Chapter 6

SUMMARY

The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them.

~ William Lawrence Bragg
6. Summary

Mosquito-borne diseases such as Malaria, Filaria, Yellow Fever, Dengue, Chikunguniya, and Japanese encephalitis cause extensive morbidity and mortality, globally. To curb the vectors of these diseases synthetic insecticides are a popular choice all over the world. However, these have a drawback of causing environmental pollution and also development of resistance in the target insects which together made the search for an alternate tool imperative. Biological control by harnessing natural enemies and entomopathogens is of interest because of their target precision, handler safety, ecological safety and host specificity. Besides, the commercial success of some entomopathogenic fungi in pest control/integrated vector control makes them an attractive option for control of disease vector.

This thesis presents analyses of the pathogenicity and virulence of promising indigenous mosquito-pathogenic fungal strains against larvae of *Anopheles stephensi*, *Culex quinquefasciatus* and *Aedes aegypti*. The invasive process of mosquito-pathogenic fungi involved, active principles and bio-safety of the promising fungi to non-target organisms were studied. Extensive review of literature was carried out on these different aspects with an attempt to consolidate available data on mosquito-pathogenic fungi.

The entomopathogenic fungi were sourced from 6 different localities of Goa and 10 isolates of fungi were recovered in pure culture either by baiting larvae or sourced from infected mosquitoes and insects. The isolation of fungi from nature and the indigenous strains obtained from Goa University Fungus Culture Collection (GUFCC) indicates that Goa has a rich bio-diversity of entomopathogenic fungi. Using the available taxonomic keys 7 fungal isolates were identified up to species level as follows:
1. Isolate GUFCC 5039: *Gliocladium roseum* Bain.

2. Isolate GUFCC 5040: *Gliocladium roseum* Bain.

3. Isolate GUFCC 5072: *Penicillium citrinum* Thom


5. Isolate D4: *Aspergillus niger* Tiegh.

6. Isolate D1: *Fusarium oxysporum* Schltdl.


Bioassays of these isolates were carried out using WHO recommended standard method either to screen or test the bio-efficacy of the mosquito-pathogenic fungi. The mortalities were corrected using Abbott’s formula wherever necessary. Larval mortality was determined and corrected mortality data were subjected to one way or two-way Analysis of Variance (ANOVA) using software SPSS version 16. The lethal dose/concentration (LD50/LC50 and LD90) required to kill 50% or 90% of larvae was calculated by Probit analysis.

Preliminary testing of five fungal isolates for larvicidal activity revealed percent mortalities in the range of 25-100%. *Gliocladium* sp. isolate GUFCC 5044 caused 100% mortality on 24 h exposure, *T. atroviride* caused 70% mortality on 72 h exposure and *P. citrinum* caused 100% mortality on 48 h exposure in *Cx. quinquefasciatus* larvae and hence they were selected for further studies.

Mode of invasion studies in the three fungal isolates revealed different routes of invasion. In *Gliocladium* sp. (isolate GUFCC 5044) cuticle of the *Cx. quinquefasciatus* 3rd instar larva seemed to be the preferred invasion route. The fungus showed profuse mycelial growth on the cuticle and the haemocoel was ramified with fungal mycelia and the organ tissues were disrupted at 24 h exposure. Also, melanization was observed around invading hyphae in the midgut. In *T. atroviride* and *P. citrinum*
invasion seemed to be through the gut of the larva with rapid ingestion of the conidia, packing the gut in 2 h.

In the *An. stephensi* larvae on exposure to *P. citrinum* intense melanization was observed. The degree of melanization differed in exposed individuals. This immune response in *An. stephensi* larvae was faster compared to *Cx. quinquefasciatus* and *Ae. aegypti* larvae.

In the *Cx. quinquefasciatus* larvae exposed to *P. citrinum* extensive mycosis accompanied by emergence of hyphae through the cuticle from the head, thorax, abdomen, anal siphon traversing the gut and haemocoel was observed. Not all the larvae showed mycelial growth; some were dead without any growth (50-60%) indicating role of mycotoxins. No mycelial growth was observed in case of *Ae. aegypti* and *An. stephensi* exposed to *P. citrinum*.

Fate of fungal spores after gut-passage through *Cx. quinquefasciatus* larvae showed that the ingested spores of *T. atroviride* were viable as they grew on Corn Meal Agar (CMA) medium.

SEM studies on the *Cx. quinquefasciatus* larvae exposed to *P. citrinum* revealed that conidial attachment to the surface was abundant on the respiratory siphon and anal lobes, slightly lesser in the thoracic region and in patches on the rest of the body. *P. citrinum* conidia were found sparsely on the surface of exposed *Ae. aegypti* larvae.

SEM of dissected gut of *Cx. quinquefasciatus* showed abundant conidia of *P. citrinum* and, of these, a few were germinated with appressorium formation substantiating that infection and invasion through gut was predominant in this vector species.

SEM of the faecal pellet of *Ae. aegypti* and *Cx. quinquefasciatus* larvae showed intact *P. citrinum* conidia enveloped by peritrophic membrane.
Attenuation of virulence is seen in majority of entomogenous fungi *Gliocladium* sp. (isolate GUFCC 5044) showed change in morphology; *T. atroviride* showed reduction in virulence while *P. citrinum* was the most stable and showed no morphological change or loss of virulence over a period of three years on repeated sub culturing in artificial media. Hence *P. citrinum* was chosen for carrying out studies on bio-efficacy, metabolites and safety to NTO and enzyme study.

