant protease inhibitors with mosquitocidal activities have been reported from plants like *Moringa oleifera*, *Adenanthra pavonima* and *Cassia leiandra* (Pontual et al., 2014; Sasaki et al., 2015; Dias et al., 2017).

In this work, plant extracts were screened for identification of trypsin inhibitors, and their larvicidal activities were tested in *Aedes albopictus*. In addition, a trypsin inhibitor with mosquito larvicidal activity on *A. albopictus* was purified from the seeds of *Calopogonium mucunoides*.

2.2 OBJECTIVES OF THE STUDY

- 1) To screen plant extracts for identifying extracts containing trypsin inhibitors
- 2) To purify trypsin inhibitor from plant extract with potent trypsin inhibition
- 3) Study the effect of trypsin inhibitor on *A. albopictus* larvae

2.3 REVIEW OF LITERATURE

2.3.1 Insect plant interactions

Plants and insects are extremely diverse groups that together constitutes about half of all known multicellular species inhabiting the area from the desert to the arctic zone (Calatayud et al., 2018). The adaptations developed by either of them complemented the diversification of each group over time. The interactions between plants and insects have developed a multilayered relationship extending their impact to various micro and macro organisms associated with them (Sharma et al., 2020). The main relationships are described as mutualistic, antagonistic or commensalistic, which are diversified through insect diversification behind host plants, coevolution, co-speciation or co-cladogenesis, chemical specialisation, ecological specialisation and eco-geological colonisation (Dethier, 1954; Ehrlich & Raven, 1964; Miller, 1987; Becerra, 1997; Kergoat et al., 2005; Rundle & Nosil, 2005; Gómez-Zurita et al., 2000; Calatayud et al., 2018). The coevolution of insects with plants thus contribute a major part to their behavioural, morphological and physiological strategies to overcome the barriers set by plants (Thompson, 1991; Bernays et al., 1991; Seger & Brockmann, 1987). The initial strategies adapted by insects against plant defence was focused on their reproduction and development. Later the insect adaptations were expanded to ensure the nutritional value of their host plants and protection from abiotic and biotic stress, including natural enemies and competitions (van Veen et al., 2006). Establishing new relations with specific microbes, insects began to manipulate the plant host's metabolism for their own needs, alternating its anatomy levels,

nutritional quality and secondary metabolism and even using plant toxins for defence against predators (Reichstein et al., 1968; Duffey, 1980; Paine et al., 1997; Hogenhout & Bos, 2011; Zhang et al., 2016; Lieutier et al., 2017). Another major development is the insect resistance to the insecticidal proteins, like protease inhibitors secreted by plants. Developing protease inhibitors (PIs) insensitive to proteases, detoxification of PIs and hydroxylation of PIs by other proteases, etc., are some adaptive strategies developed by the insects (Divekar et al., 2023).

2.3.2 Plant proteases

Proteases are a group of proteolytic enzymes involved in the hydrolysis of peptide bonds in a protein molecule (Beynon & Bond, 2001). Proteases are found to be sharp scissors and involved in catalytic reactions of proteolytic processing that give rise to new protein products (Neurath & Walsh, 1976). They are distributed in nearly all plants, animals and microorganisms (Ryan, 1990; Supuran et al., 2002; Haq et al., 2004; Mosolov & Valueva, 2005). Plant proteases have additional roles in plant growth and development, including maintenance of homeostasis, senescence, abscission, fruit ripening, apoptosis, immune responses, etc. (Grudkowska & Zagdańska, 2004; van der Hoorn & Jones, 2004; Díaz-Mendoza et al., 2014; Liu et al., 2018; Tornkvist et al., 2019). Proteases have a pivotal role in plant germination through hydrolysis and mobilisation of proteins from seeds and cereal grains (Martinez et al., 2019). Thus, this group of enzymes can be described as the key protagonists of plant growth and development.

2.3.3 Classification of plant proteases

Proteases were initially classified into endopeptidases, which target internal peptide bonds, and exopeptidases (aminopeptidases and carboxypeptidases), the action of which is directed by the NH₂ and COOH termini of their corresponding substrates (López-Otín & Bond, 2008). This general classification of proteases is based on the site of catalytic action. Endopeptidases are involved in the cleavage of peptide bonds in middle of the proteins, whereas the exopeptidases are involved in cleaving terminal bonds. The term ‘protease’ is applicable to both endopeptidase and exopeptidase, whereas ‘proteinase’, specify the group of endopeptidases. A new classification was described when more studies on proteases gave new insights. Based on the residues present at the active site, plant proteases are divided into serine, cysteine, aspartyl and metalloproteases. In the first two types, the nucleophiles are the amino acids, serine and cysteine, respectively, whereas in the last two, an activated water group does the function. Based on sequence homology and relationships, proteases are again categorised into families and clans, which led to development of MEROPS database (Rawlings & Bateman, 2021).

2.3.3.1 Serine proteases

Serine proteases are the largest group of plant proteases. They are also present in other eukaryotes, bacteria and viruses. Serine proteases have serine residue as the nucleophile in their active site which attack the carbonyl moiety of substrate peptide, forming an acyl-enzyme intermediate (Hedstrom, 2002). The nucleophilicity of serine residue is

dependent on a catalytic triad formed of serine, aspartate and histidine residues (Page & Di Cera, 2008). Mostly serine proteases are endopeptidases and include trypsin like proteases and subtilisin family. The molecular weight of plant serine proteases vary from 19 to 110 kDa, with optimum pH activity ranging from 7 to 11 and temperature between 30 to 80°C, although exceptions occur (Antão & Malcata, 2005). Serine proteases are mainly involved in degradation reactions and have broad substrate specificities including esterolytic and amidase activity. Serine proteases can be purified by ammonium sulphate precipitation, gel filtration, ion exchange and affinity chromatography. From plants, serine proteases are extracted from roots, stems, leaves, flowers, fruits, seeds and even latex. Other than protein degradation, serine proteases are involved in other physiological processes like tissue differentiation, hypersensitivity, signal transduction, pathogenesis, programmed cell death, senescence and seed germination (Tornero et al., 1997; Keita et al., 1999; Beers et al., 2000; Laplaze et al., 2000).

Serine proteases had been extracted from the seeds of *Glycine max* and *Oryza sativa*; from the latex of *Euphorbia supina*, *Taraxacum officinale*, *Synadenium grantii* and *Artocarpus heterophyllus*; from the flowers, stems, leaves and roots of *Arabidopsis thaliana*; from the storage roots of *Ipomoea batatas* and *Zea mays*; from the sprouts of *Pleuroblastus hindsii*; from the leaves of *Nicotiana tabacum*, *Phaseolus vulgaris* and *Lycopersicon esculentum*; and from the fruits of *Cucumis melo*, *Cucurbita ficifolia* and *Maclura pomifera* (Antão & Malcata, 2005).

2.3.3.2 Cysteine proteases

Cysteine proteases occurs ubiquitously in all organisms, with their function ranging from nutrient acquisition to developing host immune responses and in pathogenesis (N. Yang et al., 2023). Cysteine protease acts by hydrolysis that always involve the catalytic triad of cysteine, histidine, asparagine or aspartate and, in addition, a fourth residue, which is usually glutamine. Glutamine is important for stabilising the acyl intermediates formed during the catalytic process and takes precedence over cysteine activity sites (Rawlings & Barrett, 1994; Zastrow & Pecoraro, 2014). Cysteine proteases are distributed in prokaryotic and eukaryotic organisms and are mainly active at acidic pH. Cysteine proteases are subdivided into many families based on their amino acid sequence. Cysteine proteases are involved in nutrient metabolism and tissue invasion. Caspases, a family of cysteine proteases with catalytic cysteine and histidine dyad, play an important role in apoptosis in plants (del Pozo & Lam, 1998). The pharmacological activity of plant cysteine proteases, emphasising their role in mammalian wound healing, immuno-modulation, digestive conditions, and neoplastic alterations have been reported (Salas et al., 2008). Cysteine proteases have been isolated from *Carica papaya*, *Ananas comosus*, *Ficus glabrata*, *Ipomea batatas*, etc., and are even used in commercial industries and in the preparation of medicines (Guevara & Daleo, 2018).

2.3.3.3 Aspartyl proteases

Aspartyl or aspartic acid proteases are endopeptidases cleaving peptide bonds within a protein by the use of activated water molecules. Aspartyl proteases, unlike serine or cysteine proteases, act like acid-base reaction during the catalysis. There are two aspartate residues at the catalytic site of aspartyl proteases; one activates a water molecule by abstracting a proton, so that the water nucleophile attacks the carbonyl group of the succeeding aspartate residue. Aspartyl proteases are mostly active at acidic pH and is inhibited by pepstatin A. Aspartyl proteases are synthesised as zymogens and get activated at lower pH. Most of the eukaryotic aspartyl proteinases are single-chain enzymes having a molecular mass of approximately 35 kDa and they contain two highly conserved regions (Asp-Thr/Ser-Gly) (Munro & Hube, 2002). Pepsin, Cathepsin D, rennin etc. are examples of aspartyl proteases. Aspartyl proteases find application in food, beverages, medical and pharmaceutical industries (Theron & Divol, 2014).

2.3.3.4 Metalloproteases

Metalloproteases are enzymes in protein metabolism that act with the help of a metal ion at the catalytic site held by histidine, aspartate and glutamate residues. The metal ion polarizes the amide carbonyl in the peptide bond and use an activated water molecule for nucleophilic attack (van der Hoorn, 2008). Mostly, the metal ion in use is zinc, but sometimes cobalt or nickel is also used. Matrix metalloproteases and metalloprotease disintegrins are two related families that has implications in cell proliferation and metastasis (Chang & Werb, 2001).

A novel metalloprotease from banana peel with 40 kDa size is found to have potential effect on breaking collagen/ peptide bonds (Gurumallesha et al., 2019).

2.3.4 Role of proteases in insects

Proteases hydrolyse peptide bonds in protein molecules making the amino acids freely available for the metabolic processes. Insects produce a number of proteases, most of them have digestive roles. But there are some more involved in other biological pathways to coordinate the overall development. Such proteases often function in cascade pathways where one activates the other leading to signal amplification and ultimately generating large number of effector molecules (Kanost & Clem, 2012). The blood coagulation and complement cascade in mammals is an example of such pathways that do occur in insect embryonic development and immune responses (Krem & Di Cera, 2002; Cerenius et al., 2010). Cocoonase is the first purified insect protease involved in the hydrolysis of silk proteins in cocoon, helping the emergence of adult silk moths (Kafatos, Tartakoff, et al., 1967; Kafatos, Law, et al., 1967). Cysteine proteases involved in the digestion of yolk protein have been reported from *Drosophila melanogaster* and *Musca domestica* (Medina et al., 1988; Ribolla & De Bianchi, 1995). A serine carboxypeptidase synthesised as zymogen in the fat body of *Aedes aegypti* is taken up by oocytes and is activated during embryo formation (Cho et al., 1991). Besides their protease activity, a serine protease expressed in pupal yellow body of *Sarcophaga peregrina* possesses antibacterial activity (Nakajima et al., 1997; Tsuji et al., 1998). Serine protease zymogens are involved in the cascade leading to melanisation

in insects, which is a response to wounding and infection (Kanost & Clem, 2012). An alkaline serine protease purified from *Periplaneta americana* is found to have potential application in bioformulations or as additives in detergents and stain removers (Sanatan et al., 2013). Class II associated invariant chain peptide (CLIP) proteases are group of serine proteases involved in the innate immunity in insects (Kanost & Jiang, 2015). Cysteine cathepsins and caspases are cysteine proteases with immune function (Jagdale et al., 2017). Thus, the whole the group of proteases contribute to vital processes like development, reproduction and defence.

2.3.5 Protease inhibitors

Protease inhibitors (PIs) are molecules interfering the activity of proteases, enabling a system of regulation in protein degradation. Protease inhibitors (PIs) are broadly distributed across plants, animals, and microorganisms (Valueva & Mosolov, 2004; Christeller, 2005; Haq et al., 2004; Supuran et al., 2002; Mosolov & Valueva, 2005; Mosolov et al., 2001). Protease inhibitors are called as antinutritional factors, as they are antagonistic to protease action. Mostly PIs are small molecular weight proteins and involve in different physiological processes, including nutrient mobilisation, growth and development, reproduction, cell signalling, homeostasis and defence mechanisms. Plant protease inhibitors (PPIs) provide defence against herbivores as they inhibit the digestive enzymes of pests. Two mechanisms have been described to account for PIs action. The first one is similar to enzyme-substrate complex reaction where; the inhibitor competes with enzymes for active site (Clemente et al., 2019; Cid-Gallegos et al., 2022). The second one is

the interaction between the protease and inhibitor that induces the cleavage of an internal peptide bond in the structure of the PIs, triggering an irreversible conformational change (Clemente et al., 2019; Cid-Gallegos et al., 2022). A lot of PIs have been discovered from plants with diverse structure and function against different insect proteases. Many of them are well characterised and have many biotechnological applications.

Protease inhibitors are mostly of plant origin but are even present in animals, fungi and microbes. Microbes are a good source of PIs and an option for cost-effective preparation by fermentation and potential use in commercial industries. Viral genomes also encode genes for protease inhibitors (Rawlings, 2010). Aspartic protease inhibitor from *Bacillus* species is an inhibitor of HIV-1 protease, pepsin and fungal aspartyl protease (Vathipadiekal et al., 2010). Pepstatin A from *Actinomycetes* species is an inhibitor of aspartyl proteases (Cadicamo et al., 2013). Ecotins from animal and plant related microorganisms are having protease inhibitory action and contribute to their pathogenicity to hosts (De Meyer & Carlier, 2023). Protease inhibitors having antibacterial activity is reported from the shrimps *Marsupenaeus japonicus* and *Penaeus monodon* (Amparyup et al., 2008; Y.-R. Zhao et al., 2014). Protease inhibitors are reported from animal venoms like, in king cobra, sea anemone and scorpion (He et al., 2008; Peigneur et al., 2011; R. Zhao et al., 2011).

2.3.6 Protease inhibitors classification

Protease inhibitors can be classified into four families based on the type of proteases they inhibit, and they are serine protease inhibitors, cysteine

protease inhibitors, aspartyl protease inhibitors and metalloprotease inhibitors (M. J. Laskowski & Kato, 1980).

2.3.6.1 Serine protease inhibitors

As the name suggests, serine protease inhibitors (SPIs) are antagonistic to serine proteases and mostly act when the protease action is toxic to the plant itself. Serine protease inhibitors are present in various plant parts including leaves, seeds, tubers, etc. Most of the SPIs described so far belong to three major plant families; Leguminosae, Solanaceae and Gramineae (Richardson, 1991). Serine protease inhibitors are classified into Kunitz-type inhibitors (KTI), Bowman-Birk inhibitors (BBI), cereal trypsin/ α amylase inhibitors, mustard trypsin inhibitors, Potato type I and II inhibitors, serpins and squash serine inhibitors (M. Laskowski & Qasim, 2000; De Leo et al., 2002; Bateman & James, 2011). Kunitz-type inhibitors possess one or two polypeptide chains with 20 kDa each and have four cysteine residues forming two intrachain disulfide bonds. Kunitz-type inhibitors inhibit trypsin activity. In the Bowman-Birk inhibitors, single-chain polypeptides of 8 kDa is present which is cysteine-rich with seven disulfide bonds, and two active domains for the inhibition of trypsin or chymotrypsin. Serpins are irreversible competitive inhibitors of serine proteases having a molecular mass of around 39–43 kDa. Serine protease inhibitors are found to be effective in inhibiting extracellular serine proteases produced by phytopathogenic microbes and also suppress their growth and infection by induced expression of SPIs (Habib & Fazili, 2007). The group of SPIs have a potential effect on plant defence and find application in pest control. Serine protease inhibitors are also involved in mobilisation of storage

proteins, cell signalling and apoptosis, development, regulation of endogenous enzymes, flowering, seed germination and defence against pests (M. Clemente et al., 2019).

