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Aspergillus Niger Metabolites Efficacies Against the Mosquito Larval (C*ulex Quinquefasciatus, Anopheles Stephensi* and A*edes Aegypti*) Population after Column Chromatography

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Abstract: Problem statement: Mosquito is a very common vector of several tropical diseases such as malaria, filariasis, dengue and chikungunya. Till todate, many xenobiotics have been used to control mosquito population and protect people from mosquito born diseases but increasing rate of resistance against these xenobiotics is a major concern in the disease control management. In contrast, extensive use of these xenobiotic may lead to cause opurtunistic disease like cancer. Therefore, biological control can thus be an effective and environmental friendly approach, which can be used as an alternative to minimize the mosquito population. Fungi and fungus-derived products are highly toxic to mosquitoes, yet have low toxicity to nontarget organisms. *Aspergillus niger* is a cosmopolitan fungus which is primarily isolated from soil and decomposing plant materials. **Approach:** In the present investigation, this fungus was cultured on Czapek Dox Broth (CDB) for extracellular metabolites production. The *A. niger* was maintained at 25°C under 75 ± 5% humidity for 15 days. The extracellular metabolites were filtered through Whatman no.-1 filter papers. The purification of crude metabolites was done with different ratio of ethanol and metabolites (ethanol: metabolites = 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8 and 1:9, respectively) using column chromatography. All fractions were applied for larvicidal efficacy against the larvae of *Culex quinquefasciatus, Anopheles stephensi* and *Aedes aegypti*. The larvicidal efficacy was performed at six-log concentrations for 24, 48 and 72h. **Results:** The mortalities values were obtained by probit analysis. One potential ratio was selected for efficacy study among all ratios. The 2:8 ratio was found effective against the larvae of *Cx. quinquefasciatus* and *An. stephensi*. Whereas, a 4:6 ratio was found effective against the larvae of *Ae. aegypti*. The larvae of *Cx. quinquefasciatus* were found highly susceptible to the purified metabolites of *A. niger* than the larvae of *An. stephensi* and *Ae. aegypti*. **Conclusion:** The metabolites of *A. niger* may be environmental safer and a biocontrol agents for mosquitoes population in tropical countries where these vectors are significant.

Key words: *A. niger*, biocontrol agent, culex *quinquefasciatus*, *anopheles* stephensi, *Ae. aegypti*, column chromatography, disease-carrying mosquito, lower-income countries, dengue virus, filter papers

INTRODUCTION

The mosquito is a common insect found around the world. There are about three thousand five hundred species of mosquitoes. Mosquitoes are the major vector of diseases. *Anopheles* species are the most important species as they are capable vector for malaria parasites. Approximately half of the world's population is at risk of malaria, particularly those living in lower-income countries. It infects more than five hundred million people per year and kills more than one million WHO, 2011.

Culex mosquitoes are painful and persistent biters and are responsible for filariasis. These mosquitoes are very common in Indian sub-continent. Lymphatic Filariasis, commonly known as elephantiasis, is a painful and profoundly disfiguring disease. The disease is caused by three species of nematode thread-like worms known as *Wuchereria Bancrofti*, *Brugia malayi* and *Brugia timori*. An estimated one hundred twenty

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million people in tropical and subtropical areas of the world are infected with lymphatic filariasis; of these, almost twenty five million men have genital disease (most commonly hydrocele) and almost fifteen million, mostly women, have lymphoedema or elephantiasis of the leg. Approximately 66% of those at risk of infection live in the WHO South-East Asia Region and 33% in the African Region WHO, 2010.