The bio-efficacy of isolate *P. citrinum* was assessed by performing bioassays against 3rd instar larvae of the three vector species. The highest dose that each vector species was exposed to was $10 \times 10^6$ spores/ml in *Cx. quinquefasciatus* larvae, in *An. stephensi* larvae $20.02 \times 10^6$ spores/ml and in *Ae. aegypti* larvae $89.44 \times 10^6$ spores/ml. This dose in *Cx. quinquefasciatus* larvae caused average mortalities of 72% and 88% on 24 h and 48 h exposure respectively; in *An. stephensi* larvae 54.66% and 84% respectively and in *Ae. aegypti* larvae 48%, 61.33% and 62.7% average percent mortality on 24 h, 48 h and 72 h exposure respectively; showing thereby that *Cx. quinquefasciatus* larvae were most susceptible followed by *An. stephensi* and the least susceptible were *Ae. aegypti*, the latter requiring a much higher dose.

The metabolites of *T. atroviride* tested against *Cx. quinquefasciatus* larvae were larvicidal with LC$_{50}$ value of $26.36 \mu l/ml$ obtained on 24 h exposure. On screening, *P. citrinum* metabolites resulted in 30% mortality in *Ae. aegypti*, 60% in *An. stephensi* and 82.5% in *Cx. quinquefasciatus* larvae on 24 h exposure in dose range of 4-10 $\mu l/ml$.

The results of bioassays with metabolites from submerged cultures of *T. atroviride* and *P. citrinum* showed production of larvicidal toxins. It is assumed that similar toxins could be produced by the conidia in the gut of the larvae.
The age of the conidia seemed to play an important role in the virulence of toxic metabolites. Hence, for separation of active fractions of *P. citrinum* metabolite, 14 d culture was used as this was found to be more virulent compared to the one from 20 d culture. *P. citrinum* metabolites extracted in methanol were highly larvicidal producing 100% mortality in *Cx. quinquefasciatus* on 48 h exposure and *An. stephensi* larvae were found slightly less susceptible as indicated by 94.1% mortality.

Being promising, methanol extract of *P. citrinum* on further separation and partial purification was tested for larvicidal activity against larvae of *Cx. quinquefasciatus* which was the most susceptible to both conidia and metabolites. The Pet ether fraction at a dose of 0.73 mg ml⁻¹ showed high larvicidal activity against *Cx. quinquefasciatus* with 98% mortality on 48 h of exposure. The chloroform fraction caused 43% mortality on 48 h exposure at a dose of 0.87 mg ml⁻¹.

Purification of the active fractions methanol, pet ether and chloroform carried by TLC in the present work revealed multiple spots using 30% ethyl acetate in Pet Ether as the solvent. The second solvent system i.e. 25% ethyl acetate in Pet Ether each fraction loaded showed two spots each.

As the mortality in the chloroform fraction was below 50%, it was not investigated further. However, the remaining surviving larvae exposed to this fraction did not pupate indicating growth inhibitory activity and this area warrants further investigation.

The spectral pattern of the initial NMR of unprocessed Pet ether fraction and the NMR of organic layer obtained after processing of Pet ether fraction positively corresponded. IR spectra of the aqueous layer clearly showed the absence of a prominent –OH group pointing that the active compound is not an acid. Further
elucidation of the structure of the active compound needs to be carried out and structure-activity relationships studied.

*P. citrinum* showed highest protease activity (7.78 U ml\(^{-1}\) min\(^{-1}\)) at 168 h of incubation and *G. roseum* showed lower activity (4.82 U ml\(^{-1}\) min\(^{-1}\)) at 168 h of incubation. *P. citrinum* when assayed for chitinase, the enzyme production gradually increased with the passage of time and highest enzyme activity (0.012 U min\(^{-1}\)) was obtained after 72 h of incubation and thereafter decreased at 96 h.

Evaluation of bio-safety of *P. citrinum* to non target organisms (NTO) was done by field collection and acclimatization of larvivorous fish *Aplocheilus blocki* in the laboratory and then exposing them to the conidial suspension of *P. citrinum*. Similarly, field collection, establishment and acclimatization in the laboratory of non-target Heteropteran water bugs *L. fossarum fossarum* was done followed by their exposure to conidia and metabolites of *P. citrinum*. In both the NTOs, there was no apparent effect of the *P. citrinum* conidial suspension though low grade mortality (about 4%) was seen in the water bugs. Further, the exposure of *L. fossarum* to metabolites of *P. citrinum* extracted with methanol did not result in any sluggishness or mortality.

As has been mentioned earlier, there was no decline in the pathogenicity of *P. citrinum* on prolonged subculturing on artificial media. The conidial suspension stored in the refrigerator was active even after one year. Also, the virulence of metabolites of *P. citrinum* did not diminish after storage in the refrigerator for a period of thirteen months. The results of larvicidal bioassays showed that all the three test vector species larvae used in this study were susceptible to *P. citrinum* conidia as well as metabolites with susceptibilities in increasing order from *Ae. aegypti, An. stephensi* to *Cx. quinquefasciatus.*
Enzyme assays have revealed protease and chitinase production. Effect on NTOs was minimal. Hence *P. citrinum* can be considered as an indigenous good vector control candidate and as such appears to have good scope for development into a mosquito larvicide. It could be formulated and commercialized after extensive and multicentric field trials and confirmation of environmental safety and against NTOs.