2.3.6.2 Cysteine protease inhibitors

Cysteine protease inhibitors are also called as ‘cystatins’, which act on the proteolysis mediated by cysteine proteases as competitive inhibitors that control several developmental processes. Animal cystatins are divided into three types based on their size, sequence, and post translational modifications, which are stefins, cystatins and kininogens (Kędzior et al., 2016). Stefins are mostly intracellular with single-chain polypeptides of molecular mass of about 11 kDa without a signal peptide, disulfide bonds and carbohydrate groups (Dubin, 2005). Cystatins are single-chain polypeptides, generally having a molecular mass of about 13–14 kDa with two conserved disulfide bridges and a signal peptide for extracellular trafficking (Dubin, 2005). Kininogens are large blood plasma glycoproteins of molecular mass about 60–120 kDa having three tandemly repeated type 2-like cystatin domains, two of which inhibit papain-like enzymes (Dubin, 2005). Phytocystatins, a specific family of plant cystatins from monocots and dicots, are reported to have size ranging from 12–86 kDa (Lima et al., 2015).

2.3.6.3 Aspartyl protease inhibitors

Aspartyl protease inhibitors (APIs) are molecules that hinder the activity of aspartyl proteases. Based on the molecular nature, APIs are divided into two families, proteinaceous and small molecular inhibitors (Dash et

al., 2003). Aspartyl protease inhibitors reported from plants are all proteinaceous and include inhibitors from tomato, wheat, potato, squash, etc., and from microbes such as *Bacillus* and *Streptomyces* species (Vishnu & Rao, 2012). Aspartyl protease inhibitor purified from *Vigna radiata* seeds has been found to be linear, hydrophobic, pH stable and thermos stable peptide with a size of 1.66 kDa (Kulkarni & Rao, 2009).

2.3.6.4 Metalloprotease inhibitors

Metalloprotease inhibitors are small molecules that intrude upon the metalloprotease action. They are found in different organisms. In plants, two families of metalloprotease inhibitors are described: metallo-carboxypeptidase inhibitors and cathepsin D inhibitors. The former include inhibitors from tomato and solanaceous plants which are small peptides with a size of about 4 kDa and act competitively with a broad spectrum carboxypeptidases (Habib & Fazili, 2007). A metalloprotease inhibitor is found to accumulate in plant parts in response to injuries along with other inhibitor families (Ryan, 1990).

2.3.7 Protease inhibitors from plants

Proteases are important enzymes involved in various physiological processes that lead to proper growth and development of an organism. Protease inhibitors are antagonistic to protease activity. In plants, protease inhibitors are produced in various parts and have a crucial role in providing defence against herbivory. In plants, protease inhibitors are typically found in storage organs or other aerial parts (De Leo et al., 2002) or are often induced in response to injury or attacks by insects or pathogens (Ryan, 1990). Plant protease inhibitors (PPIs) have found to

be involved in various biological processes like nutrient mobilisation, immune responses, homeostasis, etc. Recent studies revealed the potential of PPIs in commercial industries including food and beverages, medicine etc. Plant protease inhibitors are reported from *Glycine max*, *Sinapis alba*, *Brassica hirta*, *Oryza sativa*, *Helianthus annuus*, *Solanum tuberosum*, *Zea mays*, *Momordica cochinchinensis*, *Triticum aestivum*, *Acacia confuse*, etc. (Habib & Fazili, 2007).

2.3.8 Purification of plant protease inhibitors

Homogeneity of proteins should be assured for making use of their potential applications. Several methods have been described for the separation and purification of proteins. Differential precipitation and chromatographic techniques like adsorption chromatography, ion exchange chromatography, gel filtration chromatography and affinity chromatography are generally employed for protein purification. These techniques make use of differences in chemical, physical and structural properties of target protein with others (Labrou, 2014).

2.3.8.1 Precipitation

Precipitation techniques are employed as a preliminary step in protein purification. Salting out, isoelectric precipitation and organic solvent precipitation are common in use.

Salting out is the technique that uses high concentration of salts, which can induce selective precipitation of proteins. It can be used for proteins which easily aggregate from those that are highly soluble (Englard & Seifter, 1990). Ammonium sulphate is most commonly used in salting

out because of its high solubility and low cost. Addition of salt to protein solutions mediate the attractive and repulsive electrostatic interactions, non-electrostatic protein- ion interactions and ion-solvent interaction which in together act to either increase or decrease protein solubility in the solution (Dahal & Schmit, 2018). However, removal of salts after separation needs to be done and is mostly done by dialysis. Salting out using ammonium sulphate was used in fractional precipitation of plant crude extracts for purifying protease inhibitors (Patinhara Parambil & Vadakkadath Meethal, 2022).

Isoelectric precipitation is the method employing precipitation of proteins at their isoelectric point, which has the lowest solubility. The pH of the solutions is either increased or decreased according to target protein. It is an economic and convenient method in food industry (Labrou, 2014).

Organic solvent precipitation is based on the ability of organic solvents to reduce the dielectric constant of aqueous solutions which increase attraction between opposite charged groups and results in aggregation and precipitation of proteins (Labrou, 2014).

2.3.8.2 Chromatography

Chromatography is the most common method used in the high-resolution purification of protease inhibitors. It is usually done after fractional precipitation of crude plant extract using ammonium sulphate. Depending upon the properties of the protein to be purified, different kinds of chromatography can be used like, size exclusion, adsorption etc. Size exclusion, ion exchange and affinity chromatography are

mostly done for plant protease inhibitor purification (Saxena et al., 2010; Ashouri et al., 2017; Karray et al., 2020;).

2.3.8.2.1 Size exclusion chromatography

Size-exclusion chromatography (SEC) or gel filtration or gel permeation chromatography, makes use of differences in hydrodynamic diameter of proteins in the sample (Fischer, 1980). The sample is passed through a column packed with an inert porous material that possesses appropriate pore size distribution and volume and separation occurs due to differences in the intra-particulate void volume explored by the sample components of different molecular dimensions (Freitag & Horváth, 1996). Larger molecules cannot enter the void spaces, whereas smaller ones have access to all pores. This method is often used as a step following other chromatographic separations (Welling et al., 1986). Biochemical purification methods using size exclusion is more convenient and popular because of its high recovery, even for sensitive proteins, and the ability to remove undesirable aggregates (Hagel & Janson, 1992). Size exclusion commonly uses crosslinked dextran such as Sephadex, modified agarose and polyacrylamide-based gels that do not permit high pressures, but column packings of high mechanical stability is employed in size exclusion-high performance liquid chromatography (Freitag & Horváth, 1996).

2.3.8.2.2 Ion exchange chromatography

Ion-exchange chromatography (IEC) is one of the most widely applied chromatographic technique that assures recovery of biological property of the purified proteins. The ion exchange column consists of an ion

exchanger which may be an anion or cation exchanger depending upon the fixed charged particles at its surface. The charged groups in ion exchanger bind to substances with opposite charges and will be retained in the column facilitating its separation from the rest. Diethyl aminoethyl (DEAE) and carboxymethyl ligands are weak ion exchangers, whereas sulfonic acid or quaternary ammonium groups are strong ion-exchangers most often used in IEC (Freitag & Horváth, 1996). Gradient elution with increasing salt concentration is most widely used in elution. As the salt concentration of the eluent is increased, attraction between the protein molecules and the stationary phase is diminished (Freitag & Horváth, 1996).

2.3.8.2.3 Affinity chromatography

Affinity chromatography exploits several specific reversible biological properties of macromolecules with particular substrates or inhibitors for their separation purposes (Cuatrecasas et al., 1968). Most of the biological processes are based on specific ionic and non-ionic interactions arising from the molecular level arrangement of interacting moieties (Freitag & Horváth, 1996). A bio-specific ligand can be attached to the sorbent using standard immobilization techniques. While highly specific binding is the essence of affinity chromatography, the binding between the ligand and the product to be isolated should not be too strong, which thereby facilitate the elution of the bound protein to be readily achieved by changing parameters such as salt concentration or pH, or by addition of a competitive inhibitor in solution (Cuatrecasas et al., 1968). Affinity tags like poly-histidine, Glutathione-S-transferase, etc., can be used for purifying recombinant proteins.

Stationary phases immobilized with metal ions are widely used for protein purification. This concept of immobilized metal affinity chromatography (IMAC) was developed based on the affinity of divalent metal ions to amino acids like histidine and cysteine (Porath et al., 1975). These metal ions can be fixed to the chelating agent and used in the column. The IMAC technique is based on the interaction between a metal-ion electron acceptor (Lewis acid) and an electron donor (Lewis base) on the surface of a protein (Porath et al., 1975). Transition metal ions of intermediate polarizability such as Cu^{2+} , Ni^{2+} , Zn^{2+} , and Co^{2+} are particularly used since they may interact only with the nitrogen, oxygen and sulphur in amino- and imino-groups (Porath et al., 1975). In IMAC, the elution involves lowering the pH of the mobile phase to 6.0 at which the histidine residues are protonated. The technique was accelerated by the advancements in recombinant techniques and by the invention of an improved chelating ligand, nitrilotriacetic acid (NTA) in the 1980s (Hochuli et al., 1987). Mild competing agents like glycine, histidine, or imidazole, or an organic modifier is frequently used for the elution (Freitag & Horváth, 1996).

2.3.9 Applications of plant protease inhibitors

The role of protease inhibitors in health and disease control began to be investigated during the 1950s (Vogel et al., 1968). Trypsin was the target enzyme in most of the trials, as it is a major gut protease in various organisms. Cell line experiments and animal studies had shown promising insights of protease inhibitors in treating human illnesses. Protease inhibitors can be used in treating diseases such as obesity, cardiovascular diseases, autoimmune diseases, and different types of

cancer at different molecular levels like transcription, post-transcription, translation, post-translation, and secretion of cancer cell (Cid-Gallegos et al., 2022).

2.3.9.1 Plant protease inhibitors in therapeutics

Plant protease inhibitors have demonstrated antimicrobial properties against bacteria, fungi, and some viruses (J. Y. Kim et al., 2009). They are also known to possess anti-inflammatory, antioxidant, and anticancer properties, highlighting their potential in therapeutic applications (Li et al., 2011; Arbogast et al., 2007; Mehdad et al., 2016).

Serpins isolated from barley grains (*Hordeum vulgare*) are antagonistic to serine proteases in human plasma, leucocytes, pancreas, fungal trypsin, etc., and also inhibit blood clotting factors like factor VIIIa, Xa, thrombin and plasma kallikrein (Dahl et al., 1996). Trypsin inhibitor from *Glycine max* and a trypsin-chymotrypsin inhibitor from Amaranth seeds found to have anti-carcinogenic activity (Messina & Barnes, 1991; Tamir et al., 1996; Chen et al., 2005; Hsieh et al., 2010; Borodin et al., 2013). Serine protease inhibitor from *Bauhinia bauhinioides* and *B. rufa* are reported to reduce oedema formation in perfused rabbit lungs (Neuhof et al., 2003). Another trypsin inhibitor from *B. rufa* inhibit human plasma kallikrein, which is involved in establishing prostate cancer (Nakahata et al., 2006). Protease inhibitor from buckwheat (*Fagopyrum sculentum*) is found to have antiproliferative effects on T-acute lymphoblastic leukaemia cell lines (Park & Ohba, 2004). The anti-carcinogenic activity of a Bowman-Birk inhibitor from *Vigna unguiculata* seeds shown its potential in inducing apoptosis and

lysosome membrane permeabilization on breast cancer cell lines (Joanitti et al., 2010). Adhesion, invasion and migration of gastric cell lines have been affected by the activity of trypsin inhibitor from *Enterolobium contortisiliquum* (de Paula et al., 2012). Bowman-Birk inhibitors from legumes like chick pea, soybean, lentils and pea is found to have anti-inflammatory and anti-carcinogenic effects on the gastrointestinal tract (A. Clemente & Arques, 2014). Antiproliferative activities towards leukaemia and lymphoma cell line is reported in purified trypsin inhibitor from *Phaseolus vulgaris* (M. Li et al., 2017).

Antimicrobial effects of protease inhibitors from plants have been described. Antimicrobial activity of *Coccinia grandis* protease inhibitors have been reported against *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Proteus vulgaris*, *Klebsiella pneumoniae* and pathogenic fungi like *Candida albicans*, *Aspergillus flavus*, *Mucor indicus*, *Cryptococcus neoformans* and *Penicillium notatum* (Satheesh & Murugan, 2011). Antibacterial activity of the *Acacia nilotica* trypsin inhibitor is reported against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa* (Mehmood et al., 2020). *Adenanthra pavonima* trypsin inhibitor possess antibacterial effect on *S. aureus*, *E. coli*, *P. aeruginosa*, *Klebsiella oxytoca* and *K. pneumoniae* and antifungal effects on *Candida albicans* and *C. tropicalis* (Almeida et al., 2020).

The anti-diabetic effect of amylase and α -glucosidase inhibitors from *Nigella sativa* and *Punica granatum* has been reported (Tiji et al., 2021; Laaraj et al., 2022). Anti-hyperglycaemic, pancreatic α -amylase, lipase and intestinal α -glucosidase inhibitory activities is reported from the

seed extract of *Cannabis sativa* confirming its potential in type 2 diabetes treatment (Haddou et al., 2023). Dual biological activity of a trypsin inhibitor from *Capsicum annuum* is reported with antifungal role against *Candida albicans* and a hypoglycaemic property with non-toxicity to human cells, making it a suitable component in antifungal preparations (Cotabarren et al., 2023).

Antiviral activity of protease inhibitors has been reported from many plants against viral proteases. Anti-HIV reverse transcriptase activity was reported by trypsin inhibitor from *Vicia faba*, *Glycine max* and *Acacia confusa* (Lin & Ng, 2008; Lam & Ng, 2010). Anti-HIV 1 reverse transcriptase and antitumour activity against human cell lines were reported from the seed extracts of *Bauhinia variegata* and *Glycine max* (Fang, Wong, Bah, et al., 2010; Fang, Wong, & Ng, 2010). Trypsin inhibitor from *Capsicum baccatum* showed antiviral activity on yellow mosaic virus, suppressing its infection (Moulin et al., 2014). Serine protease inhibitor from *Cucumis metuliferus* gives resistance to potyvirus in *Nicotiana benthamiana* (Mishra et al., 2020). Serpins can modulate various kinds of proteases and thereby plays a role in haematological, cardiovascular and immunological responses (Varkoly et al., 2023). This study also relates viral-derived serpins to be used as a potential drug candidate in various human disorders.

2.3.9.2 Plant protease inhibitors for pest control

Plants, being producers, are mostly the source of food for many organisms. An increased consumption by any one consumer group can have an indirect effect on the consumption of others. Insects, adapted

along with plants over time used to feed on plant and its products for their survival. With an increase in pest population, plants also developed new strategies to resist insect pests. One such approach is the production of plant protease inhibitors. Plant protease inhibitors are molecules that interrupt proteases, the hydrolytic enzymes responsible for proteolysis. The role of protease inhibitors against insect pest was first reported from soybean, which was found toxic to the larvae of *Tribolium confusum* (Lipke et al., 1954). Resistance to insect pests by transferring genes encoding protease inhibitor was first demonstrated by Hilder et al., 1987. Trypsin inhibitor gene from *Vigna unguiculata* was transferred to tobacco, raising the resistance of tobacco to various lepidopteran, coleopteran and orthopteran pests (Hilder et al., 1987). Ability of protease inhibitors in suppressing pathogenic nematode infection was reported against *Globodera tabacum*, *G. pallida* etc. (Williamson & Hussey, 1996). Serine protease inhibitors widely distributed in plants are competitive inhibitors and act against proteases in a similar standard mechanism (M. J. Laskowski & Kato, 1980). Purified soyabean trypsin inhibitor and potato inhibitor II found to inhibit protein metabolism of *Heliothis zea* and *Spodoptera exigua* larvae (Broadway & Duffey, 1986). Identification of cysteine and serine proteases in gut extracts of many insects paved a way for developing protease inhibitor-mediated control over insect pest populations (Kitch & Murdock, 1986; Houseman et al., 1989).

