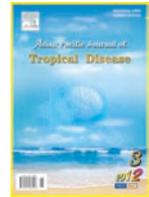




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# Larvicidal effect of *Verticillium lecanii* metabolites on *Culex quinquefasciatus* and *Aedes aegypti* larvae

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## ABSTRACT

**Objective:** To investigate the efficacy of *Verticillium lecanii* metabolites after filtration and purification through the chromatographic techniques against the larvae of *Culex quinquefasciatus* (*Cx. quinquefasciatus*) and *Aedes aegypti* (*Ae. aegypti*). **Methods:** This fungus was cultured on potato dextrose broth in the laboratory at 25 °C, while the relative humidity was maintained at (75±5)% for 15 d. Filtration process of metabolites was done using whatman-1 filter paper, column chromatography and flash chromatography. Larvicidal efficacy was performed at six different concentrations with different effective volume ratios (ethanol/metabolites: 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8 and 1:9) for a period of 24, 48 and 72 h, respectively. **Results:** Among all ratios, the 4:6 ratio was found effective against the larvae of *Cx. quinquefasciatus* and 1:9 ratio was found effective against the larvae of *Ae. aegypti*. The first, second, and third instars of *Cx. quinquefasciatus* were found more susceptible to the metabolites than the fourth instars. However, the first instars of *Ae. aegypti* were found more susceptible than the other instars. **Conclusions:** Larvicidal efficacy has been pioneered by us for the first time and performed against all instars of *Cx. quinquefasciatus* and *Ae. aegypti*. The filtration and purification made metabolites more effective than the crude metabolites. The metabolites of *Verticillium lecanii* could be an environmentally safer larvicide source for the control of mosquito larvae.

## 1. Introduction

Mosquitoes belong to order Diptera. There are about 3 500 species of mosquitoes existing in the world. *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 120 million people in tropical and subtropical areas of the world are infected with lymphatic filariasis; of these, almost 25 million men have genital disease (most commonly hydrocele) and almost 15 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[1].

*Aedes* mosquitoes on the other hand are also painful and persistent biters. *Aedes aegypti* (*Ae. aegypti*) is responsible for spreading dengue and chikungunya. Dengue is prevalent throughout the tropics and subtropics. The WHO 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. Dengue fever and dengue hemorrhagic fever are caused by dengue virus which belongs to genus *Flavivirus*, family Flaviviridae and includes serotypes 1, 2, 3 and 4 (Den-1, Den-2, Den-3 and Den-4)[2]. Mosquito control is a vital public-health practice throughout the world and especially in the tropics. It is essential to control mosquito population to prevent people from mosquito born diseases. These diseases can

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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 larvicides play a vital role in controlling mosquitoes in their breeding sites. Two insecticidal bacteria, *Bacillus thuringiensis* sp. israelensis and *Bacillus sphaericus*, have been used as larvicides to control larvae of nuisance and vector mosquitoes in many countries<sup>[3]</sup>. 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. The secondary metabolites of entomopathogenic fungi chrysosporium<sup>[4–6]</sup> and fusarium<sup>[7]</sup> have been screened as a potential larvicide successfully. *Verticillium lecanii* (*V. lecanii*) fungus has now been tested as a biocontrol agent of *Culex quinquefasciatus* (*Cx. quinquefasciatus*) and *Ae. aegypti*. This fungus was cultured on potato dextrose agar (PDA). The present communication describes the larvicidal effect of extracellular metabolites of *V. lecanii* after purification against all instars of *Cx. quinquefasciatus* and *Ae. aegypti*. This can be another way to avoid resistance problem effectively while using new fungal larvicide.

## 2. Materials and methods

### 2.1. Fungal strain

The fungal strain of *V. lecanii* (MTCC 3692) was obtained from the Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India. This strain was routinely maintained in our laboratory on PDA medium at 25 °C.

