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Metabolites of *Metarhizium anisopliae* against Malaria Vector and Non Target Organisms

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Abstract

Vector borne diseases are affecting millions of people annually. Entomopathogenic fungi toxicity on malaria vectors increase mortality rates, and are less effective on nontarget organisms, thus representing a control measure that could be used in integrated programmes. Before metabolites of entomopathogenic fungi can be integrated into control programmes, an effective delivery system must be developed.

In the laboratory, *Metarhizium anisopliae* were cultured in complete broth media. All instars of *Anopheles stephensi* and aquatic non-target organisms were passively treated with culture metabolites by different concentrations. The mortality values were subjected by probit analysis for a period of 24, 48 and 72 hours, respectively.

Fugal filtrated metabolites significantly increased mortality in the exposed larvae of malaria mosquitoes and had a less adverse effect on nontarget organisms. The highest LC_{99} (663.77 ppm) values were observed in fourth instars of *A. stephensi* and found lowest LC_{99} (254.09 ppm) values in first instars. First instars tolerated applications better than other instars. A positive correlation was found between concentrations of fungal filtrate and percentages mortalities for the all instars of malaria vector. All nontarget organisms were not found susceptible at lower concentrations. Our study marks the first to use of extracellular secondary metabolites of entomopathogenic fungus against all instars of *A. stephensi* and nontarget organisms. The good biological stability of extracellular metabolites makes this a promising alternative to mycelium and conidial based larvicides. It could be regarded as fungal –based natural larvicides for the use of vector control strategy.

Keywords: Metabolites; *Metarhizium anisopliae*; *Anopheles stephensi*

Introduction

Malaria is a vector borne disease of public health concern in tropical and subtropical parts of the world, affecting millions of people annually. Despite progress in drugs and vaccines development, no effective and acceptable multivalent vaccines are currently licensed. The drugs are becoming less effective due to increasing resistance. The most successful method to minimize the incidence of mosquito borne diseases is to eradicate and control the mosquito vectors, which is performed principally by systematic treatment of the breeding places through a combination of environmental management and application [1]. The aquatic part of the mosquito lifecycle consists of an egg stage, four larval stages and a pupae stage. Larval control has a convincing history of malaria eradication and recent studies have also shown this approach to be highly effective [2-7]. It is therefore worthwhile to investigate the ability of larvicides to control mosquito larvae and the feasibility of their operational use [8].

The drastic effects of chemical larvicidal-based intervention measures for the control of disease vectors have received wide public concern and have caused many problems like environmental pollution, insecticide resistance, resurgence of pest species, and toxic hazards to human and nontarget organisms.

There is therefore an increasing interest in alternative and integrated vector control methods, including biological control. Particular focus is on entomopathogenic fungi, especially species belonging to the genera *Lagenidium*, *Coelomomyces*, *Beauveria*, and *Metarhizium* spores. These general have proven effective in infecting and killing larvae of mosquitoes. Applying spores of *M. anisopliae* for the control of mosquito larvae, however, has certain limitations. Fungal spores are hydrophobic by nature so when applied in an aquatic environment, they clump together, reducing the area that is effectively covered. As a result, massive amounts of fungal spores are required [8]. In addition, fungal spores are more exposed to UV radiation and high temperatures, which are known to negatively affect spore persistence and germination rate [9,10].

In contrast, the extracellular secondary metabolites produced by entomopathogenic fungi have become a focus of interest of insect pathologists [11]. Some entomopathogenic fungi, *Beauveria bassiana*, *Paecilocyces fumosoroseus*, and *Fusariumm oniliforme* produce mosquito larvicidal compounds like cyclodepsipeptide, including Beauvericin [11] and the enniatin complex [12]. In India, extracellular secondary metabolites of *Lagenidium giganteum* [1], *Chrysosporium* [13,14], and many actinomycetes [15] have been screened successfully in the laboratories.

To date, there are very limited reports on the efficacy of extracellular secondary metabolites of *M. anisopliae* against larvae of malaria vectors with reference to nontarget aquatic organisms studied. Owing to the importance of *M. anisopliae* metabolite properties after filtration of broth, we tried to investigate their efficacious role to control malaria vectors as well as observed adverse effects on beneficial aquatic nontarget organisms.

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Material and Methods

Maintenance of mosquito larvae in the laboratory

The colonies of *A. stephensi* were maintained in the laboratory at a temperature of 25 ± 2°C, relative humidity was 75 ± 2% and photoperiod of 14:10 (L/D).

Source and culture media of *M. anisopliae*

The fungal strain of *M. anisopliae* was obtained from the Institute of Microbial Technology, (MTCC-892) Chandigarh, India. Fungal colonies were cultured 250 ml conical flask containing 100 ml of complete broth media (0.001 g $FeSO_4$, 0.5 g KCL, 1.5 g KH_2PO_4, 0.5 g $MgSo_4$, 7 H_2O, 6 g $NaNO_3$, 0.001 g $ZnSO_4$ g, 1.5 g Hydrolyzed Casein, 0.5 g Yeast Extract, 10 g Glucose, 2 g Peptone and 1000 ml Deionized water) fungi were incubated under static condition 25 ± 2°C for 15 ± 2 days with constant aeration in BOD (Figure 1).