The major group of protease inhibitors with insecticidal toxicity is reported by serpins and cystatins (S. Singh et al., 2020). The trypsin and chymotrypsin inhibitors from a single precursor offer resistance to

Helicoverpa punctigera and *Teleogryllus commodus* (Anderson et al., 1997). The cowpea trypsin inhibitor shows deleterious effects on the larvae of tomato moth, *Lacanobia oleracea* (Bell et al., 2001). The α -amylase inhibitors from *Colocasia esculenta* and *Ipomoea batatas* can be used to control the root crop pests such as *Araecerus fasciculatus*, *Sitophilus oryzae*, *Cylas formicarius elegantulus* and *Tribolium castaneum* (Rekha et al., 2004). The α -amylase inhibitors from *Ricinus communis* is toxic to *Callosobruchus maculatus*, *Zabrotes subfasciatus* and *Tenebrio molitor* (Do Nascimento et al., 2011). Serpins from *Arabidopsis thaliana* is having potential toxicity to proteases in *Spodoptera littoralis* larvae and nymphs of *Acyrtosiphon pisum* (Alvarez-Alfageme et al., 2011). Development of the Mediterranean flour moth, *Anagasta kuehniella* is affected by trypsin inhibitor from *Entada acaciifolia* (de Oliveira et al., 2014). Serine protease inhibitors from *Vigna unguiculata* seeds affect digestive enzymes and development of *Spodoptera littoralis* (Abd El-latif, 2015). Negative impacts on the growth of *Chilo partellus* is reported by ingesting *Capsicum annuum* protease inhibitor (Jadhav et al., 2016). A bi-functional serine protease from winged bean causes gut protease inhibition on *Helicoverpa armigera* larvae (Banerjee et al., 2017). A protease inhibitor from *Spatholobus parviflorus* seeds inhibits the development of the larvae of *Spodoptera mauritia* (Patinhara Parambil & Vadakkadath Meethal, 2022). A Kunitz-type trypsin inhibitor from *Hibiscus acetocella* seeds inhibit the growth and development of *Spodoptera litura* larvae (Sajitha & Vadakkadath Meethal, 2024).

2.3.9.3 Plant protease inhibitors for mosquito control

Conventional methods of mosquito control using chemicals has contributed to environmental and health issues and the development of resistance in mosquitoes. Thus, researches began exploring new strategies that are more efficient and environment friendly. Several works were published on the role of plants and plant products with insecticidal activity in 1940s (Haller, 1940; Pepper & Carruth, 1945; Roark, 1947). Mosquitocidal activity of phytochemicals from 300 plant species was reported by Sukumar et al., 1991. Phytochemicals extracted from different plant species were reported to be toxic to *Culex quinquefasciatus* larvae (Kalyanasundaram & Babu, 1982; Evans & Raj, 1991; Karmegam et al., 1997). Mosquitocidal effects of various phytochemicals to *Aedes*, *Culex* and *Anopheles* mosquito larvae are summarized in a review by Shaalan et al., 2005 (Shaalan et al., 2005). Larvicidal effects of organic solvent extracts from plants have been described by Ghosh et al., 2012 (Ghosh et al., 2012). Root extracts of *Asparagus racemosus* showed ovicidal, larvicidal and adulticidal activity on *Culex*, *Anopheles* and *Aedes* mosquitoes (Govindarajan & Sivakumar, 2014).

Trypsin is a major gut protease that is involved in the blood digestion of mosquitoes (Lehane et al., 1996). This paves a way for utilising trypsin inhibitors as a potential tool for controlling mosquito populations. The amount and specificity in action of plant protease inhibitors can vary in different plant extracts and may reflect the differential effect on the target mosquito species. Inhibition of development and larval mortality was reported in *A. aegypti* when treated with trypsin inhibitor from

Moringa oleifera flowers (Pontual et al., 2014). *Adenanthra pavonima* trypsin inhibitor extracted from the seeds had shown a significant effect on *Aedes aegypti* larvae in survival, weight and protease activities of midgut extracts when exposed to a non-lethal concentration of inhibitor during short- and long-duration assays (Sasaki et al., 2015). Trypsin inhibitor from *Cassia leiandra* seeds shows dose-dependent acute toxicity in third instar larvae of *Aedes aegypti* (Dias & do Ceara Fortaleza, 2016). Trypsin inhibitor from the seeds of *Leucaena leucocephala* delays egg hatching, and larval development in *Aedes aegypti* (Almeida Filho et al., 2017). Purified Kunitz-type trypsin inhibitor from *Cassia leiandra* inhibits *Aedes aegypti* gut protease and can be used for their control (Dias et al., 2017). Inhibition of gut protease activity was reported in *Aedes albopictus* larvae when treated with serine protease inhibitors (Sajna et al., 2019). Inhibition of gut protease activity of fourth instar *A. albopictus* larvae was reported by treating plant extracts containing trypsin inhibitors (Sara et al., 2020). The toxicity of extracts from *Lawsonia inermis*, *Annona muricata* and *Calotropis gigantea* containing trypsin inhibitors against different instars of *A. aegypti* was reported (N et al., 2021). These observations indicate the potential application of plant protease inhibitors in mosquito control.

2.3.9.4 Transgenic plants expressing protease inhibitors

Plant genetic transformation is a potential tool to understand genome regulation and allows for manipulation of genes that could otherwise not be done by classical breeding (Haq et al., 2004). Transformation experiments using the protease inhibitor gene enhance the resistance to

insect pests and also act as a way for higher production of inhibitor proteins for multiple applications.

Genes encoding cowpea trypsin inhibitor and pea lectin introduced into tobacco plants enhanced their resistance to *Heliothis virescens* larvae (Boulter et al., 1990). The soybean KTI₃ gene was inserted to *Populus nigra* using *Agrobacterium tumefaciens*-mediated transformation, and larvae of the insect pests *Clostera anastomosis* and *Lymantria dispar* fed with transgenic leaves shown larval mortality due to protease inhibition (Confalonieri et al., 1998). A cDNA clone which encodes for multidomain precursor of protease inhibitor from *Nicotiana alata* was transferred to tobacco and peas, which express the active inhibitor and cause mortality of *Helicoverpa armigera* larvae that ingested the transgenic plant (Charity et al., 1999). Transgenic indica and japonica rice varieties expressing barley trypsin inhibitor caused a significant reduction in the survival rate of rice weevil, *Sitophilus oryzae* (Alfonso-Rubí et al., 2003). Over expression of two inhibitors, potato protease inhibitor I and carboxypeptidase inhibitor in tomato varieties showed enhanced resistance to *Heliothis obsoleta* and *Liriomyza trifolii* larvae (Abdeen et al., 2005). A variant of potato plants expressing barley cystatin caused reduced weight gain in the larvae of *Leptinotarsa decemlineata* and *Spodoptera littoralis* (Alvarez-Alfageme et al., 2007). Better crop protection was observed in cotton balls against *Helicoverpa armigera* with the combined effects of potato type I and type II inhibitors from *Nicotiana alata* and *Solanum tuberosum*, respectively (Dunse et al., 2010). Chymotrypsin-like sequences that provide protection against some Bt toxins in *Ostrinia furnacalis* were derived

from their transcriptome data, and RNAi using dsRNA for those chymotrypsin-like sequences enhanced mortality of *O. furnacalis* larvae (Guan et al., 2017). Transgenic sugar cane plants overexpressing sugar cane peptidase inhibitor showed increased resistance to larvae of *Sphenophorus levis* (Schneider et al., 2017). Although recombinant protease inhibitors are effective in increasing pest resistance, they also contribute to unintentional pleiotropic effects and the productivity of concerned plant varieties. Prolonged closure of stomata was reported in *Arabidopsis* following the overexpression of recombinant protease inhibitors triggered Absciscic acid-hypersensitivity (Bi et al., 2017). The overexpression of recombinant protease inhibitors may also inhibit small ubiquitin-like modifier (SUMO) proteins involved in the regulation of the normal functioning of metabolism (Hou et al., 2018; Morrell & Sadanandom, 2019; Mangena, 2022).

Apart from recombinant protease inhibitors for crop protection, several new genome editing methods has been used to improve plant resistance against many kinds of pathogens (Secgin et al., 2022). Zinc-finger proteases and CRISPR/Cas system are genetic engineering techniques in use. The CRISPR/Cas system depends upon an oligo-directed mutagenesis with sequence-specific nucleases and can also be used for the introduction of a sequence of choice by homology-directed repair (HDR) (Miladinovic et al., 2021; Aksoy et al., 2022). The introduced mutations thus can alter amino acid sequences resulting alterations in the physical and biological properties of proteins.

2.3 MATERIALS AND METHODS

2.3.1 MATERIALS

Trypsin, azocasein, ammonium sulphate, sodium dodecyl sulphate, sodium carbonate, sodium bicarbonate, sodium chloride, sodium hydroxide, Tris, Tris HCl, Trichloro acetic acid (TCA), Tetramethylene diamine (TEMED), glycerol, methanol, glacial acetic acid and formaldehyde were purchased from Sisco Research Laboratories Limited, Mumbai, India. Amicon 3 kDa protein concentrator was purchased from Merck, Germany. CNBr activated Sepharose 4B and ammonium per sulphate were purchased from Sigma Aldrich, USA. Proteinase K, dialysis membrane, acrylamide, Bis acrylamide, Coomassie brilliant blue R-250, Coomassie brilliant blue G-250, β -mercaptoethanol and bovine serum albumin (BSA) were purchased from Himedia laboratories private Limited, Mumbai, India. All other reagents used were analytical grade.

2.3.2 METHODS

2.3.2.1 Collection of plants and preparation of plant extracts

The plant parts used for the study were collected from localities of Calicut University Botanical Garden, in Malappuram district, and Kovoov in Kozhikode district. They were kept in separate zip lock covers at -20°C until use. Plants were identified by Dr. A K Pradeep, Associate Professor, Department of Botany, University of Calicut. The collected plant parts were washed thoroughly in running water, distilled water and bicarbonate buffer. They were soaked in bicarbonate buffer, pH 9.0 overnight prior to homogenisation. The soaked plant parts were homogenised in bicarbonate buffer (1 g tissue per mL buffer), pH 9.0 and centrifuged at 10,000 x g for 10 minutes at 4°C. The soluble proteins were recovered in the supernatant and kept at -20°C until use.

2.3.2.2 Protease assay

The protease assay was done according to modified Charney & Tomarelli, 1947 method.

Reagents

1. Bicarbonate buffer, pH 9.0

100 mM sodium carbonate solution was prepared and the pH was adjusted to 9.0 by 100 mM sodium bicarbonate solution.

2. Azocasein

Azocasein solution was prepared by dissolving 5 mg of azocasein in 375 μ L bicarbonate buffer, pH 9.0.

3. Trypsin

Trypsin solution was prepared by dissolving 1 mg bovine trypsin in 1 mL 1 mM HCl.

4. Trichloro acetic acid (TCA)

5% TCA solution was prepared by dissolving 5g TCA in distilled water and made up to 100 mL.

5. NaOH

500 mM NaOH was prepared by dissolving 2 g of NaOH in distilled water and made up to 100 mL.

Procedure

In protease assay, bovine trypsin was the enzyme used and azocasein, an azo dye conjugated to casein was used as substrate. The final concentration of trypsin and azocasein were 0.25 μ g/ μ L and 3.43 μ g/ μ L, respectively in a total volume of 20.2 μ L. The reaction mixture was incubated at 37°C for 30 minutes after adding azocasein. After incubation, 80 μ L of 5% TCA was added to the mixture to stop the reaction and centrifuged at 10000 x g for 10 minutes. The supernatant of volume 50 μ L from each sample was taken and mixed with 150 μ L of 500mM NaOH. Proteolytic degradation of azocasein results in liberation of azo dye into the supernatant which can be quantitatively

measured by recording absorbance at 440 nm in a microplate reader. All assays were done in duplicate and repeated thrice with appropriate controls.

2.3.2.3 Screening of plant extracts for trypsin inhibitor

This was done by protease inhibition assay using trypsin as the enzyme and azocasein as substrate.

2.3.2.3.1 Protease inhibition assay

Plant extract was pre-incubated at room temperature with trypsin for 10 minutes and then azocasein was added (final concentration of trypsin and azocasein were 0.25 µg/ µL and 3.43 µg/µL, respectively). Assay was proceeded further as described in protease assay. The assay was repeated thrice and done in duplicates.

Calculation of percentage inhibition:

For calculating percentage inhibition, the absorbance of the extract alone was subtracted from that of the test (trypsin + plant extract). This was divided by absorbance of trypsin alone and then multiplied with 100. The whole value was then subtracted from 100 to get percentage inhibition of inhibitor alone. The calculation uses following formula

Percentage inhibition =

$$100 - \left(\frac{\text{absorbance of inhibitor} - \text{absorbance of control}}{\text{absorbance of trypsin alone}} * 100 \right)$$

2.3.2.4 Proteinase K treatment of plant extracts

Plants having 50% or above trypsin inhibition was taken for proteinase K treatment to check the proteinaceous nature of inhibitor. For this, plant extracts were incubated with proteinase K in 9:1 ratio at 56°C overnight. After incubation, the mixture was boiled at 96°C for 5 minutes to inactivate proteinase K and then centrifuged at 10000 x g for 10 minutes. The supernatant from each sample were then subjected for trypsin inhibition assay.

2.3.2.5 Culture and maintenance of *Aedes albopictus* larvae

Aedes albopictus larvae were collected from the field and allowed to grow to adults in the laboratory. The larvae were grown in distilled water and fed with yeast granules. They were identified with the help of Dr Raghu, Senior Scientist, Centre for Disease Control, Kozhikode.

2.3.2.6 Purification of plant protease inhibitor

2.3.2.6.1 Selection of plant extract

Plant extracts with proteinaceous trypsin inhibitor were examined for their toxicity to *Aedes albopictus* larvae. For this, 25 numbers of *A. albopictus* larvae were treated in 100 mL distilled water, with 1 mL plant extract prepared from 1 gram wet tissue in 1 mL distilled water. The highest percentage mortality was observed for larvae treated with *Calopogonium mucunoides* seed extract. Hence, *C. mucunoides* seed extract which demonstrated high trypsin inhibition and mosquito larvicidal activity was selected for purification.



Fig. 2.3.1 *Calopogonium mucunoides* Desv. plant

***Calopogonium mucunoides* Desv**

Kingdom	:	Plantae
Phylum	:	Tracheophyta
Class	:	Magnoliopsida
Order	:	Fabales
Family	:	Fabaceae
Genus	:	<i>Calopogonium</i>
Species	:	<i>mucunoides</i>

Calopogonium mucunoides Desv. also called as wild groundnut or calopo is mainly used as a cover crop in young forests. It is an annual or short-lived perennial herb, with brown hairs on stem. The upper part of

the stem is twining giving rise to trifoliate green leaves, slender raceme inflorescence of small violet flowers and the seeds are formed within hairy pods.

2.3.2.6.2 Purification of trypsin inhibitor from *Calopogonium mucunoides* seeds

Trypsin inhibitor from *C. mucunoides* seeds was purified by ammonium sulphate fractionation, size exclusion chromatography, ion exchange chromatography and trypsin affinity chromatography.

2.3.2.6.2.1 Preparation of crude extract

Calopogonium mucunoides seeds were crushed and soaked in 100 mM bicarbonate buffer, pH 9.0 overnight (1g/mL buffer). Soaked seeds were homogenized and the homogenate was continuously stirred for three hours at 4°C. It was then centrifuged at 10000 x g for 10 minutes to extract soluble proteins. The crude extract thus obtained was stored at -20°C until use.

2.3.2.6.2.2 Protein estimation

Estimation of protein in the *C. mucunoides* crude extract was determined by Bradford's dye binding method (Bradford, 1976).

2.3.2.6.2.3 Ammonium sulphate fractionation of crude extract

The crude extract prepared was subjected to ammonium sulphate fractionation using different concentrations of ammonium sulphate (Englard & Seifter, 1990). This was done sequentially by adding different concentrations of ammonium sulphate (0-30%, 30-50% and 50-70%). Differential precipitation was done at 4°C. Supernatant after

30% ammonium sulphate precipitation was used for 50% ammonium sulphate precipitation and its supernatant taken for 70% ammonium sulphate precipitation. All the precipitates were dissolved in minimum quantity of 100 mM bicarbonate buffer, pH 9.0 and transferred to separate dialysis bags and dialysed against 100 mM bicarbonate buffer at 4°C for 24 hours with four changes of buffer. After dialysis, sample in each bag was centrifuged at 10000 x g for 10 minutes at 4°C and supernatant taken. The protein in the supernatant was estimated using Bradford's dye binding method (Bradford, 1976). Trypsin inhibition assay was done to calculate percentage trypsin inhibition. *Aedes albopictus* larvae were treated with different ammonium sulphate fractions at a final protein concentration of 7.2 µg/mL. Of the different fractions, 0-30% ammonium sulphate fraction was used for purification of trypsin inhibitor.

2.3.2.6.2.4 Size exclusion chromatography

Size exclusion chromatography was performed before ion exchange as the 0-30% ammonium sulphate fraction was coloured and the colour was sticking onto the ion exchange resin. Size exclusion chromatography of 0-30% ammonium sulphate fraction was done in a SEC 650 gel filtration column in Biorad NGC Quest plus FPLC system.