### 2.2. Preparation of broth and culture of *V. lecanii*

*V. lecanii* was cultured on potato dextrose broth (PDB). Five 250 mL conical flask, each containing 100 mL of PDB (Infusion of potatoes 200.0 g, dextrose 20.0 g, deionized water 1 000 mL) were autoclaved at 137.9 kPa for 20 min. The broth was supplemented with chloramphenicol at a final concentration of 50 µg/mL as a bacteriostatic agent. *V. lecanii* colonies growing on the PDA plates were transferred to each flask using the inoculation needle. The conical flasks inoculated with *V. lecanii* were incubated at 25 °C for 15 d.

### 2.3. 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 powder. The colonies of *Cx.*

*quinquefasciatus* and *Ae. aegypti* were maintained in the laboratory at a temperature of 25 °C, with a relative humidity of (75±5)% and 14 h photoperiod. The larvae of *Cx. quinquefasciatus* and *Ae. aegypti* were maintained in separate enamel containers.

### 2.4. Isolation and purification of extracellular metabolites

Cell free culture filtrates of *V. lecanii* were obtained by filtering the broth through successive Whatman-1 filter papers after incubation period. Thereafter, the metabolites were purified by column chromatography. In the experiment, the sample was prepared by 4 mL sample in 1 mL solvent (ethanol/deionized water) and was chromatographed on a silica gel (100–200 mesh size). Elution were done with various volume ratios of ethanol and metabolites (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8 and 1:9, respectively) and it was purified thrice. Then, 5-mL fractions were collected from all ratios. Once again, these fractions were purified by flash chromatography.

### 2.5. Larvicidal investigation of purified metabolites against *Cx. quinquefasciatus* larvae

To investigate larvicidal activity, filtered metabolites which were strained through Whatman-1 filter paper and purified through column chromatography were applied separately. Different ratios of ethanol and metabolites were assessed against the first, second, third, and fourth instars of *Cx. quinquefasciatus* and *Ae. aegypti*.

### 2.6. Bioassays

Larvicidal activity of metabolites of *V. lecanii* against *Cx. quinquefasciatus* and *Ae. aegypti* was assessed by using the standard method<sup>[8]</sup>. All mosquito larvae of *Cx. quinquefasciatus* and *Ae. aegypti* were separated and placed in a container with microbe free deionized water. After that, different test concentrations of the metabolites in 100 mL 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 100 mL of test concentration. Similarly, the control was run to test the natural mortality, except concentrations of culture medium used instead of the fungal filtrates. Thereafter, we could further examine the mortality which was determined after 24, 48 and 72 h of the treatment, the experiment time. No food was offered to the larvae during the experiments. Experiments were replicated thrice to validate the results.

### 2.7. Data management and statistically analysis

The data on the efficacy were subjected to the probit analysis<sup>[9]</sup>. The control mortality was corrected by Abbott's formula<sup>[10]</sup>. The relationships between probit and log concentrations were established as probit equations.

### 3. Results

The findings were significant when metabolites could effectively control larval populations of mosquito with increasing filtration. The efficacies were observed after Whatman–1 filter paper, column chromatography and then flash chromatography, separately.

#### 3.1. *V. lecanii* metabolites against *Cx. quinquefasciatus* after Whatman–1 filtration

The Whatman filtered metabolites were found effective against the larvae of *Cx. quinquefasciatus*. The fourth instar larvae were found more susceptible to the metabolites than the other instars. The susceptibility of *Cx. quinquefasciatus* at each instar stage to the metabolites after 72 h exposure is represented in Table 1. The probit regression equations for each larval stage of *Cx. quinquefasciatus* are also shown in Table 1. In control group, no mortality could be observed. The observed lethal concentrations had shown the degree of susceptibility of *Cx. quinquefasciatus* to fungal metabolites was in order of the first instar < the second instar < the third instar < the fourth instar, after Whatman–1 filtration.

