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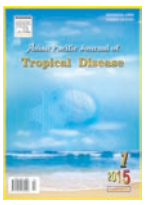

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Virulency of *Verticillium* sp. against mosquito vectors for malaria, filarial, and dengue

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ABSTRACT

Objective: Entomopathogenic fungi like *Beauveria bassiana*, *Metarhizium anisopliae* have been significantly pathogenic for mosquito vectors. Although few have been used for control. Moreover, the genus *Verticillium* encompasses a cosmopolitan group of ascomycete fungi. It is a major plant pathogen and parasitic on other fungi and insects.
Methods: The culture filtrates released from the *Verticillium lecanii* (MTCC 3692) were grown potato carrot broth media. These filtrates were purified with Whatman-1 filter paper and flash chromatograph respectively.
Results: The results demonstrated LC_{50}, LC_{90}, and LC_{99} values of 0.6, 4.2, and 4.86, for *Culex quinquefasciatus* , and 1.3, 2.32, and 3.36 (μL/cm^2) for *Anopheles stephensi* after exposure for 10 h. LT_{90} values were 6.76 for *Culex quinquefasciatus* and *Anopheles stephensi* 3.54 for *Anopheles stephensi*. Moreover, the *Aedes aegypti* were completely susceptible at all selected doses.
Conclusions: The fungal culture filtrates of *Verticillium* sp. can reduce malaria, dengue, and filarial transmission on a par with chemical insecticides providing efficient delivery system can be developed.

1. Introduction

A pressing need exists for additional tools in insect control, particularly as few new chemical pesticides are under development[1]. Moreover, rapidly emerging insecticide resistance is creating an urgent need for new active ingredients to control the adult mosquitoes that transmit malaria[2]. In this regard the fungal spores of entomopathogenic fungi have shown considerable promise by causing substantial mortality within 7-14 days of exposure. These fungal conidia have been significantly reduced disease vectors species of mosquitoes[3]. The critics have argued that 'slow acting' fungal biopesticides are incapable of delivering mosquito control in different parts of the world. Moreover, the potential of entomopathogenic fungi *Metarhizium anisopliae* for the control of adult *Aedes aegypti* (*Ae. aegypti*) has confirmed under field conditions[3].

Eight fungal entomopathogens have screened for the ability to kill anopheline mosquitoes. The fungi were applied by spraying containers of mosquitoes with an oil formulation of infectious spores. Upon contact with a mosquito, the fungal spores (conidia) start to develop and invade the mosquito, after which the fungus multiplies and kills its host within two weeks. Moreover, fungus infected mosquitoes were less likely to take subsequent blood meals than uninfected mosquitoes. Moreover, Blanford *et al.* and Scholte *et al.*[4,5] studies have been provided exciting new data that it is concerned the development of a new weapon in the war against malaria. These results were encouraging for further research on mosquito control. Entomopathogenic fungi shown promise as effective agents against adult mosquitoes. In addition, transgenic fungi have expressed anti-plasmodium effector molecule that can target the parasite inside its vectors[6]. Recently, *Metarhizium* has been engineered to act against malaria to directly kill the disease agent with in mosquito vectors and also effectively block onwards transmission[7].

The genus *Verticillium* encompasses a cosmopolitan group of ascomycete fungi. It is normally considered to be nonpathogenic in humans. *Verticillium* produces an antifungal compound vergosin and an antitumor antibiotic, as well as a wide variety of additional compounds used by various industries. The health effects resulting from fungal exposure may be dependent on dosage and cause of

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exposure and vary on individuals. The growth of the pathogenic fungi can cause opportunistic diseases. They thrive more under humid, nutrient available areas and with favourable temperatures. They have strong anti-fungal activity. The antifungal metabolites of *Verticillium* sp. inhibited biomass growth of itself and pathogens in liquid culture. The strong antifungal activities against the phytopathogenic fungi *Verticillium* sp. have been protected to the host by producing secondary metabolites[8].