The steps followed for size exclusion chromatography were;

- The column was equilibrated with 100 mM bicarbonate buffer prior to sample application
- A total of 1.67 mg protein was loaded onto column

- Column was run using 100 mM bicarbonate buffer, pH 9.0 containing 150 mM NaCl at a flow rate of 1 mL min⁻¹
- 250 µL fractions were collected

The protein concentration of elutes were determined using Bradford' dye binding method (Bradford, 1976). Trypsin inhibition assay was done to calculate percentage trypsin inhibition of elutes. Elutes with higher trypsin inhibition were pooled and concentrated using amicon pro affinity concentrator. Concentrated size exclusion sample used for next level of purification. Protein purity was checked by loading elutes onto 10% SDS-PAGE (Laemmli, 1970).

2.3.2.6.2.5 Ion exchange chromatography

Pooled and concentrated size exclusion fraction containing the inhibitor was further purified by ion exchange chromatography. This was done using Source Q resin (anion exchanger) column in a Biorad NGC Quest plus FPLC system with a flow rate of 2 mL min⁻¹.

The steps followed for ion exchange chromatography;

- Column equilibration: The 1 mL column was equilibrated with 100 mM bicarbonate buffer, pH 9.0 by passing 5 column volume of buffer
- Sample application: A total of 1.02 mg protein was loaded onto column with a flow rate of 2 mL min⁻¹

- Column wash: Column was washed with 5 column volumes of 100 mM bicarbonate buffer, pH 9.0 prior to elution. This was done to remove unbounded proteins from the column
- Elution of proteins: Elution of proteins were done in a gradient manner using 100 mM bicarbonate buffer containing 0.0 to 1M NaCl

The protein concentration of elutes were determined using Bradford's dye binding method (Bradford, 1976). Trypsin inhibition assay was done to calculate percentage trypsin inhibition of elutes. Protein purity was checked by loading elutes onto 10% SDS-PAGE (Laemmli, 1970).

2.3.2.6.2.6 Trypsin affinity chromatography

Trypsin affinity chromatography was done by batch method for purifying ion exchange elutes. This was done using cyanogen bromide (CNBr) activated Sepharose 4B resin coupled to trypsin in batch method.

2.3.2.6.2.6.1 Coupling of trypsin with Cyanogen bromide activated Sepharose 4B

- Trypsin affinity resin was prepared by coupling 10 mg trypsin to CNBr activated Sepharose 4B
- 250 mg CNBr activated Sepharose 4B was swelled in double distilled water for 8 hours

- It was then washed with the coupling buffer (100 mM NaHCO₃ solution containing 500 mM NaCl, pH 8.3) prior to coupling with trypsin
- 1 mL Trypsin solution was prepared by dissolving 10 mg trypsin in coupling buffer
- Trypsin solution was added slowly to the swelled resin and mixed well
- After coupling, excess trypsin was washed away using coupling buffer
- To block trypsin unbound active sites, if any remaining, the medium was transferred to 100 mM Tris buffer, pH 8.0 and allowed to stand for an hour
- The medium was finally washed with alternating buffers, 100 mM CH₃COOH/CH₃COONa, pH 4.0 containing 500 mM NaCl and 100 mM Tris buffer, pH 8.0 containing 100 mM NaCl in 4 cycles

2.3.2.6.2.6.2 Loading of ion exchange fraction to trypsin Sepharose resin

Ion exchange purified fraction with highest trypsin inhibition was loaded onto 400 µL trypsin Sepharose resin.

The steps involved in batch method of trypsin affinity chromatography were;

- The trypsin Sepharose resin was equilibrated with 100 mM Tris buffer, pH 8.0
- A total of 50 µg protein was mixed with resin and incubated for 45 minutes
- Resin was washed with 100 mM Tris buffer, pH 8.0 until protein free
- Elution was done using 0.1 M HCl and elutes were immediately neutralized with 0.1 M NaOH

The protein concentration of elutes were determined using Bradford's dye binding method (Bradford, 1976). Trypsin inhibition assay was done to calculate percentage trypsin inhibition of elutes. Protein purity was checked by loading elutes onto 10% SDS-PAGE (Laemmli, 1970).

2.3.2.7 Determination of molecular weight of purified inhibitor

2.3.2.7.1 SDS-PAGE

Denaturing PAGE using SDS was done to determine molecular weight of purified inhibitor. All the elutes obtained in respective chromatographic techniques were run on SDS-PAGE (Laemmli, 1970) to check the protein purity in successive stages. Samples were loaded onto 10% SDS-PAGE along with a protein marker. The molecular weight corresponding to purified inhibitor was calculated with reference to the size of protein marker loaded.

Reagents

1. Acrylamide solution (30%)

A stock solution of acrylamide (30%) was prepared by dissolving 15 grams of acrylamide and 0.4 grams of bis- acrylamide in distilled water and made up to 50 mL. the solution was filtered and stored at 4°C in an amber coloured bottle.

2. Preparation of buffers

○ Buffer 1, pH 8.8

Tris (0.614 M) buffer is prepared in water by dissolving 7.43 g Tris and made up to 100 mL. The pH of the solution is adjusted to 8.8 with HCl and 164 mg SDS is added to it.

○ Buffer 2, pH 6.8

Tris (0.147 M) buffer is prepared in water by dissolving 1.78 g Tris and made up to 100 mL. The pH of the solution is adjusted to 6.8 with HCl and 108 mg SDS is added to it.

○ Chamber buffer, pH 8.3

Chamber buffer was prepared in distilled water at final concentration of 0.025 M Tris, 0.192 M glycine and 0.1% SDS. The pH of the solution was adjusted to 8.3.

3. Ammonium per sulphate solution (APS)

This was prepared by dissolving 15 mg ammonium persulphate in 1 mL distilled water.

4. 6X loading dye

6X loading dye was prepared in distilled water at final concentrations of 375 mM Tris HCl (pH 6.8), 6% SDS, 48% glycerol, 9% β mercaptoethanol and 0.03% bromophenol blue.

5. Fixative

Fixative was prepared by mixing 75 μ L formaldehyde with 100 mL of 50% methanol.

6. Staining solution

Staining solution was prepared by dissolving 60 mg of Co-omassie brilliant blue R-250 powder in a solution containing 44 mL methanol, 44 mL distilled water and 12 mL glacial acetic acid.

7. Destaining solution

Destaining solution was prepared by mixing 50 mL methanol, 75 mL glacial acetic acid and 875 mL distilled water.

Preparation of gel

A mini gel of 10% acrylamide was prepared consisting separating gel and spacer gel. They are prepared as follows

Separating gel (10%)

- Acrylamide (30%) : 3 mL
- Buffer 1, pH 8.8 : 5.5 mL
- TEMED : 10 μ L
- APS (15mg/mL) : 450 μ L

Spacer gel (3%)

- Acrylamide (30%) : 500 μ L
- Buffer 2, pH 6.8 : 4.25 mL
- TEMED : 5 μ L
- APS (15 mg/mL) : 250 μ L

Sample loading and running the gel

Protein samples were mixed with 6X loading dye and incubated for 5 minutes at boiling water bath. Samples were then loaded onto the gel after filling with chamber buffer. The gel was run in 15 mA until the dye reaches the separating gel, and then at 25 mA, till the dye reaches the bottom of the gel. Gel was then transferred to fixative and kept for 45 minutes. After that gel was transferred to staining solution and kept for 45 minutes and then transferred to destaining solution and left until destained.

2.3.2.8 Calculation of half-maximal inhibitory concentration (IC_{50})

Half-maximal inhibitory concentration was calculated using crude, 0-30% ammonium sulphate fraction and concentrated size exclusion fraction. For calculating IC_{50} , different concentrations of double diluted inhibitor containing samples were mixed with 1 mg/mL trypsin and incubated for 10 minutes. The residual trypsin activity was determined by protease assay using azocasein as the substrate.

2.3.2.9 Toxicity studies on *Aedes albopictus* larvae

The toxicity of the inhibitor on *Aedes albopictus* larvae was studied following WHO protocol (WHO, 2005). For this, 25 second instar larvae were taken in 100 mL distilled water. They were fed with yeast granules. Larvae were exposed to different concentrations of inhibitor containing crude, 0-30% ammonium sulphate fraction and concentrated size exclusion fraction. Assays were done at room temperature. Larval mortality was observed after 48 hours. The LC₅₀ (48 hours) was calculated using probit regression analysis in SPSS version 23.

2.4 RESULTS

2.4.1 Screening plant extracts for identifying trypsin inhibitors

Trypsin is the major gut protease in many organisms, including mosquitoes. Trypsin inhibitors are molecules that inhibit the activity of trypsin. Trypsin inhibitors are produced in the plant body as a defensive molecule against herbivory. Thus, trypsin inhibitors from plant extracts can be used as an effective control measure against mosquito larvae. In this study, 45 plant extracts were screened to identify extracts containing trypsin inhibitors. The list of plants screened are included in table 2.4.1.

Table 2.4.1 list of plant extracts screened for identifying trypsin inhibitors

Sl No.	Plant name (Family)	Common name/ Vernacular name	Plant part used	Percentage trypsin inhibition (mean $\pm$ SE)
1	<i>Calopogonium mucunoides</i> Desv. (Fabaceae)	Wild ground nut	Seeds	87.99 $\pm$ 0.38
2	<i>Lantana camara</i> L. (Verbanaceae)	Aripooov	Seeds	85.35 $\pm$ 1.36
3	<i>Mucuna pruriens</i> (L.) DC. (Fabaceae)	Velvet bean	Seeds	70.03 $\pm$ 0.46
4	<i>Coffea arabica</i> L. (Rubiaceae)	Coffee	Leaves	69.85 $\pm$ 1.87
5	<i>Ocimum tenuiflorum</i> L. (Lamiaceae)	Holy basil, Tulasi	Leaves	62.10 $\pm$ 0.76
6	<i>Globba sessiliflora</i> Sims (Zingiberaceae)	Dancing ladies	Leaves	61.74 $\pm$ 2.91

Sl No.	Plant name (Family)	Common name/ Vernacular name	Plant part used	Percentage trypsin inhibition (mean $\pm$ SE)
7	<i>Mussaenda erythrophylla</i> Schumach and Thonn (Rubiaceae)	Ashanti blood	Leaves	56.05 $\pm$ 1.76
8	<i>Eryngium foetidum</i> L. (Apiaceae)	Mexican coriander	Leaves	55.09 $\pm$ 3.74
9	<i>Cinnamomum cassia</i> (L.) J.Presl (Lauraceae)	Chinese cinnamon	Leaves	52.81 $\pm$ 1.45
10	<i>Plectranthus amboinicus</i> Lour. (Lamiaceae)	Indian mint	Leaves	52.81 $\pm$ 1.45
11	<i>Zingiber officinale</i> Roscoe (zingiberaceae)	Ginger	Leaves	51.96 $\pm$ 1.70
12	<i>Mussaenda luteola</i> Delile (Rubiaceae)	Yellow mussaenda	Leaves	49.90 $\pm$ 6.44
13	<i>Thunbergia erecta</i> (Benth.) T.Anderson (Acanthaceae)	Bush clock vine	Leaves	49.90 $\pm$ 6.44
14	<i>Vanilla planifolia</i> Andrews (Orchidaceae)	West Indian vanilla	Leaves	49.02 $\pm$ 3.71
15	<i>Manihot esculenta</i> Crantz (Euphorbiaceae)	Cassava	Leaves	44.07 $\pm$ 0.62
16	<i>Chassalia curviflora</i> (Wallich) Thwaites (Rubiaceae)	Curved flower woody chassalia	Leaves	44.06 $\pm$ 1.13

Sl No.	Plant name (Family)	Common name/ Vernacular name	Plant part used	Percentage trypsin inhibition (mean $\pm$ SE)
17	<i>Grewia nervosa</i> (Lour.) G. Panigrahi (Malvaceae)	Shiral	Fruits	41.16 $\pm$ 2.70
18	<i>Piper betle</i> L. (Piperaceae)	Betel	Leaves	40.75 $\pm$ 0.62
19	<i>Hemigraphis alternata</i> (Burm. f.) T. Anderson (Acanthaceae)	Waffle plant	Leaves	40.04 $\pm$ 2.14
20	<i>Scheicheria oleosa</i> (Lour.) Oken (Sapindaceae)	Kusum	Leaves	38.37 $\pm$ 0.20
21	<i>Argyreia pomacea</i> (Roxb.) Choisy (Convolvulaceae)	Mustai	Leaves	33.69 $\pm$ 1.34
22	<i>Murraya koenigii</i> (L.) Spreng. (Rutaceae)	Curry tree	Leaves	33.27 $\pm$ 2.28
23	<i>Justicia wynadensis</i> (Nees) T. Anderson (Acanthaceae)	Maddu soppu	Leaves	31.84 $\pm$ 1.81
24	<i>Catharanthus roseus</i> (L.) G. Don (Apocynaceae)	Periwinkle	Leaves	28.06 $\pm$ 3.06
25	<i>Kopsia fruticose</i> (Roxb.) A. DC. (Apocynaceae)	Shrub vinca	Leaves	27.69 $\pm$ 0.79
26	<i>Litchi chinensis</i> Sonn. (Sapindaceae)	Lychee	Seeds	24.74 $\pm$ 0.40
27	<i>Lawsonia inermis</i> L. (Lythraceae)	Mehandi	Leaves	24.32 $\pm$ 1.25

Sl No.	Plant name (Family)	Common name/ Vernacular name	Plant part used	Percentage trypsin inhibition (mean $\pm$ SE)
28	<i>Simarouba glauca</i> DC. (Simaroubaceae)	Paradise tree	Leaves	21.20 $\pm$ 1.87
29	<i>Carica papaya</i> L. (Caricaceae)	Omakkaya	Leaves	21.17 $\pm$ 1.83
30	<i>Pancratium parvum</i> Dalzell (Amaryllidaceae)	Forest lily	Bulb	20.55 $\pm$ 0.65
31	<i>Tinospora cordifolia</i> (Willd.) Miers (Menispermaceae)	Heart leaved moonseed	Leaves	18.85 $\pm$ 0.77
32	<i>Hibiscus rosasinensis</i> L. (Malvaceae)	Shoeback plant	Leaves	17.53 $\pm$ 0.39
33	<i>Laggera alata</i> (D. Don) Sch. Bip. Ex Oliv. (Asteraceae)	Winged stem laggera	Leaves	17.48 $\pm$ 0.28
34	<i>Clerodendrum viscosum</i> Vent. (Lamiaceae)	Hill glory bower	Leaves	16.07 $\pm$ 0.88
35	<i>Mimusops elengi</i> L. (Sapotaceae)	Spanish cherry	Seeds	13.87 $\pm$ 1.44
36	<i>Callisia repens</i> (Jacq.) (Commelinaceae)	Turtle wine	Leaves	12.83 $\pm$ 0.40
37	<i>Asparagus racemosus</i> Willd. (Asperagaceae)	Shatavari	Seeds	11.91 $\pm$ 2.16
38	<i>Clitoria ternatea</i> L. (Fabaceae)	Blue pea	Leaves	11.42 $\pm$ 2.88
39	<i>Leea indica</i> (Burm. f.) Merr.	Bandicoot berry	Leaves	10.30 $\pm$ 0.38

Sl No.	Plant name (Family)	Common name/ Vernacular name	Plant part used	Percentage trypsin inhibition (mean $\pm$ SE)
	(Vitaceae)			
40	<i>Olea dioica</i> (Roxb.) (Oleaceae)	Edana	Leaves	9.89 $\pm$ 0.10
41	<i>Combretum indicum</i> (L.) DeFilipps (Combretaceae)	Rangoon creeper	Leaves	8.26 $\pm$ 3.41
42	<i>Lophopetalum wightianum</i> Arn. (Celastraceae)	Spindle tree	Leaves	7.55 $\pm$ 1.10
43	<i>Conyza sps.</i> (Asteraceae)	Horseweeds	Leaves	6.79 $\pm$ 1.23
44	<i>Thevetia peruviana</i> (Pers.) K. Schum. (Apocynaceae)	Yellow oleander	Leaves	4.95 $\pm$ 1.56
45	<i>Pithecellobium dulce</i> (Roxb.) Benth. (Fabaceae)	Madras thorn	leaves	0.00

Values are mean $\pm$ standard error (n= 3)

Out of 45 plants screened, 11 plants showed more than 50% trypsin inhibition. Highest inhibition was observed by *Calopogonium mucunoides* seed extract with a percentage inhibition of 87.99 $\pm$ 0.38. The plants having more than 50% trypsin inhibition were subjected to proteinase K treatment to check whether the inhibitor is proteinaceous or not.