#### 3.2. *V. lecanii* metabolites (4:6 volume ratio) against *Cx. quinquefasciatus* after column and flash chromatography

The results of efficacy (not shown) showed the highest mortality of *Cx. quinquefasciatus* after 72 h of exposure when ethanol/metabolites was at 4:6 volume ratio. Tables 2 and 3 show the lethal concentrations of all fractions of *V. lecanii* metabolites against all instars of *Cx. quinquefasciatus*

after column chromatography and flash chromatography as well as their confidential limits and probit equations. After column chromatography, the second and third instars had 100% mortality to the metabolites (Table 2). In control group, no mortality could be observed. The observed lethal concentrations had shown the degree of susceptibility of *Cx. quinquefasciatus* to fungal metabolites was in order of the second instar > the third instar > the first instar > the fourth instar, after column chromatography purification.

After flash chromatography, the first, second, and third instars had 100% mortality to the metabolites (4:6 volume ratio) after 72 h of exposure (Table 3). However, the sensitivity of the fourth instars declined. In control group, no mortality could be observed. The observed lethal concentrations had shown the degree of susceptibility of *Cx. quinquefasciatus* to fungal metabolites was in order of the first instar > the second instar > the third instar > the fourth instar, after flash chromatography purification.

#### 3.3. *V. lecanii* metabolites against *Ae. aegypti* after Whatman–1 filtration

The metabolites of *V. lecanii* after filtration through Whatman–1 filter paper had mortality for all larval instars of *Ae. aegypti*. The first instar larvae of *Ae. aegypti* were found more susceptible to the metabolites than the other instars. The susceptibility of *Ae. aegypti* at each instar stage to the metabolites after 72 h exposure is represented in Table 4. The probit regression equations for each larval stage of *Ae. aegypti* are also shown in Table 4. No mortality was recorded in control group. Nevertheless, the third and fourth instars of *Ae. aegypti* had no mortality for *V. lecanii* metabolites

**Table 1**

Probit equations and susceptibility of *Cx. quinquefasciatus* larvae against whatman–1 filtrated extracellular metabolites of *V. lecanii* after 72 h of exposure.

Instar	Probit equation	LC <sub>50</sub> (ppm)	LC <sub>90</sub> (ppm)	LC <sub>99</sub> (ppm)
First	$Y = 0.08 + 2.79X$	80.00 (78.83–81.17)	169.82 (168.59–171.05)	407.38 (406.00–408.76)
Second	$Y = 0.08 + 2.84X$	60.00 (58.86–61.14)	151.35 (150.12–152.58)	354.81 (353.47–356.15)
Third	$Y = 0.11 + 2.81X$	54.95 (53.81–56.09)	154.88 (153.65–156.11)	371.53 (370.15–372.91)
Fourth	$Y = 0.09 + 2.93X$	40.00 (38.86–41.14)	128.82 (127.62–130.02)	293.81 (292.61–296.43)

The data in brackets are 95% confidential limits.

**Table 2**

Probit equations and susceptibility of *Cx. quinquefasciatus* larvae against extracellular metabolites of *V. lecanii* (ethanol:metabolite = 4:6, v/v) purified by column chromatography after 72 h of exposure.

Instar	Probit equation	LC <sub>50</sub> (ppm)	LC <sub>90</sub> (ppm)	LC <sub>99</sub> (ppm)
First	$Y = 0.10 + 2.87X$	60.00 (58.86–61.14)	141.25 (140.05–142.45)	331.13 (329.79–332.47)
Second	--	--	--	--
Third	--	--	--	--
Fourth	$Y = 0.10 + 2.66X$	100.00 (98.80–101.20)	208.92 (207.64–210.20)	524.80 (523.33–526.27)

The data in brackets are 95% confidential limits. -- means 100% mortality was observed.

**Table 3**

Probit equations and susceptibility of *Cx. quinquefasciatus* larvae against extracellular metabolites of *V. lecanii* (ethanol:metabolite = 4:6, v/v) purified by flash chromatography after 72 h of exposure.