Maintenance of nontarget organisms

Daphnia pulex, *Cyclopes*, *Lymnea auriculeta*, tadpoles of *Rana tigrina* were collected from nearby water bodies and were maintained in the laboratory. They were fed on suspension media spirulina and Deionized water (250 g + 50 ml). Media were changed three times in a week. The day night cycle consisted of 16 hours light and 8 hours of darkness. Young organisms were served as the experimental organisms in 200 ml beakers.

Filtration of extracellular metabolites

Cell free culture filtrates were obtained by filtering the broth through successive whatman No.1 filter paper after incubation. Fungal metabolites present in the filtrate were used to examine the larvicidal activities of *M. anisopliae*.

Bioefficacy for target organisms

All mosquito larvae of *A. stephensi* were separated and placed in separate containers (60 cm × 40 cm × 20 cm), containing microbe free Deionized water. After that, different test concentrations of metabolites in 100 ml water were prepared in 250 ml beakers. Bioassays were conducted separately for each instar of mosquitoes at five selected log concentrations. Log concentrations for *M. anisopliae* were 2.35, 2.65, 2.83, 2.95 and 3.05 ppm. To test the larvicidal activity, 20 larvae of each stage were separately exposed to 100 ml of test concentrations. Similarly, the control was run to test the natural mortality, except concentrations of culture medium used instead of the fungal filtrates.

Mortality and survival were determined after 24, 48 and 72 hours of the exposure. During experimental time, no food was offered to the larvae. The experiments were replicated thrice to validate results. All test containers were tightly covered with pierced aluminum foil and placed at room temperature without sunlight.

Bioefficacy for nontarget organisms

Adverse toxicity effects of the extracellular metabolites of *M. anisopliae* were also tested against four aquatic nontarget organisms i.e. *D. pulex*, *Cyclopes*, *L. auriculeta*, and tadpoles of *R. tigrina* at room temperature. First, *D. pulex*, and *Cyclopes* were collected in 250 ml beakers which contain 100 ml of deionized water with different concentrations, viz. 0.1,0.2,0.3,0.4, and 0.5 ml. Fifty organisms of each species were placed in different beakers for the bioassay of study. Ten organisms of *L. auriculeta* and tadpoles of *R. tigrina* were collected too and placed in 250 ml beakers of filtered metabolites concentrations viz.1, 2, 4, 8 and 16 ml. Mortality was recorded after 24, 48 and 72 hours.

Datamanagment and statistical analysis

The efficacy study of filtrate metabolites of *M. anisopliae* were assessed against larvae of *A. stephensi*. Experimental test that demonstrated more than 20% control mortality were discarded and repeated when control mortality reached between 5-20% observed, and was corrected by Abbott's formula [16,17]. The concentrations produce 50%, 90% and 99% mortality in larvae (LC_{50}, LC_{90} and LC_{99} respectively) were calculated with their fiducial limits at 95% confidence level. The relation between probit equation and probit regression lines were drawn for each of the larval stages (Figure 2).

Results

The LC values of crude extracellular metabolites of *M. anisopliae* reported in present study were found effective against all four instar of *A. stephensi*. 10% mortality was observed just after 24 hours in 1st instar. The LC_{99} values of *M. anisopliae* metabolites were higher at 685.84 ppm for the 3rd instar and lowest at 51.286 ppm for the 4th instar (Table 1). on the other hand, adverse effects of cell free culture filtrate of *M. anisopliae* were observed on selected nontarget organisms at five concentrations after 24, 48, and 72 hours of exposure. In control, 4% mortality was observed, which was corrected by Abbott's formula.

The chi square values for the test in 3rd instars of *A. stephensi*

Figure 1: Cultures colonies of *Metarhizium anisopliae* in complete broth media after 15 days of growth.

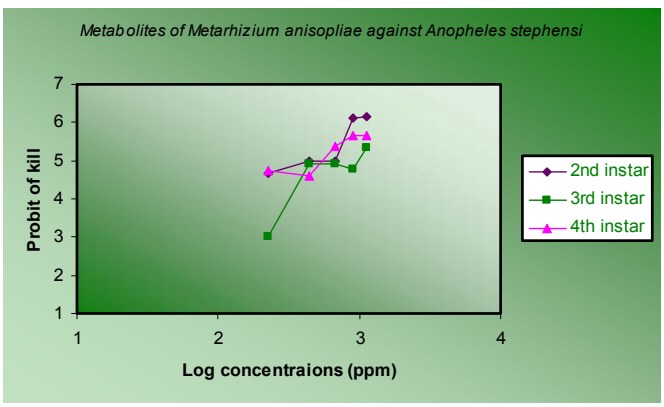

Figure 2: Comparative relationship between probit of kill and log concentrations of *M. anisopliae* filtrate metabolites showing probit regression line in larvae of *An. stephensi* in the laboratory.