2.4.2 Proteinase K treatment of plant extracts

To check the proteinaceous nature of inhibitor, 11 plant extracts having more than 50% trypsin inhibition were digested with proteinase K. Trypsin inhibition assay was done using the extracts before and after the

treatments. Differences in the percentage inhibition were calculated. Six extracts were found to have proteinaceous inhibitor and they are *Calopogonium mucunoides*, *Mucuna pruriens*, *Coffea arabica*, *Ocimum tenuiflorum*, *Globba sessiliflora* and *Eryngium foetidum* (table 2.4.2).

Table 2.4.2 Proteinase K treatment of plant extracts

Sl No.	Plant name	Before proteinase K treatment mean % inhibition $\pm$ SE	After proteinase K treatment mean % inhibition $\pm$ SE	p value	Proteinaceous inhibitor
1	<i>Calopogonium mucunoides</i>	87.99 $\pm$ 0.38	18.97 $\pm$ 0.12	0.002	+
2	<i>Lantana camara</i>	85.35 $\pm$ 1.36	82.44 $\pm$ 0.41	0.101	-
3	<i>Mucuna pruriens</i>	70.03 $\pm$ 0.46	17.06 $\pm$ 0.05	0.005	+
4	<i>Coffea arabica</i>	69.85 $\pm$ 1.87	15.89 $\pm$ 0.19	0.010	+
5	<i>Ocimum tenuiflorum</i>	62.10 $\pm$ 0.76	17.51 $\pm$ 0.03	0.005	+
6	<i>Globba sessiliflora</i>	61.74 $\pm$ 2.91	17.91 $\pm$ 0.08	0.021	+
7	<i>Mussaenda erythrophylla</i>	56.05 $\pm$ 1.76	53.94 $\pm$ 0.03	0.223	-
8	<i>Eryngium foetidum</i>	55.09 $\pm$ 3.74	12.45 $\pm$ 0.38	0.025	+
9	<i>Cinnamomum cassia</i>	52.81 $\pm$ 1.45	44.05 $\pm$ 0.11	0.056	-
10	<i>Plectranthus amboinicus</i>	52.81 $\pm$ 1.45	48.71 $\pm$ 0.34	0.085	-
11	<i>Zingiber officinale</i>	51.96 $\pm$ 1.70	41.69 $\pm$ 0.93	0.080	-

Values are mean $\pm$ standard error (n=3). Plus sign (+) indicates that the inhibitor is proteinaceous in nature while minus sign (-) indicates non-proteinaceous nature.

In the case of plant extracts with proteinaceous inhibitors, there was a drastic reduction in trypsin inhibition after the proteinase K treatment. Loss of inhibition was due to hydrolysis of proteinaceous inhibitor by proteinase K. Non-proteinaceous inhibitors were present in five extracts; *Lantana camara*, *Mussaenda erythrophylla*, *Cinnamomum cassia*, *Plectranthus amboinicus* and *Zingiber officinale* (table 2.4.2).

2.4.3 Larvicidal activity of proteinaceous trypsin inhibitors to *Aedes albopictus*

Larvicidal activity of proteinaceous trypsin inhibitors were checked. *Aedes albopictus* larvae were treated with the plant extracts containing proteinaceous trypsin inhibitors. Highest percentage mortality was observed in larvae treated with *C. mucunoides* extract (100 ± 0.00) followed by *Coffea arabica*, *Mucuna pruriens*, *Globba sessiliflora*, *Eryngium foetidum* and *Ocimum tenuiflorum* (table 2.4.3). Hence, for the purification of inhibitor, *C. mucunoides* extract was selected as it causes high mortality in *A. albopictus* larvae.

Table 2.4.3 Larvicidal activity of plant extracts with proteinaceous inhibitors

Plant extract	Percentage mortality after 48 hours (mean $\pm$ SE)
<i>Calopogonium mucunoides</i>	100 ± 0.00
<i>Coffea arabica</i>	66.67 ± 3.53
<i>Mucuna pruriens</i>	50.67 ± 2.67
<i>Globba sessiliflora</i>	38.67 ± 1.33
<i>Eryngium foetidum</i>	18.67 ± 1.33
<i>Ocimum tenuiflorum</i>	5.33 ± 1.33

2.4.4 Purification of trypsin inhibitor from *Calopogonium mucunoides* seeds

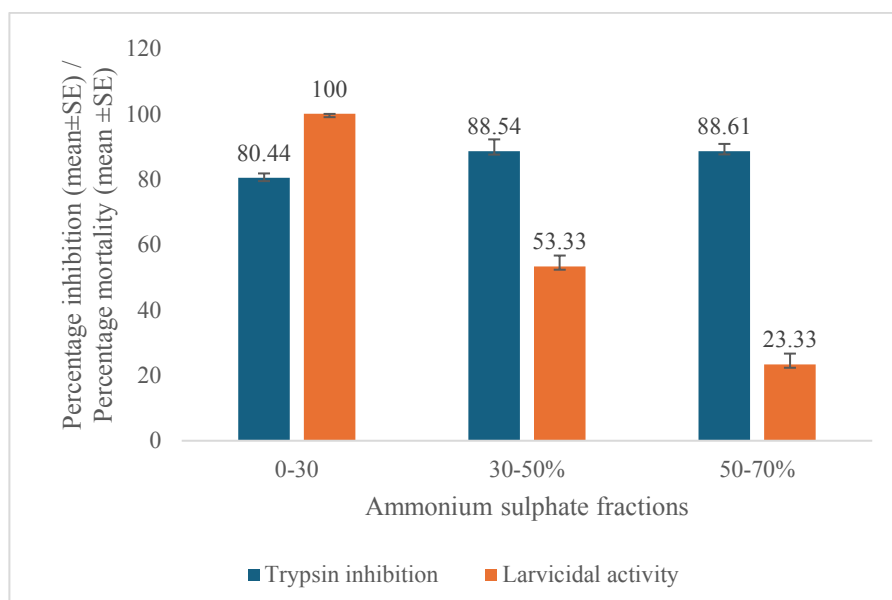
Crude extract prepared from *C. mucunoides* seeds was used for purification of inhibitor. The methods employed were ammonium sulphate fractionation, gel filtration chromatography, ion exchange chromatography and trypsin affinity chromatography.

2.4.4.1 Ammonium sulphate fractionation for enrichment of trypsin inhibitor

From the seeds, crude extract was prepared and subjected to ammonium sulphate precipitation. Differential precipitation was done with 0-30%, 30-50% and 50-70% of ammonium sulphate. Protein concentration of the 0-30%, 30-50% and 50-70% ammonium sulphate fractions were 12.2 $\mu\text{g}/\mu\text{L}$, 8.05 $\mu\text{g}/\mu\text{L}$ and 7.2 $\mu\text{g}/\mu\text{L}$, respectively. Trypsin inhibition was the highest for 50-70% ammonium sulphate fraction ($88.61 \pm 2.21\%$) followed by 30-50% ammonium sulphate fraction ($88.54 \pm 3.66\%$) and 0-30 % ammonium sulphate fraction ($80.44 \pm 0.00\%$) (table 4.4) (figure 2.4.1). Larvicidal activity of all ammonium sulphate fractions were checked on *Aedes albopictus* at same protein concentration (7.2 $\mu\text{g}/\text{mL}$). The highest larvicidal activity was observed for 0-30% ammonium sulphate fraction ($100.00 \pm 0.00\%$) followed by 30-50% ammonium sulphate fraction ($53.33 \pm 3.33\%$) and 50-70% ammonium sulphate fraction ($23.33 \pm 3.33\%$) (Table 2.4.4) (Figure 2.4.1).

Table 2.4.4 Trypsin inhibition and larvicidal activity of ammonium sulphate fractions

Ammonium sulphate fractions	% Trypsin inhibition (Mean $\pm$ SE)	% Mortality after 48 hours (Mean $\pm$ SE) (At 7.2 μ g/mL protein concentration)
0-30%	80.44 $\pm$ 0.00	100.00 $\pm$ 0.00
30-50%	88.54 $\pm$ 3.66	53.33 $\pm$ 3.33
50-70%	88.61 $\pm$ 2.21	23.33 $\pm$ 3.33

**Fig. 2.4.1** Trypsin inhibition and larvicidal activity of ammonium sulphate fractions. The histograms represent the percentage trypsin inhibition and larvicidal activity of ammonium sulphate fractions

Trypsin inhibition of crude extract and its double diluted fractions with protein concentration was calculated (Fig 2.4.2). From the graph, IC_{50} of crude sample was found to be $0.70 \pm 0.25 \mu$ g/ μ L.

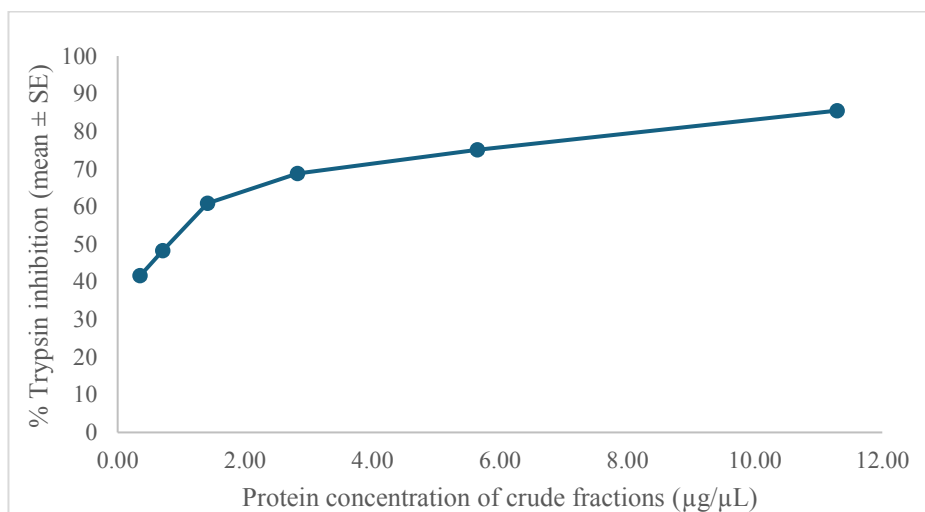


Fig. 2.4.2 Trypsin inhibition of crude extract and its double diluted fractions

Trypsin inhibition of 0-30% ammonium sulphate fraction and its double diluted fractions with protein concentration was calculated (Fig 2.4.3). From the graph, IC_{50} of 0-30% ammonium sulphate fraction was found to be $0.81 \pm 0.19 \mu\text{g}/\mu\text{L}$.

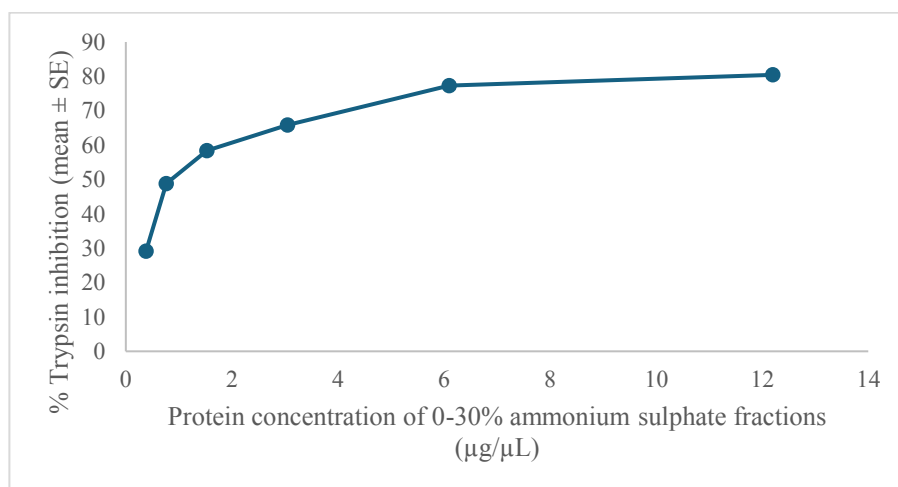


Fig. 2.4.3 Trypsin inhibition of 0-30% ammonium sulphate fraction and its double diluted fractions

2.4.3.2 Size exclusion chromatography

The 0-30% ammonium sulphate fraction was subjected to size exclusion chromatography using SEC 650 column. A total of 1.67 mg protein was loaded onto column after equilibration with 100 mM bicarbonate buffer, pH 9.0. Elution was done using 100 mM bicarbonate buffer, pH 9.0 containing 150 mM NaCl. A prominent peak and a few small peaks were observed (figure 2.4.4). The percentage trypsin inhibition of the fractions collected were calculated and the fractions 42, 43, 44 and 45 were having more than 45% inhibition and high protein concentration (table 2.4.4) (figure 2.4.5). These fractions were pooled together and concentrated.

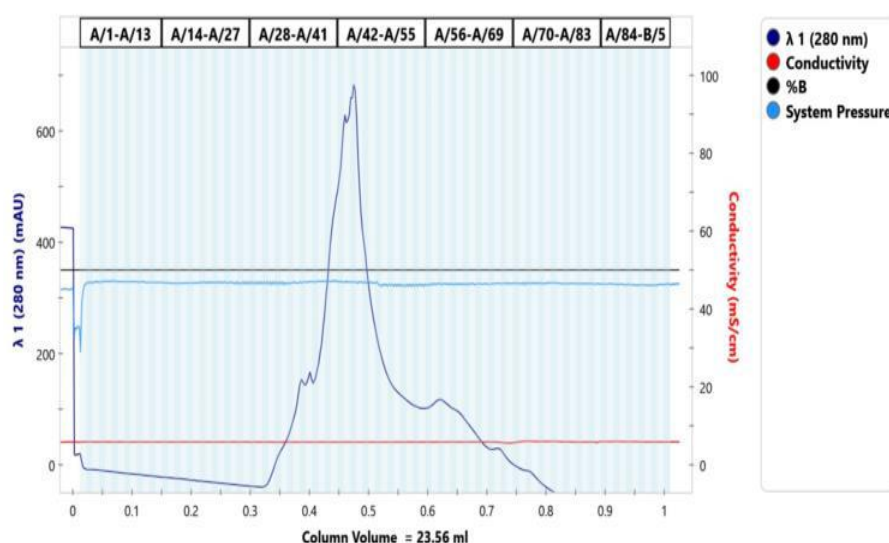


Fig. 2.4.4 Run report of size exclusion chromatography

Table 2.4.5 Percentage inhibition obtained from fractions collected during size exclusion chromatography

Fractions collected during SEC	% Trypsin inhibition (Mean $\pm$ SE)	Protein concentration ($\mu\text{g}/\mu\text{L}$)
Fraction number		
377	29.17 $\pm$ 0.40	0.01 $\pm$ 0.00
38	30.68 $\pm$ 0.13	0.02 $\pm$ 0.00
39	32.20 $\pm$ 0.15	0.08 $\pm$ 0.00
40	36.74 $\pm$ 0.20	0.14 $\pm$ 0.00
41	44.17 $\pm$ 0.20	0.31 $\pm$ 0.00
42	47.96 $\pm$ 0.26	0.35 $\pm$ 0.00
43	63.11 $\pm$ 0.33	0.50 $\pm$ 0.00
44	50.53 $\pm$ 0.42	0.43 $\pm$ 0.00
45	46.21 $\pm$ 0.15	0.33 $\pm$ 0.00

Percentage inhibition and respective protein concentration of elutes during size exclusion chromatography

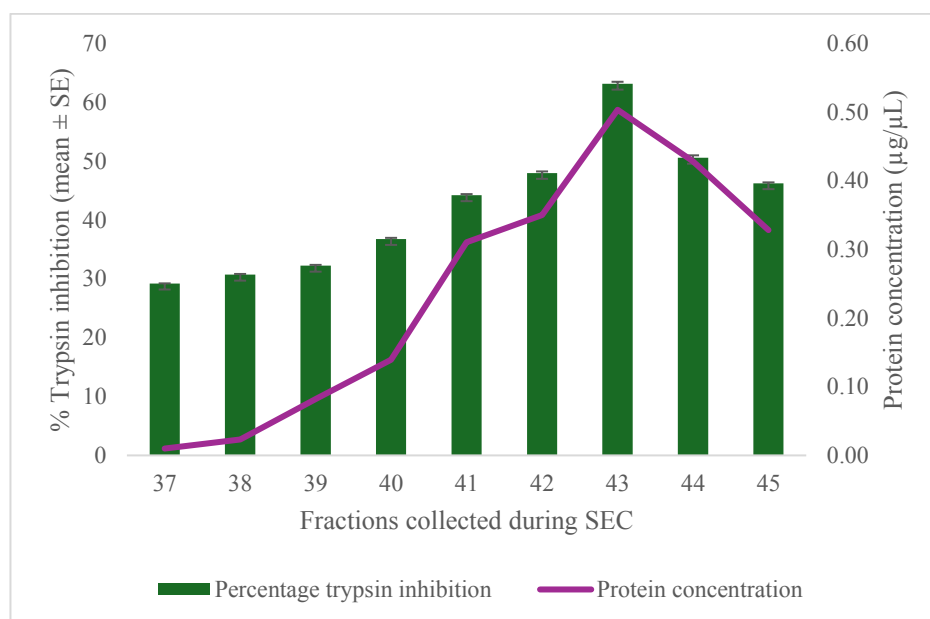


Fig 2.4.5 Trypsin inhibition and protein concentration of fractions obtained during size exclusion chromatography. The histogram represents the trypsin inhibition of elutes from gel filtration and line graph represent their corresponding protein concentration

The percentage inhibition of the concentrated sample was and 58.29 ± 0.43 at a protein concentration of $1.11 \pm 0.01 \mu\text{g}/\mu\text{L}$. The IC_{50} of concentrated fraction was calculated from the graph plotted, protein concentration Vs trypsin inhibition (figure 2.4.6). The IC_{50} of concentrated fraction was found to be $0.51 \pm 0.19 \mu\text{g}/\mu\text{L}$.