Instar	Probit equation	LC <sub>50</sub> (ppm)	LC <sub>90</sub> (ppm)	LC <sub>99</sub> (ppm)
First	--	--	--	--
Second	--	--	--	--
Third	--	--	--	--
Fourth	$Y = 0.09 + 2.75X$	80.00 (78.83–81.17)	177.82 (176.57–179.07)	426.57 (425.16–427.98)

The data in brackets are 95% confidential limits. -- means 100% mortality was observed.

**Table 4**

Probit equations and susceptibility of *Ae. aegypti* larvae against whatman–1 filtrated extracellular metabolites of *V. lecanii* after 72 h of exposure.

Instar	Probit equation	LC <sub>50</sub> (ppm)	LC <sub>90</sub> (ppm)	LC <sub>99</sub> (ppm)
First	$Y = 0.10 + 2.66X$	100.00 (98.80–101.20)	208.92 (207.64–210.20)	524.80 (523.33–526.27)
Second	$Y = 0.07 + 2.55X$	120.00 (118.77–121.23)	269.15 (267.81–270.49)	691.83 (690.29–693.37)
Third	--	--	--	--
Fourth	--	--	--	--

The data in brackets are 95% confidential limits. -- means 100% mortality was observed.

**Table 5**

Probit equations and susceptibility of *Ae. aegypti* larvae against extracellular metabolites of *V. lecanii* (ethanol:metabolite = 1:9, v/v) purified by column chromatography after 72 h of exposure.

Instar	Probit equation	LC <sub>50</sub> (ppm)	LC <sub>90</sub> (ppm)	LC <sub>99</sub> (ppm)
First	$Y = 0.09 + 3.00X$	20.00 (18.86–21.14)	123.02 (121.82–124.22)	257.03 (255.75–258.31)
Second	$Y = 0.09 + 2.95X$	40.00 (38.86–41.14)	123.02 (121.82–124.22)	281.83 (280.55–283.11)
Third	$Y = 0.10 + 2.84X$	60.00 (58.86–61.14)	147.92 (146.68–149.14)	346.73 (345.39–348.07)
Fourth	$Y = 0.09 + 2.76X$	80.00 (78.83–81.17)	173.78 (172.53–175.03)	416.86 (415.45–418.27)

The data in brackets are 95% confidential limits.

**Table 6**

Probit equations and susceptibility of *Ae. aegypti* larvae against extracellular metabolites of *V. lecanii* (ethanol:metabolite = 1:9, v/v) purified by flash chromatography after 72 h of exposure.

Instar	Probit equation	LC <sub>50</sub> (ppm)	LC <sub>90</sub> (ppm)	LC <sub>99</sub> (ppm)
First	$Y = 0.09 + 3.16X$	16.98 (15.75–18.21)	109.64 (108.50–110.78)	194.98 (193.75–196.21)
Second	$Y = 0.11 + 3.05X$	20.00 (18.86–21.14)	123.02 (121.82–124.22)	234.42 (233.17–235.67)
Third	$Y = 0.09 + 2.95X$	40.00 (38.86–41.14)	123.02 (121.82–124.22)	281.83 (280.55–283.11)
Fourth	$Y = 0.09 + 2.86X$	60.00 (58.86–61.14)	144.54 (143.31–145.77)	338.84 (337.50–340.12)

The data in brackets are 95% confidential limits.

after 72 h. The observed lethal concentrations had shown the degree of susceptibility of *Ae. aegypti* to fungal metabolites was in order of the first instar > the second instar > the third instar > the fourth instar.

### 3.4. *V. lecanii* metabolites (1:9 volume ratio) against *Ae. aegypti* after column and flash chromatography

Tables 5 and 6 show the lethal concentrations of all fractions of *V. lecanii* metabolites against all instars of *Ae. aegypti* after column chromatography and flash chromatography as well as their confidential limits and probit equations. The efficacy study showed the highest mortality at 1:9 (ethanol/metabolites) volume ratio of metabolites after 72 h of exposure (Table 5). No mortality was recorded in control group. The observed lethal concentrations had shown the degree of susceptibility of *Ae. aegypti* to fungal metabolites was in order of the first instar > the second instar > the third instar > the fourth instar, after column chromatography purification (Table 5). The same order was found after flash chromatography purification (Table 6).