Malaria is transmitted to humans by the bite of infected female mosquitoes of more than 30 Anopheline species. An estimated 3.4 billion people are risk of malaria and 1.2 billion are at high risk. In high risk areas, more than one malaria case occurs per 1 000 population[9]. Approximately 3.5 billion people live in dengue endemic countries which are located in the tropical and subtropical regions of the world[10]. Lymphatic filariasis, commonly known as elephantiasis, is a neglected tropical disease. The infection occurs when filarial parasites are transmitted to humans through *Culex quinquefasciatus* (*Cx. quinquefasciatus*). More than 1.3 billion people in eighty one countries worldwide are threatened by lymphatic filariasis[11]. Interestingly the present study evaluated *Verticillium* sp. as new adulticide for controlling of malaria, filaria and dengue vectors.

2. Materials and methods

2.1. Collection and culture of Verticillium sp. 3692

The strain of *Verticillium* sp. was obtained from Microbial Type Culture Collection and Gene Bank (MTCC-3692) Institute of Microbial Technology, Chandigarh India. *Verticillium* sp. were maintained on autoclaved Potato Carrot Broth (PCB) media (potato scrubbed and diced 20.0 g, carrot peeled and grated 20.0 g, deionized water 1000 mL and adjust pH to 7.2 with KOH). The broth was supplemented with 50 μg/mL chloramphenicol as a bacteriostatic agent. The colonies of *Verticillium* sp. were grown on PCA solid medium plates were transferred to each flask using an inoculation needle. The conical flasks, inoculated with *Verticillium* sp. were incubated at 25 ˚C for 15 days (Figure1).

Figure 1. The culture of *Verticillium* sp. 3692 in PCB maintained in the laboratory.

2.2. Filtration of culture filtrates

The culture filtrates were obtained by filtering the broth through Whatman No. 1 filter paper. These metabolites were further filtered with the flash chromatograph. In the Flash chromatograph, a plastic column were filled with silica gel, with the sample to be separated placed on top of this support. The rest of the column was filled with an isocratic or gradient solvent which, with the help of pressure, enabled the sample to run through the column and became separated. Flash chromatography used air pressure initially, to speed up the separation.

2.3. Bioassays

The bioassays were carried out with laboratory reared *Cx. quinquefasciatus*, *Ae. aegypti*, and *Anopheles stephensi* (*An. stephensi*) as per the standard procedures recommended by World Health Organization with some respected modifications[12]. The freshly emerged three days old sugar fed adults were used for the assay. The five different volumes of 1.6, 2.2, 2.7, 3.2, and 3.8 μL/cm^2 of metabolites were sprayed in a cage (25 cm length × 15 cm width × 5 cm depth) containing 25 mosquitoes. The exposed mosquitoes were kept under observation, and dead mosquitoes were discarded daily. Each bioassay including control was conducted in triplicate on different days. In the control cages deionized water was sprayed. Daily mortality counts were performed. The bioassays were carried out at room temperature with 75% ± 5% relative humidity. The negative control was deionized water with 1% PCB while the positive control was Gokilaht®-S 5EC (*d,d-trans*-cyphenothrin).

2.4. Statistical analysis

The efficacy study of the filtrate metabolites of *Verticillium* sp. were assessed against *Cx. quinquefasciatus*, *Ae. aegypti*, and *An. stephensi* by probit analysis with the statistical package IBM SPSS 19.0[13].

Figure 2. The infected *Cx. quinquefasciatus*, *Ae. aegypti* and *An. stephensi* with the culture filtrates of *Verticillium* sp. under the cage.

3. Results

In the present investigation we had evaluated the lethal effect of culture filtrates of *Verticillium* sp. against *Cx. quinquefasciatus* and *Ae. aegypti* after exposure of 12 h. The Whatman No.1 filtrates were recorded LC_{50}, LC_{90}, and LC_{99} 0.71, 4.2, and 5.2 μL/cm^2 with the mortality rate (R^2) 0.925. When these culture filtrates were again filtrated with the flash chromatograph the LC_{50}, LC_{90}, and LC_{99}, 0.6, 4.2, and 4.8 μL/cm^2 were recorded with the mortality rate 0.754. Moreover, the LT_{50}, and LT_{90} 1.94, and 6.76 h at 4.7 mL. Similarly, these filtrates were used against *An. stephensi* the LC_{50}, LC_{90}, and LC_{99} values 1.3, 2.7, and 3.36 μL/cm^2 respectively with the mortality rate (R^2) 0.851. Moreover, the flash chromatograph filtered LC_{50}, LC_{90}, and LC_{99}, were 1.3, 2.32, and 3.36 μL/cm^2 with the R^2 value 0.904. Whereas, Wahatman-1 filtrates have shown the