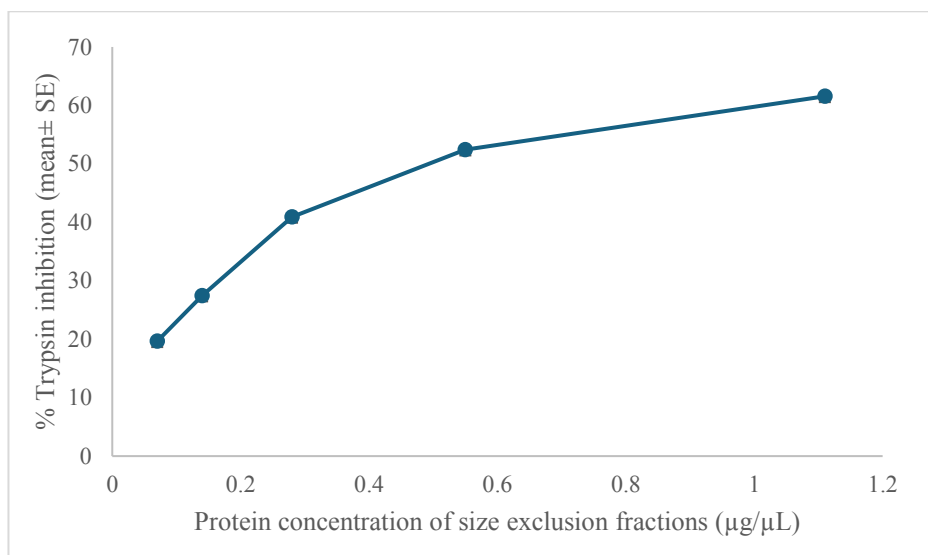


Fig. 2.4.6 Trypsin Inhibition of size exclusion fractions and its double diluted fractions

2.4.3.3 Ion exchange chromatography

The concentrated partially purified inhibitor from size exclusion chromatography was subjected to ion exchange chromatography. This was done in a Biorad NGC quest plus FPLC system with a 1 mL source Q column. A total of 1.02 mg protein was loaded onto column after column equilibration with 100 mM bicarbonate buffer, pH 9.0. Same buffer was used to wash the column before elution to remove unbound proteins. Elution of proteins was done with a gradient of NaCl in bicarbonate buffer pH, 9.0 from 0.0 to 1 M NaCl. A single prominent

peak was observed at 700 mM NaCl concentration (Figure 2.4.7). Trypsin inhibition of the fractions collected were calculated by trypsin inhibition assay. The elutes 4,5 and 6 were having more than 45% inhibition and high protein concentration (Table 2.4.6) (Figure 2.4.8).

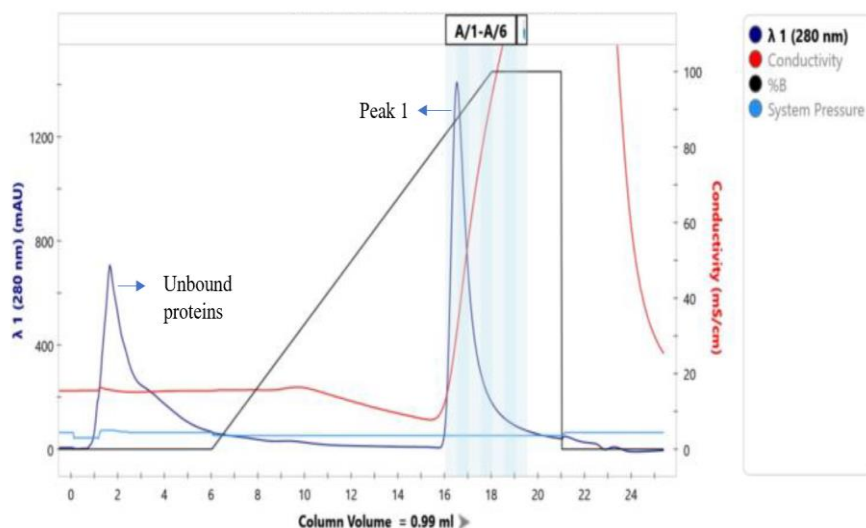


Fig. 2.4.7 Run report of ion exchange chromatography

Table 2.4.6 Percentage inhibition of elutes collected during ion exchange chromatography

Fractions during IEC	% Trypsin inhibition (Mean ± SE)	Protein concentration ($\mu\text{g}/\mu\text{L}$)
Fraction number		
1	15.94 ± 0.32	0.01 ± 0.00
2	25.43 ± 0.32	0.02 ± 0.00
3	33.58 ± 0.92	0.08 ± 0.00
4	48.66 ± 0.12	0.31 ± 0.00
5	58.39 ± 0.21	0.50 ± 0.00
6	51.70 ± 0.44	0.36 ± 0.00
7	38.81 ± 0.85	0.14 ± 0.00

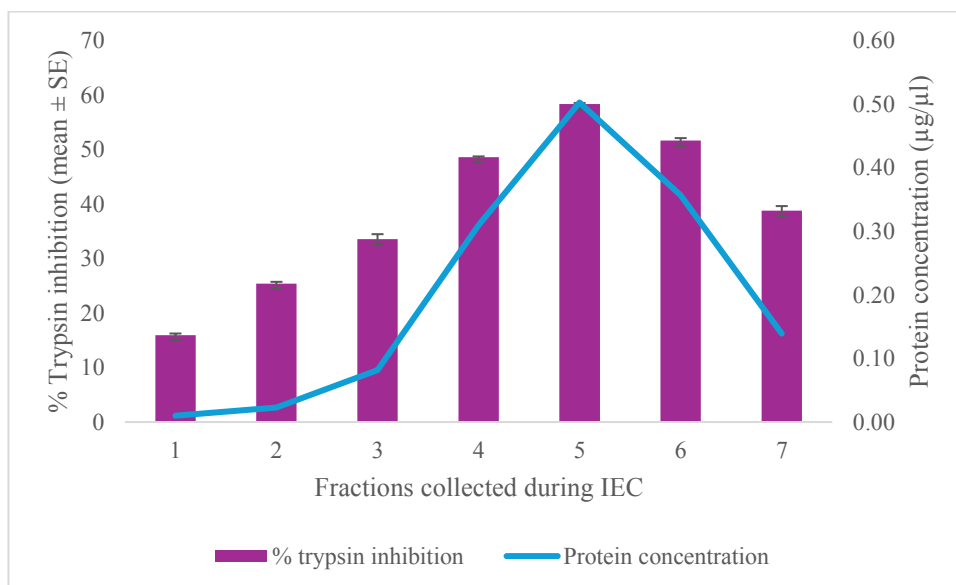


Fig. 2.4.8 Trypsin inhibition and protein concentration of elutes obtained during ion exchange chromatography. Histogram represent trypsin inhibition of elutes and line graph represent the protein concentration of corresponding fractions

Highest inhibition was obtained for the fractions 4, 5 and 6 and they were pooled together. The pooled sample had a trypsin inhibition of $54.08 \pm 0.37\%$ at a protein concentration of $0.50 \pm 0.00 \mu\text{g}/\mu\text{L}$. This pooled sample was used for purification by trypsin affinity chromatography.

2.4.3.4 Trypsin affinity chromatography

Trypsin affinity chromatography was done by batch method. Trypsin affinity resin of volume $400 \mu\text{L}$ was equilibrated with 100 mM Tris buffer, pH 8.0 and incubated with $50 \mu\text{g}$ protein ($100 \mu\text{L}$) pooled fraction from ion exchange chromatography. After elution, two fractions were collected. The trypsin inhibition of first fraction was $23.01 \pm 0.28\%$ with a protein concentration of $0.038 \pm 0.00 \mu\text{g}/\mu\text{L}$. The second fraction gave

a trypsin inhibition of $22.06 \pm 0.25\%$ with a protein concentration of $0.032 \pm 0.00 \mu\text{g}/\mu\text{L}$. These fractions were pooled together and gave a mean inhibition of $22.54 \pm 0.27\%$ at a protein concentration of $0.04 \mu\text{g}/\mu\text{L}$.

2.4.4 Determination of molecular weight of the purified inhibitor

Molecular weight of the purified inhibitor was calculated from the SDS-PAGE based on the molecular weight of standard proteins. Crude plant extract, 0-30% ammonium sulphate fraction, size exclusion fraction (after concentrating), ion exchange fraction (pooled fraction) and trypsin affinity fraction (pooled fraction) were loaded onto 10% acrylamide gel to assess the degree of purification in each step (Figure 2.4.9).

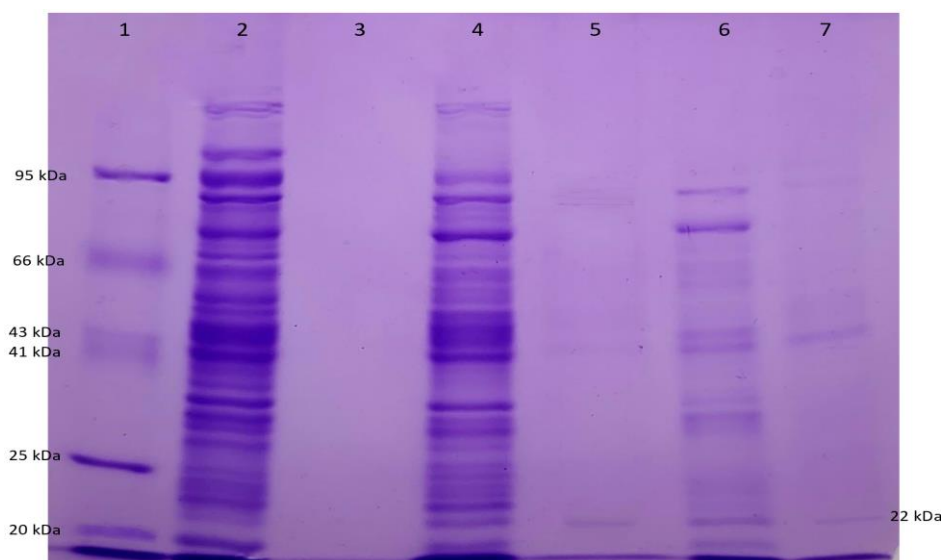


Fig. 2.4.9 SDS-PAGE showing protein profile during different stages of purification. 10% SDS-PAGE of proteins during different stages of purification of *Calopogonium mucunoides* seed extract. Lane 1. Protein marker, Lane 2. Crude extract (50 μg), Lane 3. Dye alone, Lane 4. 0-30% ammonium sulphate fraction (50 μg), Lane 5. Trypsin affinity fraction (10 μg), Lane 6. Size exclusion fraction (10 μg), Lane 7. Ion exchange fraction (10 μg)

The table 2.4.7 represents the protein concentration and trypsin inhibition of fractions in each stage of purification

Table 2.4.7 Protein concentration and trypsin inhibition of fractions in each stage of purification

Sample	Protein concentration ($\mu\text{g}/\mu\text{L}$)	Percentage trypsin inhibition (Mean $\pm$ SE)
Crude	11.94	87.99 $\pm$ 0.38
0-30% ammonium sulphate fraction	12.20	80.45 $\pm$ 1.35
Concentrated size exclusion fraction	1.11	58.29 $\pm$ 0.43
Pooled ion exchange fraction	0.50	54.08 $\pm$ 0.37
Pooled trypsin affinity fraction	0.04	22.54 $\pm$ 0.27

2.4.5 Toxicity studies

Toxicity of the trypsin inhibitor containing samples were checked on second instar *A. albopictus* larvae as per WHO protocol (WHO, 2005). For this, 25 larvae were introduced into 100 mL distilled water and treated using crude, 0-30% ammonium sulphate fraction and size exclusion fraction (concentrated fraction).

2.4.5.1 Toxicity of crude extract to *A. albopictus* larvae

Toxicity of crude extract was checked in *A. albopictus* larvae. For this, larvae were treated with crude and its serially double diluted fractions. A graph was plotted with the percentage mortality with respective

protein concentrations (Figure 2.4.10). The LC_{50} (48 hours) of crude was found to be $0.50 \pm 0.01 \mu\text{g/mL}$.

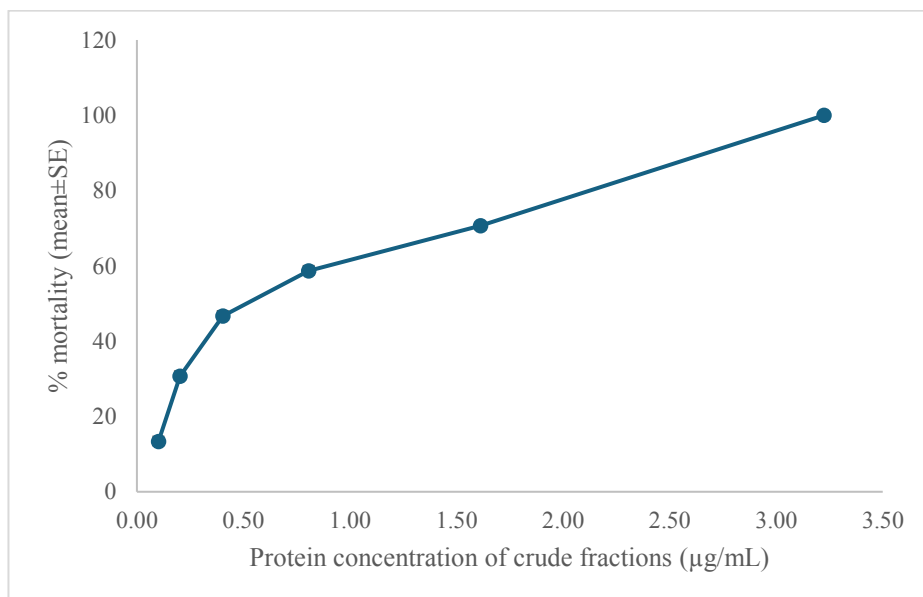


Fig. 2.4.10 Larvicidal activity of crude fractions on *A. albopictus* larvae

2.4.5.2 Toxicity of 0-30% ammonium sulphate fraction to *A. albopictus* larvae

Toxicity of 0-30% ammonium sulphate fraction was checked in *A. albopictus* larvae. For this, larvae were treated with 0-30% ammonium sulphate fraction and its serially double diluted fractions. A graph was plotted with the percentage mortality with respective protein concentrations (Figure 2.4.11). The LC_{50} (48 hours) of crude was found to be $0.48 \pm 0.01 \mu\text{g/mL}$.