Aedes mosquitoes on the other hand are also painful and persistent biters. *Aedes aegypti* is responsible for spreading Dengue and Chikungunya. Dengue is prevalent throughout the tropics and subtropics. The World Health Organization estimates that around 2.5 billion people are at risk of dengue. Infections have dramatically increased in recent decades due to increased urbanization, trade and travel. No effective drug or vaccine is available so far .Only solution is to prevent the disease-carrying mosquito from breeding and biting humans. Dengue is the most important mosquito spread viral disease and a major international public health concern. It is a self limiting disease found in tropical and sub-tropical regions around the world, predominantly in urban and semi-urban areas. DF/DHF is caused by dengue virus which belongs to genus Flavivirus, family Flaviviridae and includes serotypes 1-4 (Den-1, Den-2, Den-3 and Den-4) WHO, 2010. Mosquito control is a vital public-health practice throughout the world and especially in the tropics. These diseases can be controlled by targeting the causative parasites and pathogens. It is easier to control vectors than parasites. The chemical control was one of the most widely used conventional methods for mosquito control since chemical pesticides are relatively inexpensive and usually produce immediate control. It is known that larvicide play a vital role in controlling mosquitoes in their breeding sites. Two insecticidal bacteria have been used as larvicides to control larvae of nuisance and vector mosquitoes in many countries, *Bacillus thuringienesis* sp. *Israelensis* and *B. sphaericus* (Wirth *et al*., 2010). Unfortunately, the development of resistance against these chemicals in various mosquito populations has also been reported.

It is now essential to control mosquito population so that people can be protected from mosquito born diseases. Therefore, biological control can thus be an effective and environmental friendly approach, which can be used as an alternative to minimize the mosquito population. Fungi and fungus-derived products are highly toxic to mosquitoes, yet have low toxicity to nontarget organisms. Extracellular secondary metabolites from many fungi have been screened for larvicidal activity against mosquitoes. The secondary metabolites of entomopathogenic fungi *Chrysosporium* (Priyanka *et al*., 2001; Priyanka and Prakash, 2003; Verma and Prakash, 2010; Soni and Prakash, 2010), *Fusarium* (Prakash *et al*., 2010) have been screened as a potential larvicides successfully. *A. niger*, fungus has now been tested as a biocontrol agent of *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti*. This fungus was grown on Czapek Dox Agar (CDA). The present communication describes the larvicidal effect of extracellular metabolites of *A. niger* after purification by column chromatography against all instars of *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti*. This can be another way to avoid resistance problem effectively minimized while using new fungal larvicide.

MATERIALS AND METHODS

Fungal strain: The fungal strain of *A. niger* (MTCC 2587) was obtained from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India. This strain was routinely maintained in our laboratory on Czapek Dox Agar (CDA) medium at 25°C (Fig. 1).

Preparation of broth and culture of *A. niger*:

A modified culture broth of was prepared for the maintanance of *A. niger*. *A. niger* was grown on Czapek Dox Broth (CDB). Five 250 ml conical flask, each containing 100 ml CDB (Sucrose 30 g, Sodium nitrate 3 g, Dipotassium phosphate 1 g, Magnesium sulphate 0.05 g, Potassium chloride 0.05 g, Ferrous sulphate 0.01 g and Deionized water 1000 m L^{-1}) were autoclaved at 20 psi for 20 min. The broth was supplemented with 50 μg mL^{-1} chloramphenicol as a bacteriostatic agent. *A. niger* colonies grown on the CDA plates were transferred to each flask using the inoculation needle. The conical flasks inoculated with *A. niger* were incubated 25°C for 15 days (Fig. 2).

Maintenance of mosquito larvae in laboratory: Mosquito larvae were collected from various localities, including urban, rural and semi-urban regions of Agra (27°, 10'N, 78°05'E), India and reared in deionized water containing glucose and yeast power. The colonies of *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti* were maintained in the laboratory at a temperature of 25°C, with a relative humidity of 75±5% and 14h photoperiod. The larvae of *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti* were maintained in separate enamel containers as per the standard method.

Isolation and purification of extracellular metabolites: Cell free culture filtrates of *A. niger* were obtained by filtering the broth through successive

Whatman-1filter papers after incubation period. Thereafter, the metabolites were purified by column chromatography. In the experiment, the sample was prepared by 4ml sample in 1 mL solvent (ethanol/deionized water) and was chromatographed on a silica gel (100-200 mesh size). Elution were done with various ratios of ethanol and metabolites (ethanol/metabolites-9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8 and 1:9, respectively) and purified it thrice. Then, 5-ml fractions were collected from all ratios.