Table 1

Virulent effect of Whatman-1 and flash chromatograph filtered culture filtrates of *Verticillium* sp. 3692 against *Cx. quinquefasciatus* (Say), *An. stephensi* (Liston), and *Ae. aegypti* (Linn.).

Mosquito	LC_{50} (μL/cm^2)[c]	LC_{90} (μL/cm^2)[c]	LC_{99} (μL/cm^2)[c]	LT_{50} (h)[d]	LT_{90} (h)[d]	LC_{50} (μL/cm^2)[c]	LC_{90} (μL/cm^2)[c]	LC_{99} (μL/cm^2)[c]	LT_{50} (h)[d]	LT_{90} (h)[d]
	Whatman-1					Flash Chromatograph				
Cx. quinquefasciatus	0.74	4.20	5.20	3.98	8.91	0.60	4.20	4.86	1.94	6.76
	(0.11-1.36)	(3.66-4.86)	(4.60-5.80)	(2.91-5.05)	(6.92-10.9)	(0.030-1.28)	(3.66-4.86)	(4.03-5.24)	(0.80-3.82)	(5.62-7.90)
An. stephensi	1.30	2.70	3.36			1.30	2.32	3.36	1.58	3.54
	(0.81-1.90)	(2.12-3.22)	(2.80-3.91)			(0.816-1.92)	(1.77-2.88)	(2.80-3.90)	(0.41-2.75)	(2.37-4.41)
Ae. aegypti	*	*	*	*	*	*	*	*	*	*

*100% mortality.

100% mortalities against *Ae. aegypti* after exposure of 7 h (Table 1). Moreover, infected *Cx. quinquefasciatus*, *Ae. aegypti* and *An. stephensi* with the culture filtrates of *Verticillium* sp. were observed under the cage (Figure 2). The significant percent mortality were observed at tested concentrations after exposure of 7 h (Figures 3 and 4).

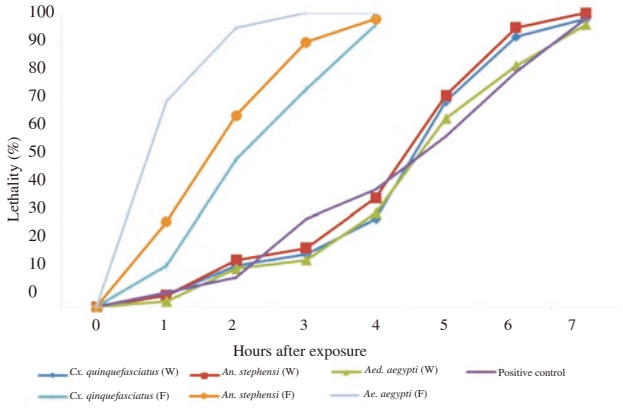

Figure 3. Mortality of (*Verticillium* sp). against *Cx. quinquefasciatus*, *An. stephensi*, and *Ae. aegypti* plotted as function of time post exposure.

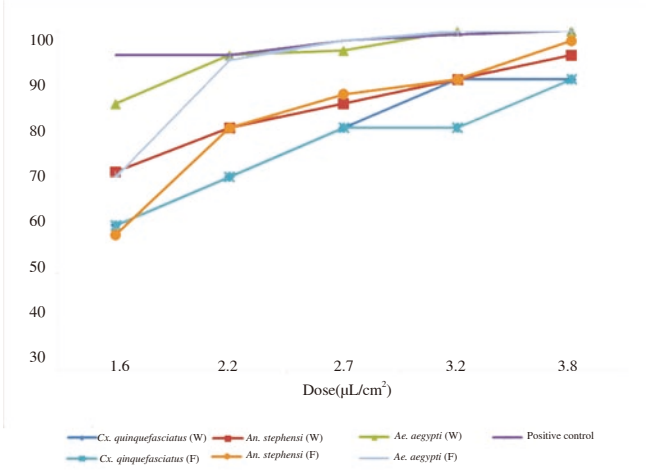

Figure 4. Effects of culture filtrates of *Verticillium* sp. against *Cx. quinquefasciatus*, *An. stephensi*, and *Ae. aegypti* at different doses.