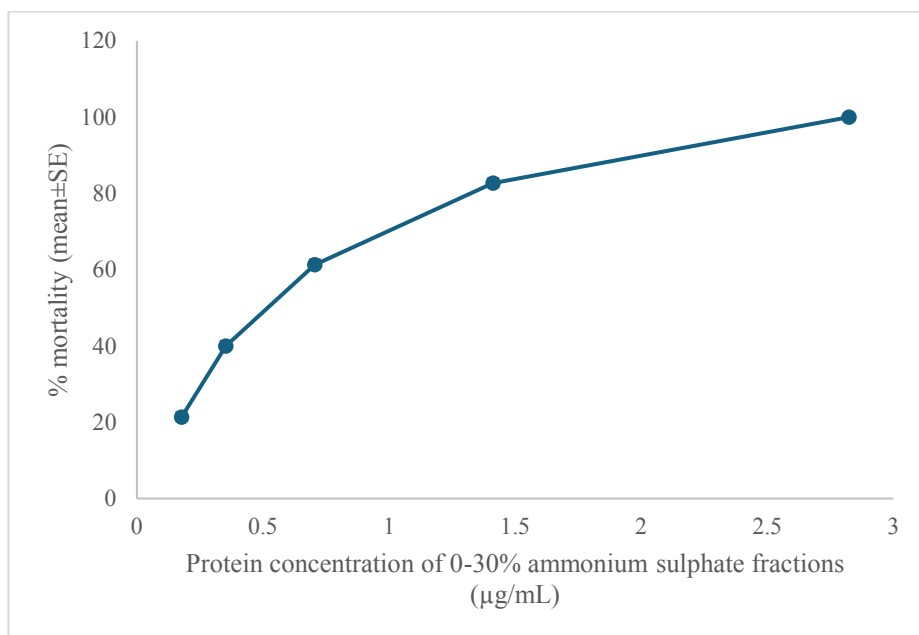


Fig. 2.4.11 Larvicidal activity of 0-30% ammonium sulphate fractions on *A. albopictus* larvae

2.4.5.3 Toxicity of size exclusion fraction to *A. albopictus* larvae

Toxicity of size exclusion fraction was checked in *A. albopictus* larvae. For this, larvae were treated with size exclusion fraction and its serially double diluted fractions. A graph was plotted with the percentage mortality with respective protein concentrations treated (Figure 2.4.12). The LC_{50} (48 hours) of crude was found to be 0.32 ± 0.00 µg/mL.

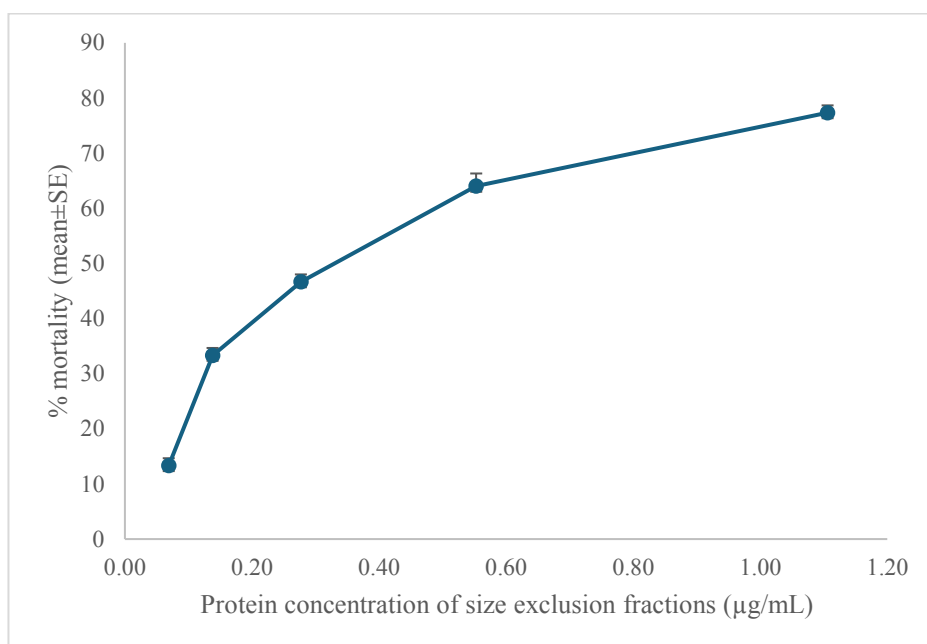


Fig. 2.4.12 Larvicidal activity of size exclusion fractions on *A. albopictus* larvae

2.5 DISCUSSION

Proteases are hydrolytic enzymes involved in protein metabolism. Proteases are also involved in the regulation of fate, localisation and activity of many proteins and modulating protein-protein interactions (López-Otín & Bond, 2008). The role of protease is hence directed to various biological processes like gene expression, angiogenesis, wound healing, immunity, apoptosis, etc. (López-Otín & Bond, 2008). Protease inhibitors are molecules that inhibit the activity of protease enzymes. Protease inhibitors act as a control mechanism on the overexpression or increased cellular concentration of proteases and hence have a key role in the maintenance of biological system. Protease inhibitors are present in microbes, plants, animals, etc. Plant protease inhibitors (PPIs) have potential application in pest control, as they target the digestive proteases of insect pests. Apart from this, PPIs have wide application in therapeutics and other biotechnological applications.

2.5.1 Screening of plant extracts for identifying trypsin inhibitors

In plants, protease inhibitors are typically found in storage organs or other aerial parts (De Leo et al., 2002). There are reports of presence of trypsin inhibitors purified from leaves, flowers and seeds of plants even though seeds are used in most studies (Tamir et al., 1996; Bhattacharyya et al., 2006; Satheesh & Murugan, 2011; Pontual et al., 2014; Dias & do Ceara Fortaleza, 2016). In this study, different parts of the plant, like leaves, seeds and fruits, were used to prepare plant extracts. A total of 45 plant extracts were screened for identifying extracts containing trypsin inhibitors. Out of the 45 plant extracts, 11 extracts had shown

trypsin inhibition more than 50%. The highest trypsin inhibition ($87.99 \pm 0.38\%$) was observed for the extract from seeds of *Calopogonium mucunoides* Desv., followed by seed extract of *Lantana camara* L. ($85.35 \pm 1.36\%$). The other 9 plant extracts with more than 50% trypsin inhibition were *Mucuna pruriens* (L.) DC. ($70.03 \pm 0.46\%$), *Coffea arabica* L. ($69.85 \pm 1.87\%$), *Ocimum tenuiflorum* L. ($62.10 \pm 0.76\%$), *Globba sessiliflora* Sims ($61.74 \pm 2.91\%$), *Mussaenda erythrophylla* Schumach and Thonn ($56.05 \pm 1.76\%$), *Eryngium foetidum* L. ($55.09 \pm 3.74\%$), *Cinnamomum cassia* (L.) J.Presl ($52.81 \pm 1.45\%$), *Plectranthus amboinicus* Lour. ($52.81 \pm 1.45\%$) and *Zingiber officinale* Roscoe ($51.96 \pm 1.70\%$). Seed extracts were used for *C. mucunoides*, *L. camara* and *M. pruriens*, and in all other cases, leaves were used for preparing extracts.

The plant extracts with more than 50% trypsin inhibition were subjected to proteinase K digestion, and six of the eleven extracts were found to have proteinaceous trypsin inhibitor. These are the extracts from *C. mucunoides*, *M. pruriens*, *Coffea arabica*, *O. tenuiflorum*, *G. sessiliflora* and *E. foetidum*.

Mucuna pruriens, commonly known as velvet bean is a climbing shrub and reported as a potential traditional medicine with anti-neurotoxic and antioxidant activities (Lampariello et al., 2012). The seed extract from *M. pruriens* gave trypsin inhibition to an extent of $85.35 \pm 1.36\%$. A trypsin inhibitor with antifungal properties was purified from the seeds of *M. pruriens* (Chandrashekharaiyah, 2013). A heat stable α -amylase inhibitor and a novel bowman birk inhibitor with a specific activity of 202.31 TIU/mg protein has been purified from *M. pruriens* (Bharadwaj

et al., 2018; Lone et al., 2021). Thus, it is possible that a particular plant can have a variety of protease inhibitor molecules which accounts for its special properties like antioxidant, antimicrobial, anti-carcinogenic etc. (J.-Y. Kim et al., 2005; Y. Liu et al., 2023).

Coffea arabica is an economically important plant widely used in food and beverages. The leaf extract from *C. arabica* gave a trypsin inhibition of $69.85 \pm 1.87\%$. Legumin-like proteins with insecticidal properties were isolated from *C. arabica* and *C. racemosa* seeds (M. B. Coelho et al., 2010). A lipid transfer protein with α -amylase inhibitor properties was purified from *C. canephora* seeds (Zottich et al., 2011). There are no reports on purified trypsin inhibitor from *C. arabica*, but hypoglycaemic effects of bioactive compounds in their leaf extract have recently been reported (G. O. Nascimento S. P. D. Marques & Trevisan, 2023).

Ocimum tenuiflorum, or holybasil, is a perennial plant widely used in ayurvedic medicines. The leaf extract from *O. tenuiflorum* was found to have $62.10 \pm 0.76\%$ trypsin inhibition. Its leaves and flowers are used as traditional medicines. *Ocimum tenuiflorum* and *O. sanctum* have been reported to have antiproliferative, antioxidant and antimicrobial activities (Utispan et al., 2020; Srichok et al., 2022; Sasi Kumar & D'Souza, 2024). There are no reports of purified trypsin inhibitors from *O. tenuiflorum*.

Globba sessiliflora is an ornamental herb species. The leaf extract from *G. sessiliflora* gave trypsin inhibition to an extent of $61.74 \pm 2.91\%$. The essential oils from the rhizomes and aerial parts of *G. sessiliflora* are

found to have myorelaxant, antioxidant, antipyretic, anti-inflammatory and antinociceptive properties (Ravendra Kumar & Mathela, 2012) (R. Kumar & Szczepaniak, 2017). The presence of a trypsin inhibitor with larvicidal activity to *Aedes aegypti* larvae is reported in *G. sessiliflora* (N et al., 2021). Biological activities of volatile extracts from *G. schomburgkii* and *G. pendula* are also studied (DOUNGCHAWEE et al., 2019; PHUONG et al., 2020).

Eryngium foetidum is a member of Apiaceae family with known pharmacological effects. The leaf extract from *E. foetidum* gave a trypsin inhibition of $55.09 \pm 3.74\%$. Anti-inflammatory and antioxidant properties of *E. foetidum* has been reported (MEKHORA et al., 2012; THOMAS et al., 2017). The leaf extract of *E. foetidum* has inhibitory activity on gut proteases of *Aedes albopictus* larvae (SARA et al., 2020). The four species of *Eryngium*, *E. pseudothoriifolium*, *E. thoriifolium*, *E. davisii*, and *E. falcatum*, are found to have cytotoxic effects on prostate carcinoma and endometrial cancer cells and antimicrobial effects on some bacteria and fungi (CELIK et al., 2019). Antiproliferative activity of *E. caucasicum* has been reported in a melanoma cancer cell line (FOROUHANDEH et al., 2022).

Calopogonium mucunoides, commonly called as wild ground nut, is a twining member of Fabaceae widely used as a cover crop. Their seeds are used as forage for animals during the dry season. The seed extract from *C. mucunoides* gave trypsin inhibition to an extent of $87.99 \pm 0.38\%$. The presence of a proteinaceous trypsin inhibitor in *C. mucunoides* is reported (NORIOKA et al., 1988) but not purified. The

antimicrobial and antidiarrheal properties *C. mucunoides* extract are studied (Enechi & Abugu, 2016; Emenike & Chukwuebuka, 2021).

2.5.2 Larvicidal activity of plant extracts containing trypsin inhibitors

Trypsin inhibitors target the protease, trypsin, the major gut protease in most insects, and hence can be used in the control of insect pests. Mosquito larvicidal potential of plant trypsin inhibitors has been reported (Pontual et al., 2014; Sasaki et al., 2015; Dias et al., 2017). In this study, plant extracts containing trypsin inhibitors were used to check larvicidal activity in *Aedes albopictus*. *Aedes albopictus* larvae were treated with extracts from *Calopogonium mucunoides*, *Coffea arabica*, *Mucuna pruriens*, *Globba sessiliflora*, *Eryngium foetidum* and *Ocimum tenuiflorum*. Larval mortality was observed in all extracts tested with varying degree. The highest mortality was observed for plant extract from seeds of *C. mucunoides* (100.00 ± 0.00 %) followed by *Coffea arabica* (66.67 ± 3.53 %), *M. pruriens* (50.67 ± 2.67 %), *G. sessiliflora* (38.67 ± 1.33 %), *E. foetidum* (18.67 ± 1.33 %) and *O. tenuiflorum* (5.33 ± 1.33 %). It is reported that protease inhibitors are mostly found in storage organs like seeds and tubers and they are found to have role in seed development and germination (Ussuf et al., 2001). So, it is possible that extracts prepared from seeds may possess more amount of protease inhibitors. However, there may be other factors like the specificity of inhibitors to cause insecticidal activity. The difference observed in the mosquito larvicidal activity of plant extracts screened in the study may be accounted for by the affinity of trypsin inhibitor present in the plant to trypsin and its concentrations.

In this study, *C. mucunoides* seed extract is found to have a proteinaceous trypsin inhibitor with the highest trypsin inhibition and larvicidal activity against *A. albopictus*. Therapeutic values of leaves of *C. mucunoides* are explored (Chizitere Emenike & Chukwuebuka, 2022). Substantial antioxidant and anti-inflammatory activity are reported from the ethanol extract of *C. mucunoides* leaves (Egele et al., 2025). Since there are no reports on purification of trypsin inhibitor from this plant, it was selected for purification.

2.5.3 Purification of trypsin inhibitor from the seeds of *Calopogonium mucunoides*

The seed extract of *Calopogonium mucunoides* gave trypsin inhibition to an extent of $87.99 \pm 0.38\%$. This plant was selected for purification as it contains proteinaceous trypsin inhibitor and there is no report of its larvicidal activity against *A. albopictus*. The proteinaceous nature of inhibitor is advantageous, as it can be used for expressing recombinant trypsin inhibitors.

The crude extract of *C. mucunoides* was subjected to ammonium sulphate fractionation, size exclusion chromatography, ion exchange chromatography and trypsin affinity chromatography to purify the trypsin inhibitor. Ammonium sulphate is commonly used in salting out process, as it induces precipitation of proteins in solutions at high concentrations (Duong-Ly & Gabelli, 2014). This process removes non-protein contaminants in the crude and concentrate trypsin inhibitor proteins in the extract. For differential precipitation, 3 different

concentrations of ammonium sulphate were used (0-30%, 30-50% and 50-70%).

The highest trypsin inhibition, $88.61 \pm 2.21\%$, was obtained for the fraction with 50-70% ammonium sulphate, followed by $88.54 \pm 3.66\%$ inhibition for 30-50% ammonium sulphate fraction and $80.44 \pm 0.00\%$ inhibition for 0-30% ammonium sulphate fraction. An increased inhibition in 30-50 and 50-70% ammonium sulphate fraction than 0-30% fraction has been reported in many studies (Oukasha & El-latif, 2015; Sajitha & Vadakkadath Meethal, 2024). In addition, second instar *Aedes albopictus* larvae were treated with these ammonium sulphate fractions, and toxicity analysed. Among the three fractions treated, 0-30% fraction showed the highest toxicity, with 100% mortality of larvae within 48 hours. The 30-50% ammonium sulphate fraction gave a mortality of $53.33 \pm 3.33\%$ and 50-70% ammonium sulphate fraction gave a mortality of $23.33 \pm 3.33\%$. So, based on the toxicity to mosquito larvae, 0-30% ammonium sulphate fraction was chosen for further purification. Application based purification of proteins are already reported (Remans et al., 2022).

The 0-30% ammonium sulphate fraction was further purified by size exclusion chromatography. For this, 1.67 mg protein was loaded into gel filtration column and eluted with 0.1 M bicarbonate buffer, 150 mM NaCl. During elution a single prominent peak and some small peaks were obtained. The elutes were screened by trypsin inhibition assay, and those having high inhibition were pooled and concentrated and further purified by ion exchange chromatography.

Ion exchange chromatography was done in a FPLC system with a 1 mL source Q column and 1.02 mg protein was loaded for purification. Protease inhibitors were purified by ammonium sulphate precipitation, size exclusion and ion exchange chromatography from soap nut (Gandreddi et al., 2015). On ion exchange chromatography, proteins eluted as single peak. But on SDS-PAGE, it was seen that the peak contains multiple protein bands. Thus, further purification was done in trypsin affinity chromatography. Elution yielded a 22 kDa protein band in 10% SDS-PAGE. The purified inhibitor was named as CmTI (*Calopogonium mucunoides* trypsin inhibitor) and inhibited trypsin to an extent of 22.54 ± 0.27 % at a protein concentration of $0.04 \mu\text{g}/\mu\text{L}$. The yield of the trypsin inhibitor was comparatively lower with $0.1 \mu\text{g}$ pure inhibitor per gram wet weight of seed. Thus, further characterisation including kinetic analysis has not been done. The gene coding for the CmTI needs to be cloned and expressed in a suitable expression vector such as bacteria, yeast or algae for large scale purification and further characterisation and toxicity analysis.