Larvicidal investigation of purified metabolites: To investigate larvicidal activity of filtered metabolites through Whatman-1 filter paper and purified through column chromatography were applied with different ratios of ethanol and metabolites. These purified fractional ratios were assessed against first, second, third and fourth instars of *An. stephensi, Cx. quinquefasciatus* and *Ae. aegypti*. Among all ratios the 4:6 ratio was found effective against the larvae of *Ae. aegypti*, 2:8 ratio was effective against the *An. stephensi and Cx. quinquefasciatus* larvae.

Bioassays: Larvicidal activity of metabolites of *A. niger* against *An. stephensi, Cx. quinquefasciatus* and *Ae. aegypti* was assessed by using the standard method. All mosquito larvae of *An. stephensi, Cx. quinquefasciatus* and *Ae. aegypti* were separated and placed in a container with microbe free deionized water.

Fig. 1: The culture of *A. niger* maintained on Czapek dox agar (CDA) medium at 25°C

Fig. 2: The culture of *A. niger* maintained on Czapek Dox Broth (CDB) medium at 25°C

After that different test concentrations of the metabolites in 100mL deionized were prepared in 250-ml beakers. Bioassays were conducted separately for each instar at six different log test concentrations (1.30, 1.60, 1.78, 1.90, 2.00 and 2.08 ppm) of purified metabolites. To test the larvicidal activity of extracellular purified metabolites, 20 larvae of each stage were separately exposed to 100m L^{-1} of test concentration. Similarly, the control was run to test the natural mortality, except concentrations of culture medium used instead of the fungal filtrates (Koch and Pasture). Thereafter, we could further examine the mortality which was determined after 24, 48 and 72h of the treatment, the experiment time. No food was offered to the larvae during the experiments. Experiments were replicated thrice to validate the results.

Data management and statistically analysis: The data on the efficacy were subjected to the probit analysis. The control mortality was corrected by Abbott's formula. The relationship between probit and log concentrations were established as probit equations and probit regression lines were drown for each of larval stage.

RESULTS

The findings were significant that while increasing filtration, metabolites could effectively control larval populations of mosquito. The efficacies were observed after column chromatography purification.

***A. niger* metabolites against mosquito larvae:** The purified metabolites were applied against the all larval instars of *An. stephensi, Cx. quinquefasciatus* and *Ae. aegypti* for bioefficacy test. The larvae of *Cx. quinquefasciatus* were found most effective against the *A. niger* (2:8 ratio) metabolites than the *An. Stephensi* and *Ae. Aegypti*. The degree of susceptibility of mosquito larvae against the *A. niger* metabolites were in the order of *Cx. Quinquefasciatus* > *An. Stephensi* > *Ae. Aegypti*.

***A. niger* (2:8 ratio) metabolites against the *Cx. Quinquefasciatus* larvae:** All larval instars of *Cx. Quinquefasciatus* have shown mortality for *A. niger* metabolites. The first, second and third instar larvae of *Cx. Quinquefasciatus* have shown 100% mortality for the *An. Niger* metabolites. Whereas, the third and fourth instar larvae were found less susceptible to the metabolites. The LC_{50} 60 ppm, LC_{90} 154.88 ppm and

LC_{99} 363.07 ppm were observed for the third instar larvae of *Cx. Quinquefasciatus* with their probit equations and confidential limits after 72h (Table 1). The probit regression lines drown for each of larval stage of *Cx. Quinquefasciatus* (Fig. 3). In control group no mortality could be observed. The observed LC values have shown the degree of susceptibility of fungal metabolites amongst the four larval stages of *Cx. Quinquefasciatus* in order of first instar > second instar > third instar > fourth instar.