4. Discussion

The laboratories and field studies have been successfully demonstrated that entomopathogenic fungi can efficiently kill vectors of malaria, dengue, and filarial[14-18,1]. The biopesticides have been used of comprising entomopathogenic fungi, such as *Beauveria bassiana* and *Metarhizium anisopliae*. These fungi may decrease disease transmission by reducing mosquito vector longevity and also occur worldwide[19]. Many isolates have not been tested for virulence against mosquitoes. In this concern in our Laboratories, seventy eight isolates of entomopathogenic fungi representing twenty species were screened as potential biological control agents of *Ae. aegypti*, *An. stephensi*, and *Cx. quinquefasciatus* larvae and adults. Now we have one more species of *Verticillium* sp. has found considerable virulence against adults of *Ae. aegypti*, *Cx. quinquefasciatus*, and *An. stephensi* in the laboratories. Most of these fungal strains have shown lethal effect after exposure of 24, 42 and 72 h. The culture filtrates *Aspergillus niger* has been found pathogenic in 7.0 h against *An. stephensi*, *Ae. aegypti*, and *Cx. quinquefasciatus*[17]. In the present investigations, the mortality rates were 100% against *Ae. aegypti*, while 95% against *Cx. quinquefasciatus* and *An. stephensi* after exposure of 7 h. Based on these efficacy data this fungal strain can be selected, for the control of adult mosquitoes with observed doses.

Beauveria bassiana has been expressed trypsin modulating oostatic factor exhibited increased virulence against *Anopheles gambiae* and *Ae. aegypti* related to the wild type strain. Utility of entomopathogenic fungi has expressed mosquito specific molecules to improve their biological control activities against mosquito vectors of diseases[20]. The pathogenic fungi produce a wide variety of toxic metabolites, which vary from low molecular weight products of secondary metabolism to complex cyclic peptides and proteolytic enzymes[21]. The fungal metabolites can be more effective by joint action of numerous toxins and enzymes[17]. Another point to consider regarding bioassays is that an exposure time of 24 h, which is adequate for standard screening, may not be achievable in the field. While it may be possible for mosquitoes in resting boxes, such as those designed for using against exophilic *Anopheles arabiensis* in Tanzania, to stay exposed to fungus-treated surfaces for several hours or more, mosquitoes landing on a visual target may only rest on spores for as little as a few minutes[22]. Entomopathogenic fungus *Leptolegnia chapmanii* has shown significant reduction in the population of *Ae. aegypti*[23]. Recently, virulency for engineered *Metarhizium* or similar pathogens, and that all available information regarding the population ecology of the combined mosquito fungus system has been considered[7]. Field applications of entomopathogenic fungi should take it into account, one potential solution is to use a higher dose to account for reduced exposure time. In *Aspergillus niger* the "ochratoxin" mycotoxin can be fast acting metabolites for controlling of adult mosquitoes[17]. The nonchemical approaches to the management of malaria, dengue, and filaria are limited by site specificity of available methods. Similarly, fungi biopesticides have significant potential to achieve reduction in transmission comparable with those achieved with existing instant kill insecticides[24]. An understanding of the modes of action of these methods will likely improve their range and efficacy. And efforts should continue to fully explore the operational feasibility

of this alternative approach for mosquito control. The myco-control technology is encouraging for extensive future research. Further investigations need to be excreted to translate laboratory promising results of many of the microbial agents into field control agents[25]. An important area of future research also involves examining *Verticillium* sp. for the determinants of race specificity. Similarly all these novel findings could be implemented with a time application with its fast acting impact against *An. stephensi*, *Cx. quinquefasciatus*, and *Ae. aegypti* population.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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Several fungi have recently been used for adult mosquito control. The authors attempt to use new taxa of fungus for this purpose.

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