## 4. 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 a 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 mycelium-based biocontrol agents for the mosquito population. Fungal biocontrol agents have reduced inputs of harmful synthetic chemical pesticide in agriculture, horticultural, and forest system.

A number of entomopathogenic fungi have been so far used effectively to control mosquito vector for the last few decades. The efficacy of *Metarhizium anisopliae* ICPE-30 and *Beauveria bassiana* I93-825 (IMI 391510) ( $2 \times 10^{10}$  conidia/m<sup>2</sup>) applied on mud panels (simulating walls of traditional Tanzanian houses), black cotton cloth and polyester netting was evaluated against adult *Anopheles gambiae*[11]. They concluded that both fungal isolates reduced mosquito survival on immediate exposure up to 28 d after application.

The role of fungi *Beauveria bassiana* (Balsamo) metabolites for controlling malaria and filaria in tropical countries have been evaluated[12]. These metabolites were found to be more effective on *Anopheles stephensi* (*An. stephensi*) than *Cx. quinquefasciatus* larvae. Further, the pathogenicity of *Fusarium oxysporum* (*F. oxysporum*) against the larvae of *Cx. quinquefasciatus* (Say) and *An. stephensi* (Liston) in laboratory have been tested[7]. They could observe that the extracellular metabolites of *F. oxysporum* in Czapek Dox broth were the most effective against the first and fourth instars of *An. stephensi*. The third and fourth instars of *Cx. quinquefasciatus* were more sensitive than the 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 *Cx. quinquefasciatus* larvae. This may be due to the size of culex which increases more surface area. In these experiments, the metabolites

were applied directly to all instars after filtration through Whatman no-1 paper. However, in our study, we have filtered the metabolites through the column chromatography and then further by flash chromatography. Additionally, *Chrysosporium tropicum* metabolites have been observed to be effective against mixed population of adult mosquito (*Cx. quinquefasciatus*, *An. stephensi*, and *Ae. aegypti*) after purification with flash chromatography[5]. The cultural filtrate of *Culicinomyces clavissporus* has also been tested as mycoadulcicide against the *Cx. quinquefasciatus*, *An. stephensi* and *Ae. aegypti*[13]. The above experiments were aimed against the adult mosquitoes, while in our experiment, the metabolites after purification with column chromatography and flash chromatography were applied against the instars of *Cx. quinquefasciatus* and *Ae. aegypti* larvae only. The results indicated that the extracellular metabolites of *V. lecanii* could be a better larvicide for vector control.

The virulence of two strains of the entomopathogenic fungus *V. lecanii* to the aphids *Myzus persicae*, *Aphis gossypii* and *Brevicoryne brassicae* was bioassayed[14]. Three new fungal metabolites were isolated and purified from the broth culture of two entomopathogenic fungi *Verticillium alboatrum* and *Verticillium leptobactrum*[15]. The obtained compounds were screened for their antibacterial, antifungal, antiviral and antitumor activity. The study illustrated the biological activities of new fungal metabolites from *Verticillium alboatrum* and *Verticillium leptobactrum*, therefore providing a potential drug and a good candidate for further studies and development. The above mentioned results of efficacy of *V. lecanii* were studied against the other insects not on the mosquitoes.

In comparison with the results mentioned above, it was perceptible that ethanol and metabolite mixed (4:6 and 1:9) filtrates, thrice filtered by column chromatography and then by flash chromatography, exerted a promising mosquito larvicidal potential as tested in this study. 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. As reported in the present study, the lethal concentrations of metabolites of *V. lecanii* after flash chromatography was found effective against *Cx. quinquefasciatus* and *Ae. aegypti* larvae. The result showed that the efficacy of *V. lecanii* metabolites also increased with increasing concentration. We can confirm here that the purified extracellular metabolites are efficacious against the mosquito larvae.

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