***A. niger* (2:8 ratio) metabolites against the *An. Stephensi* larvae:** The metabolites of *A. niger* were found effective against the larvae of *An. Stephensi*. The first instar larvae were found more susceptible to the metabolites than the other instars. The LC_{50} 20 ppm, LC_{90} 123.02 ppm and LC_{99} 257.03 ppm were observed for first instar larvae of *An. Stephensi.* In second instar larvae LC_{50} 40 ppm, LC_{90} 123.02 ppm and LC_{99} 281.83 ppm were recorded.

Table 1: Probit equations and susceptibility of *Cx. Quinquefasciatus* larvae against extracellular metabolites (2:8) of *A. niger* with 95% Confidential Limits (CL) after 72h after column chromatography

Instars	Probit equation	LC_{50}(ppm)	LC_{90}(ppm)	LC_{99} (ppm)
First	**	**	**	**
Second	**	**	**	**
Third	**	**	**	**
Fourth	Y = 0.09+2.86x	60 (58.86-61.14)	144.54 (143.31-145.77)	338.84 (337.5-340.18)

(** 100% mortality was observed)

Table 2: Probit equations and susceptibility of *An. Stephensi* larvae against extracellular metabolites (2:8) of *A. niger* with 95% Confidential Limits (CL) after 72h after column chromatography

Instars	Probit equation	LC_{50} (ppm)	LC_{90} (ppm)	LC_{99} (ppm)
First	Y=0.09+3x	20 (18.86 -21.14)	123.02 (121.82-124.22)	257.03 (255.75-258.31)
Second	Y=0.09+2.95x	40 (38.86-41.14)	123.02 (121.82-124.22)	281.83 (280.55-283.11)
Third	Y=0.09+2.81x	601 (58.86-61.14)	158.48 (157.25-159.71)	371.53 (370.15-372.91)
Fourth	Y=0.09+2.72x	80 (78.83-81.17)	186.20 (184.95-187.45)	457.08 (455.67-458.49)

Table 3: Probit equations and susceptibility of *Ae. Aegypti* larvae against extracellular metabolites (4:6) of *A. niger* with 95% Confidential Limits (CL) after 72h after column chromatography

Instars	Probit equation	LC_{50} (ppm)	LC_{90} (ppm)	LC_{99} (ppm)
First	Y=0.10+2.89x	40 (38.86-41.14)	138.03 (136.83-139.23)	316.22 (314.91-317.53)
Second	Y=0.07+3.21x	20 (18.86-21.14)	100 (98.8-101.2)	181.97 (180.77-183.17)
Third	Y=0.09+2.82x	60 (58.86-61.14)	154.88 (153.65-156.11)	363.07 (361.73-364.41)
Fourth	Y=0.11+2.74x	80 (78.83-81.17)	177.82 (176.57-179.07)	426.57 (425.16-427.98)

For third instar larvae LC_{50} 40 ppm, LC90 158.48 and LC99 371.53 ppm were observed. Whereas, for fourth instar larvae LC_{50} 80 ppm, LC_{90} 186.20 and LC99 457.08 ppm were observed with their probit equations and confidential limits after 72h (Table 2). The probit regression lines drown for each of larval stage of *An. Stephensi* (Fig. 4). In control group no mortality could be observed. The observed LC values have shown the degree of susceptibility of fungal metabolites amongst the four larval stages of *An. Stephensi* in order of first instar > second instar > third instar > fourth instar.

***A. niger* (4:6 ratio) metabolites against the *Ae. Aegypti* larvae:** The metabolites of *A niger* were effective against the all instars of *Ae. Aegypti* larvae. The LC_{50} 40 ppm, LC90 138.03 ppm and LC_{99} 316.22 ppm values were calculated for the first instars.