2.5.4 Determination of molecular weight of purified inhibitor

The trypsin inhibitor, CmTI purified from *C. mucunoides* gave a single band on SDS-PAGE. The subunit molecular weight of CmTI determined from SDS-PAGE based on the molecular weight of the standard proteins was 22 kDa. Many of the plant protease inhibitors are small molecular weight proteins ranging from 5- 25 kDa (Paul & Bhattacharjee, 2024). Of the 2 categories of plant protease inhibitors, Bowman-Birk type inhibitors in general are small molecular weight proteins/peptides range in molecular weight from 8-10 kDa (Odani & Ikenaka, 1973). Some of

the Bowman-Birk inhibitors include 11.4 kDa trypsin inhibitor from *Solanum surattense*, 7 kDa protease inhibitor from *Maclura pomifera*, 8 kDa protease inhibitor from *Vigna mungo*, etc. (Herwade et al., 2021; Indarte et al., 2017; Prasad, Dutta-Gupta, et al., 2010). The other category, that is, Kunitz-type protease inhibitors, range in molecular weight from 8-22 kDa (M. Laskowski & Qasim, 2000). Some examples of Kunitz-type inhibitors include a 20 kDa trypsin inhibitor from *Inga vera*, 23 kDa inhibitor from *Theobroma cacao*, 21 kDa inhibitor from *Hibiscus acetocella* (da Silva Bezerra et al., 2016; do Amaral et al., 2022; Sajitha & Vadakkadath Meethal, 2024).

2.5.5 Toxicity studies on *Aedes albopictus* larvae

In mosquito larvae, trypsin like proteases are the predominant gut proteases. For example, in *Culex pipiens* larvae, serine proteases represent the major gut protease (Amrutha et al., 2018). Thus, inhibiting trypsin like serine protease will hamper the digestion of proteins, leading to delay in development and mortality. During purification, the trypsin inhibitor enriched fractions were tested on *A. albopictus* larvae to assess its effect on larval development and mortality. The LC₅₀ (48 hours) value for the ammonium sulphate fraction (0-30%) was 0.48 ± 0.01 µg/mL. On further purification, the size exclusion fraction was more effective with LC₅₀ (48 hours) value of 0.32 ± 0.00 µg/mL. Size exclusion fraction was further purified by ion exchange followed by affinity chromatography on trypsin-Sepharose. The yield of purified CmTI was very low (0.1 µg per mg seed), hence the purified inhibitor could not be used for toxicity studies. Thus, the size exclusion fractions were used for toxicity analysis. There are two

other high molecular weight contaminating polypeptides in size exclusion fraction other than the 22 kDa polypeptide. The intensity of high molecular weight polypeptides was much less in the size exclusion fraction compared to ammonium sulphate fraction. Considering the fact that the LC₅₀ (48 hours) value of size exclusion fraction was lower than that of ammonium sulphate fraction, the observed mortality will be the result of toxicity of 22 kDa trypsin inhibitor. Trypsin inhibitor purified from the flowers of *Moringa oleifera* by trypsin affinity chromatography had interfered the survival and development of *Aedes aegypti* larvae (Pontual et al., 2014). Proteinase inhibitor isolated from *Adenanthra pavonima* seeds had shown short-term and long-term developmental effects on *Aedes aegypti* larvae with lower rate of survival (Sasaki et al., 2015). The trypsin inhibitor purified from *Cassia leiandra* seeds caused loss of activity of midgut proteases by 50% at 4.65 µM concentration (Dias et al., 2017). The, purified CmTI has the potential to be used as a mosquito larvicide. For this, the gene coding for the inhibitor can be expressed in bacteria or yeast and after killing the cells, it can be used to feed mosquito larvae.

2.6 SUMMARY

A total of 45 plant extracts were screened for identifying trypsin inhibitors by trypsin inhibition assay and eleven extracts were found to have more than 50% trypsin inhibition. These extracts were digested with proteinase K to check whether the inhibitor is a protein or not. Out of the eleven extracts, six extracts had proteinaceous trypsin inhibitors. When the *Aedes albopictus* larvae were treated with the extracts containing proteinaceous trypsin inhibitors, highest percentage mortality was observed for the extract from the seeds of *Calopogonium mucunoides*. Incidentally, this extract had the highest trypsin inhibition ($87.99 \pm 0.38\%$) among the plant extracts tested. This plant was selected for purification of the inhibitor. Thus, the extract from *C. mucunoides* were used to purify the trypsin inhibitor by ammonium sulphate fractionation, size exclusion chromatography, ion exchange chromatography and trypsin affinity chromatography. The IC_{50} for the crude, 0-30% ammonium sulphate fraction and size exclusion fraction were 0.72 ± 0.01 , 0.78 ± 0.01 and $0.51 \pm 0.00 \mu\text{g}/\mu\text{L}$, respectively. On toxicity analysis, the crude, 0-30% ammonium sulphate fraction and size exclusion fraction were toxic to *Aedes albopictus* larvae with an LC_{50} (48 hours) of 0.50 ± 0.01 , 0.48 ± 0.01 , and $0.32 \pm 0.00 \mu\text{g}/\text{mL}$, respectively. The purified trypsin inhibitor, CmTI had a subunit molecular weight of 22 kDa. The yield of purified inhibitor, CmTI was 0.1 μg per gram of seed (wet weight). The gene coding for the inhibitor can be expressed in a suitable host system, such as bacteria, yeast, or algae in bacteria or yeast and after killing the cells, it can be used to feed mosquito larvae.

RECOMMENDATIONS

Mosquitoes are biological vectors of many infectious diseases which affect more than half of the world population. In spite of newer strategies developed for mosquito control, the outbreak of mosquito-borne diseases in different parts of the world is a common occurrence. Chemical control measures using different insecticides contributed to the development of resistance in mosquitoes. Also, the chemical insecticides are harmful to the environment and cause health hazards. Through this work, we explore two methods for mosquito control, which are effective as well as environment friendly. The first one focusses on an ovarian peptide, naturally synthesised in mosquitoes and the other one uses a new trypsin inhibitor from *Calopogonium mucunoides* seed extract.

Trypsin modulating oostatic factor (TMOF), a decapeptide isolated from the ovaries of *Aedes aegypti*, has lethal effect in adult and larval mosquitoes. We have cloned and expressed the gene coding for TMOF in bacteria after codon optimisation. *Aedes albopictus* larvae when treated with recombinant TMOF expressed in bacteria led to significant mortality within 48 hours. An active mutant of the TMOF peptide was also identified which is more lethal to *A. albopictus* larvae. The recombinant TMOF and recombinant mutant TMOF was found to have toxicity to *Culex quinquefasciatus* larvae, but not to non-target organisms like *Luprops tristis*, *Aplocheilus lineatus* and *Drosophila melanogaster*. Stability of recombinant TMOF and mutant TMOF was

also studied in different pH and temperature. An Indian patent (Patent number 554267) is obtained for this work entitled as “An insecticidal preparation having trypsin modulating oostatic factor (TMOF)”. Field trials need to be conducted to use it as an insecticide. Also, optimisation of conditions for large scale production of recombinant TMOF in bioreactors is recommended.

The recombinant TMOF and mutant TMOF also modifies the gut bacterial composition of mosquito larvae. The role of gut bacteria in the toxicity of the insecticide needs to be investigated.

From a total 45 plant extracts screened, 6 extracts containing trypsin inhibitor showed mosquito larvicidal activity. Only one of the plant extracts, *Calopogonium mucunoides*, which showed the highest trypsin inhibition, was used for further purification of the trypsin inhibitor and toxicity analysis. *Calopogonium mucunoides* crude extract, 0-30% ammonium sulphate fraction and partially purified size exclusion fraction produced significant larvicidal activity in *A. albopictus* mosquito. The toxicity studies using purified trypsin inhibitors from the other five plant extracts (*Mucuna pruriens*, *Coffea arabica*, *Eryngium foetidum*, *Globba sessiliflora* and *Ocimum tenuiflorum*) is recommended. Since the yield of purified inhibitor from *Calopogonium mucunoides* was very low, it is recommended to clone the gene coding for *C. mucunoides* trypsin inhibitor and express in a suitable host like yeast or algae to use for control of mosquito larvae. Also field studies to establish the efficacy of *C. mucunoides* trypsin inhibitor in the field is recommended.

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PRESENTATIONS AND PUBLICATIONS

Presentations

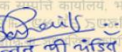
- Presented a paper in the International Conference on “Emerging and Re-emerging Infectious Vector-borne & Zoonotic Diseases: Impact of Climate Change and Global Warming on the title “Cloning of recombinant Trypsin Modulating Oostatic Factor and study of its toxicity on the larvae of *Aedes albopictus* (Skuse) (Diptera; Culicidae)” in 2023.
- Presented a paper in the 2nd International Conference on Research methodology in the title “Identification of plant extracts expressing trypsin inhibitors with potential larvicidal activity against *Aedes albopictus* (Skuse) (Diptera; Culicidae)” in 2023.

Publications

- Published a paper entitled “Bacterially expressed recombinant TMOF induces mortality and gut microbial alterations in *Aedes albopictus* larvae” in Scientific Reports
- Published a paper entitled “Isolation of a new trypsin inhibitor from *Calopogonium mucunoides* and its toxicity to the larvae of the Dengue vector, *Aedes albopictus*” in international journal of Entomology research

- Published a review paper entitled “Trypsin modulating oostatic factor and its application as a larvicide for mosquito control: A review” in international journal of mosquito research

**Certificate of Patent obtained for the insecticidal preparation
using recombinant TMOF expressed in *Escherichia coli***

 INTELLECTUAL PROPERTY INDIA GOVERNMENT OF INDIA TRADE MARKS & PATENTS OFFICE		 Government of India Ministry of Commerce & Industry		क्रम सं./SL No. ०४५१९४१६९ 	
पेटेंट कार्यालय, भारत सरकार The Patent Office, Government of India					
पेटेंट प्रमाण पत्र Patent Certificate					
(Rule 74 of The Patents Rules)					
आवेदन सं. / Application No. २०२४५१३०३७०७		फाइल करने की तारीख / Date of Filing २७/०४/२०२४			
आविष्कारकों का नाम / Name of Inventor(s) १.VADAKKADATH MEETHAL, Kannan 2.MUNDOLI, Deepthi		पेटेंटर / Patentee University of Calicut			
प्रमाणित किया जाता है कि पेटेंटी को, उपरोक्त आवेदन में ब्यापकृत AN INSECTICIDAL PREPARATION HAVING TRYPSIN MODULATING OOSTATIC FACTOR (TMOF) नामक आविष्कार के लिए, पेटेंट अधिनियम, १९७० के उपबंधों के अनुसार आज तारीख अर्थात् २०२४ के सत्ताईसवें दिन से बीस वर्ष की अवधि के लिए पेटेंट अनुदत्त किया गया है। It is hereby certified that a patent has been granted to the patentee for an invention entitled AN INSECTICIDAL PREPARATION HAVING TRYPSIN MODULATING OOSTATIC FACTOR (TMOF) as disclosed in the above mentioned application for the term of 20 years from the 27th day of April 2024 in accordance with the provisions of the Patents Act, 1970.					
					
 डॉ. पी. एन. शर्मा Controller of Patents					
दिनांक : १३/११/२०२४ Date of Grant : 13/11/2024					
Note :- The fees for renewal of this patent, if it is to be maintained, will fall due on 27th day of April 2026 and on the same day in every year thereafter.					

NCBI submissions

GenBank submission of the synthetic gene construct encoding recombinant TMOF

Synthetic construct recombinant trypsin modulating oostatic factor gene, complete cds

GenBank: PX309362.1

[FASTA](#) [Graphics](#)

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LOCUS PX309362 996 bp DNA linear SYN 17-SEP-2025
DEFINITION Synthetic construct recombinant trypsin modulating oostatic factor gene, complete cds.
ACCESSION PX309362
VERSION PX309362.1
KEYWORDS .
SOURCE synthetic construct
ORGANISM [synthetic construct](#)
other sequences; artificial sequences.
REFERENCE 1 (bases 1 to 996)
AUTHORS Vadakkadath Meethal, K. and M, D.
TITLE Direct Submission
JOURNAL Submitted (08-SEP-2025) Department of Zoology, University of Calicut, Thenhipalam, Malappuram, Kerala 673635, India
COMMENT ##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##
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BioProject submission of metagenomic analysis

Effect of recombinant trypsin modulating oostatic factor on gut microbiota of *Aedes albopictus* larvae Accession: PRJNA1322081 ID: 1322081

The gut microbiota has a significant role in pathogen transmission and mosquito biology. To check whether treatment with recombinant TMOF alters the gut microbiota, the second instar larvae of *A. albopictus* were exposed to LC10 (48 hours) concentration of recombinant TMOF and allowed to grow until the fourth instar stage. Bacterial composition of the treated larvae was assessed using 16S rRNA gene sequencing and compared with that of the control. [Less...](#)

Accession	PRJNA1322081
Data Type	Raw sequence reads
Scope	Multispecies
Submission	Registration date: 10-Sep-2025 University of Calicut

Project Data:

Resource Name	Number of Links
SEQUENCE DATA	
SRA Experiments	2
OTHER DATASETS	
BioSample	2

BioSample / SRA

BioProject: PRJNA1322081 Effect of recombinant trypsin modulating oostatic factor on gut microbiota of Aedes albopictus larvae

BioSample: SAMN51228013 Metagenome or environmental sample from gut metagenome

SRR35314951 gut metagenome of Aedes albopictus larvae treated with recombinant trypsin modulating oostatic factor

SRA accession

Status Released

Release date 2025-09-10

Created 2025-09-10 11:03

Updated 2025-09-10 13:39

Experiment	Library ID	Library strategy	Library source	Library selection	Library layout	Platform	Instrument
SRX30416822	Lib02	AMPLICON	METAGENOMIC	PCR	SINGLE	ILLUMINA	Illumina NovaSeq 6000

Experiment description 16S rRNA V3V4 region was PCR amplified using 341F and 806R primers and sequenced on Illumina NovaSeq 6000 platform

File name	File type	MD5SUM
Gut_TMOF.fastq	fastq	367a8eb4cfff545554dca7754fb35af7

Total number of spots	Total number of bases	GC percentage
64921	38255294	57.60

Organism	Percent aligned	Spot count
Microbacterium paraoxydans		3958
Klebsiella pneumoniae subsp. ozaenae		996
Kaistia adipata		460
Bosea		264
Agrococcus casei		230
Bacillus badius		156
Rubidimonas crustatorum		153
Cellulosimicrobium		100
Paenibacillus contaminans		93
Brevibacillus parabrevis		45

BioSample / SRA

BioProject: PRJNA1322081 Effect of recombinant trypsin modulating oostatic factor on gut microbiota of Aedes albopictus larvae

BioSample: SAMN51228012 Metagenome or environmental sample from gut metagenome

SRR35314952 gut metagenome of Aedes albopictus larvae

SRA accession

Status Released

Release date 2025-09-10

Created 2025-09-10 11:03

Updated 2025-09-10 13:01

Experiment	Library ID	Library strategy	Library source	Library selection	Library layout	Platform	Instrument
SRX30416821	Lib01	AMPLICON	METAGENOMIC	PCR	SINGLE	ILLUMINA	Illumina NovaSeq 6000

Experiment description 16S rRNA V3V4 region was PCR amplified using 341F and 806R primers and sequenced on Illumina NovaSeq 6000 platform

File name	File type	MD5SUM
Gut_Control.fastq	fastq	54678d7b9b8070106414b58b5c160ab9

Total number of spots	Total number of bases	GC percentage
127767	75511870	55.98

Organism	Percent aligned	Spot count
Kaistia adipata		2661
Bacillus badius		900
Microbacterium paraoxydans		806
Bosea		748
Variovorax ureilyticus		730
Methylobacterium organophilum		398
Agrococcus casei		194
Bacillus capparidis		137
Dermacoccus abyssi		135
Clostridium cavendishii DSM 21758		127