Instars	Probit equations	LC_{50}	95% CL	LC_{90}	95% CL	LC_{99}	95% CL
First Instar	**	**	**	**	**	**	**
Second instar	2.234x – 0.7873	90.364	95.724-84.965	209.89	212.09-207.69	394.45	397.45-391.45
Third instar	2.9073x – 3.4476	18.653	183.657-183.65	304.789	308.199-301.37	685.84	691.861-683.82
Fourth instar	1.6101x+0.7604	1.174	1.753-0.595	5.011	6.615-3.407	51.286	53.522-49.05

**** 100% death larvae**

Table 1: Probit equations and susceptibilities of *Anopheles stephensi,* against extracellular metabolites of *Metarhizium anisopliae* with 95% confidential limit (CL).

was statistically significant at the 95% confidence level, which has suggested that there was no significantly difference between expected and observed data. Chi-square values at 3 df were 5.88, 2.41 and 77.49 for the all three instars. chi-square value for the 2nd instar were lower than the critical value of chi at 0.05 significance levels whereas, in case of 4th instar was found statistically significant at 0.05 significant level. This has suggested that there was no significant difference between expected and observed data. The small values of chi-square confirmed the adequate representation lines for the experimental data.

Nontarget organisms, *D. pulex* exhibited 62% mortality after 72 hours at 0.3 ml concentration and at 0.5 ml, mortality were increased by 100%. Copepods found 82% affected at 0.4 ml concentration after 24 hours and 100% affected after 48 hours. Whereas, *L. aureculeta* and *R. tigrina* were not found effective at any respective concentrations.

Discussion

A number of entomopathogenic fungi have been used effectively to control the mosquito vectors for last few decades. However, studies on the effects of extracellular metabolites on mosquitoes larvae and non-target organisms appear to be very limited in comparison to the use of spores and mycelia of the fungi.

However, it is important to note here that direct use of spores or mycelia of fungi can have universal problems to nontarget organisms because these entities can be retained to the ecosystem after completing their utilization. Therefore, the use of extracellular metabolites of the fungi can thus provide better technology alternatives, which has been ignored in the past for controlling large population of mosquito larvae in different breeding sites.

The Deuteromycete fungus *M. anisopliae* is broad host – range insect pathogens that can be easily isolated from most soil using selective media [18,19]. The conidia of *M. anisopliae* have previously been tested against Vince weevil, spittle bugs, termites [20] and larvae of *A. aegypti, A. quatrain*culatus, *A. stephensi*, *C. Pipiens*, *C. restuans*, *C. salinatious*, *C. quinquefasciatus*, *Ochlerotatus atropalpus* and *Ochlerotatus taeniorhynchus* were also found susceptible in laboratories and fields [21-23]. Invasion of conidia, depend on temperature, moisture and specific nutrients on the host cuticle. Moreover, its aggressiveness varies according to strain and has been correlated with different levels and kinetics of proteolytic, lipolytic and chitinolytic activity [24]. Action of *M. anisopliae* on mosquito larvae appears to be complex and is not fully understood [25].

So far, we have discussed about invasion of conidia that tend to be exploited for mosquito control with limited use due to hydrophobic by nature so when applied in an aquatic environment, they clump together, reducing the area that is effectively covered. As a result, massive amounts of fungal spores are required. [8]. In contrast, entomopathogenic fungi produce extracellular metabolites also. The secondary metabolites of the entomopathogenic fungi *Metarhizium* [21], *Beauveria* [26], *Trichophyton*, *Tolypocladium* [27] and *Chrysosorium* [13,14] have potential insecticidal activities. Some are well established as true insecticides, such as Beauvericin, destruxin, tolypin, and cyclosporine A and C. In the present investigation, we have successfully compared the efficacy and toxicity of metabolites against larvae of *A. stephensi*. Also these metabolites were tested against selected aquatic nontarget organisms of *D. pulex*, copepods, *R. tigrina* and *L. auriculeta* for the first time.

Fungi are likely to be developed for programmatic use. Especially since fungus production and application all involve relatively simple infrastructures and processes, which could potentially be adopted in malaria endemic countries. Therefore,an endeavor to find a novel suitable alternative to spores have now been accomplished in the laboratory by selectively using extracellular secondary metabolites from entomopathogenic fungi against malaria vector. However, there remains a need to test the metabolites in large-scale field trials and to develop protocols to ensure simple and economical distribution and application in malaria endemic developing countries. Further, it can be tested in different ecological climates of the world as effective alternative as well as cost-effective candidates.

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