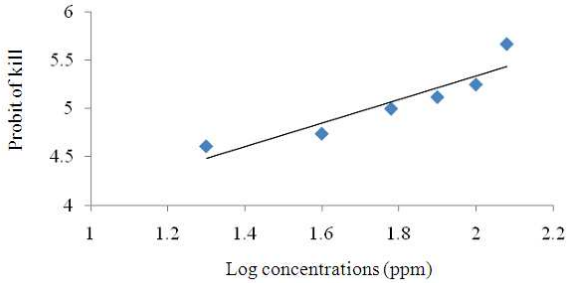

Fig. 3: Relationship between probit of kill and log concentrations of *A. niger* filtrate metabolites (2:8 ratio) showing probit regression lines in larvae of *Cx. Quinquefasciatus* after 72h in the laboratory after column chromatography

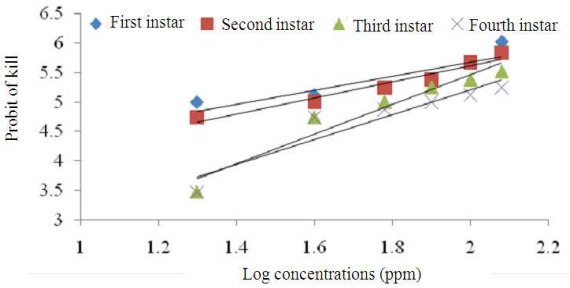

Fig. 4: Relationship between probit of kill and log concentrations of *A. niger* filtrate metabolites (2:8 ratio) showing probit regression lines in larvae of *An. Stephensi* after 72h in the laboratory after column chromatography

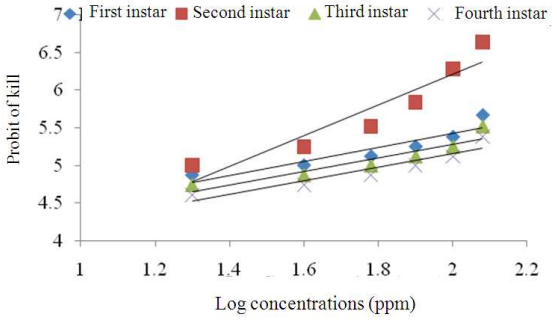

Fig. 5: Relationship between probit of kill and log concentrations of *A. niger* filtrate metabolites (4:6 ratio) showing probit regression lines in larvae of *Ae. Aegypti* after 72h in the laboratory after column chromatography

For second instars LC50 20 ppm, LC_{90} 100 ppm and LC_{99} 181.97 ppm values were calculated. In third instars LC_{50} 60 ppm, LC_{90} 154.88 ppm and LC_{99} 363.07 ppm were recorded. Whereas, for fourth instars LC_{50} 80 ppm, LC_{90} 177.82 and LC_{99} 426.57 ppm were observed with their probit equations and confidential limits after 72h (Table 3). The probit regression lines drown for each of larval stage of *Ae. Aegypti* (Fig. 5). In control group no mortality could be observed. The observed LC values have shown the degree of susceptibility of fungal metabolites amongst the four larval stages of *Ae. Aegypti* in order of first instar < second instar > third instar > fourth instar.

DISCUSSION

Unlike other mosquito control agents, the entomopathogenic fungi are unique because fungi have the ability to directly infect the host insect by penetrating into the cuticle and do not need to ingest by the insect to cause disease. There are preferential advantages when we use fungi as biocontrol agent for mosquitoes. *V. lecanii* has so far not been tested and this is the primary report on it as mosquito larvicide. The fungi have very narrow range and considerable progress has been made in recent years in development of environmentally benign spores and myceliumbased biocontrol agent for the mosquito population. Fungal biocontrol agents have reduced inputs of harmful synthetic chemical pesticide in agriculture, horticultural and forest system. The effect of three citrus species and enantiomers of α- and β-pipiens were also studied against third instar larvae of *Cx. Pipiens*. They studied that the activity of naturally occurring naphthoquinones and derivatives against *Cx. Pipiens* has shown 100% mortality. These studies were based on plant extract against mosquito larvae.

A number of entomopathogenic fungi have been so far used effectively to control mosquito vector for the last few decades. The efficacy of *Metarhizium anisopolie* ICIPE-30 and *Beauveria bassiana* I93-825 (IMI 391510) (2 × 1010 conidia m-2) applied on mud panels (simulating walls of traditional Tanzanian houses), black cotton cloth and polyester netting was evaluated against adult *Anopheles gambiae* (Mnyone *et al*., 2010). They concluded that both fungal isolates reduced mosquito survival on immediate exposure up to 28 d after application. A study with the spores of *C. lobatum* also shows 100% mortality to each instar larvae of *An. Stephensi*. However, in these studies, the spores of fungus were used but not the metabolites, whereas the present investigation is based on metabolites of the fungus.

The roll of fungi *Beauveria bassiana* (Balsamo) metabolites for controlling malaria and filarial in tropical countries have been evaluated (Singh and Prakash, 2010). They observed that these metabolites were found to be more effective on *An. Stephensi* comparatively *Cx. Quinquefasciatus* larvae. Further, the pathogenicity of *Fusarium oxysporum* against the larvae of *Culex quinquefasciatus* (Say) and *Anopheles stephensi* (Liston) in laboratory have been tested (Prakash *et al*., 2010). They could observe that the extracellular metabolites of *F. oxysporum* in Czapek Dox broth were most effective against the first and fourth instars of *An. Stephensi*. The third and fourth instars of *Cx. Quinquefasciatus* were more effective than first and second instars. The results of the present study showed that the extracellular metabolites of *F. oxysporum* were less effective against *An. Stephensi* but highly effective against *C. quinquefasciatus* larvae. This may be due to the size of *Culex* which enhanced more surface area. In these experiments the metabolites were applied directly to all instars after filtration through Whatman no-1 paper. While in our study, we have filtered the metabolites through the column chromatography. Again, the efficacy of *Chrysosporium tropicum* metabolites is effective against mixed population of adult mosquito (*Culex quinquefasciatus*, *Anopheles stephensi* and *Aedes aegypti*) after purification with flash chromatography have been observed (Verma and Prakash, 2010). The above experiment was aimed against the adult mosquitoes, while in our experiment the metabolites after purification with column chromatography were applied against the instars of *Cx. Quinquefasciatus* and *Ae. Aegypti* larvae only.

The culture filtrates of five different soil fungi *viz., Aspergillus flavus, Aspergillus parasiticus, Penicillium falicum, Fusarium vasinfectum* and *Trichoderma viride* were tested for the larvicidal activity against third instar larvae of mosquito vector *Culex quinquefasciatus*. They have found that among the five different fungi, the culture filtrates of *A. flavus* was found to be more toxic than the other four species of fungi against *Cx. Quinquefasciatus.* Pathogenicity of the fungus *A. clavatus,* isolated from the locust, *Oedaleus senegalensis*, against larvae of the mosquito *Ae. Aegypti, An. Gambiae* and *Cx. Quinquefasciatus* have been recorded (Seye *et al*., 2009). They have found that the conidia of the *A. clavatus* were effective against the larvae of *Ae. Aegypti, Cx. Quinquefasciatus* and *An. Astephensi*. These studies were based on the use of conidia against the mosquito larvae instead of metabolites. On the other hand, in our study we have used the metabolites after purification through the chromatographic technique against the mosquito larvae. The use of metabolites is safer than the conidia.

CONCLUSION

In comparison with the results mentioned above, it was perceptible that ethanol and metabolite mixed (2:8 and 4:6) filtrates, thrice filtered by column chromatography and then by flash chromatography, tested in this study exerted promising mosquito parricidal potential. These were greater than or comparable to that of previously described filtrates and their isolated compound. Hence, it can be now concluded that the use of extracellular metabolites of the fungi may provide better technology alternatives for controlling large population of mosquito larvae and adults. The LC values of metabolites of *A. niger* after flash chromatography reported in the present study was found effective against An. *Stephensi, Cx. Quinquefasciatus* and *Ae. Aegypti* larvae. The results show that as the concentration of metabolite increased, the efficacy of metabolite also increased. We can confirm here that after purification the extracellular metabolites are efficacious against the mosquito larvae which can be a biotechnological agent for exploitation.

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