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FUSARIUM OXYSPORUM F.SP. PISI AGAINST THE LARVAE OF MAJOR MOSQUITO VECTORS: A LABORATORY INVESTIGATION

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ABSTRACT

Efficacy of the metabolites of *Fusarium oxysporum* f.sp. pisi has been investigated against the mosquito larvae of *Culex quinquefasciatus, Anopheles stephensi* and *Aedes aegypti* in laboratory. *F. oxysporum* f.sp. pisi was grown on Potato dextrose broth in the laboratory at 25°C, 75±5% humidity for 15 days. Filtration process was done using whatman-1 filter paper, column chromatography and flash chromatography. Larvicidal efficacy was performed against all larval instars of *Cx. quinquefasciatus*, *An. stephensi* and *Ae. aegypti* at six different concentrations with different effective ratios (ethanol/metabolites: 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, and 1:9) by the probit analysis for a period of 24, 48, and 72 h, respectively. Among all ratios, one potential ratio was selected for efficacy study. The 5:5 ratio was found highly effective against the larvae of *Cx. quinquefasciatus*, 7:3 ratios was effective against the *An. stephensi* and 1:9 ratio was found effective against the larvae of *Ae. aegypti*. The first, second and third instar larvae of *Cx. quinquefasciatus* have shown 100% mortality, while fourth instar larvae have LC_{50} 20 ppm, LC_{90} 89.12 ppm and LC_{99} 144.54 ppm values for the metabolites. The metabolites of *F. oxysporum* f.sp. pisi has not been tested against the mosquito larvae previously. The metabolites of *F. oxysporum* f.sp. pisi could be a fungal larvicides resource for the control of mosquitoes and could be another agent for biotechnological exploitation, if found suitable in field trials.

Keywords: *Fusarium oxysporum* f.sp. pisi, Metabolites, *Culex quinquefasciatus, Anopheles stephensi, Aedes aegypti*, Fungal larvicide

INTRODUCTION

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 [1].

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 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 [2].

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, 2, 3 and 4 (Den-1, Den-2, Den-3 and Den-4) [3]. 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 borne diseases. 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* [4]. 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 borne 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 non-target organisms [5]. Metabolites of *Chrysosporium* [6-11], *Metarhizium* and *Beeauveria* [12-13], *Lagenidium* [14-15], *Verticillium* [16] and *Fusarium* [17-19] have been screened as a potential larvicides successfully against the mosquito. *F. oxysporum* f.sp. pisi metabolites has been tested against the *Cx. quinquefasciatus, An. stephensi* and *Ae. aegypti* larvae in the present study in the laboratory. The present communication describes the larvicidal effect of extracellular metabolites of *F. oxysporum* f.sp. pisi after purification by flash chromatography against all instars of *Cx. quinquefasciatus, An. stephensi and Ae. aegypti.* This can be another way to avoid resistance problem effectively minimized while using new fungal larvicide.

MATERIALS AND METHOD

Fungal strain: The fungal strain of *F. oxysporum* f.sp. pisi (MTCC 2480) 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 Potato Dextrose Agar (PDA) medium at 25^0C (Fig 1).

Preparation of broth and culture of *F. oxysporum* f.sp. pisi: The broth was prepared for culture of *F. oxysporum* f.sp. pisi by the method of Gardner and Pillai [20]. *F. oxysporum* f.sp. pisi was grown on Potato Dextrose Broth (PDB). Five 250 ml conical flask, each containing 100 ml PDB (Infusion of potatoes 200g, Dextrose 20g and deionized water 1000ml) were autoclaved at 20 psi for 20 min. The broth was supplemented with 50 µg/ml chloramphenicol as a bacteriostatic agent. *F. oxysporum* f.sp. pisi colonies grown on the PDA plates were transferred to each flask using the inoculation needle. The conical flasks inoculated with *F. oxysporum* f.sp. pisi were incubated 25^0C 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^o, 10'N, 78^o05'E), India and reared in deionized water containing glucose and yeast powder. The colonies of *An. stephensi, Cx. quinquefasciatus* and *Ae. aegypti* were maintained in the laboratory at a temperature of 25^0C, 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 [21].

Filtration and purification of extracellular secondary metabolites: Cell free culture filtrates of *F. oxysporum* f.sp. pisi 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 1ml 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. These ratios were further purified through the flash chromatography. Then, 5-ml fractions were collected from all ratios.

Larvicidal efficacy of purified metabolites against mosquito larvae: To investigate the larvicidal activity of purified metabolites through flash 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 5:5 ratio was found effective against the larvae of *Cx. quinquefasciatus,* 7:3 ratio was effective against the *An. stephensi,* and 1:9 ratio was effective against the *Ae. aegypti* larvae.

Bioassays: Larvicidal efficacy of metabolites of *F. oxysporum* f.sp. pisi against *An. stephensi, Cx. quinquefasciatus* and *Ae. aegypti* was assessed by using the standard method [22]. All mosquito larvae of *An. stephensi, Cx. quinquefasciatus* and *Ae. aegypti* were separated and placed in a separate container with microbe free deionized water. 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 100ml 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 24h, 48h 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 efficacies were subjected to the probit analysis [23]. The control mortality was corrected by Abbott's formula [24]. 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 flash chromatography purification.

***F. oxysporum* f.sp. pisi metabolites against mosquito larvae:** The purified metabolites of *F. oxysporum* f.sp. pisi 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 susceptible to the *F. oxysporum* f.sp. pisi (5:5 ratio) metabolites than the larvae of *An. stephensi* and *Ae. aegypti*. The degree of susceptibility of mosquito larvae against the *F. oxysporum* f.sp. pisi metabolites were in the order of *Cx. quinquefasciatus* > *Ae. aegypti* >*An. stephensi*.

***F. oxysporum* f.sp. pisi (5:5 ratio) metabolites against the *Cx. quinquefasciatus* larvae:** All larval instars of *Cx. quinquefasciatus* have shown mortality for *F. oxysporum* f.sp. pisi metabolites. The first, second and third instar larvae of *Cx. quinquefasciatus* have shown 100% mortality for the *F. oxysporum* f.sp. pisi metabolites. Whereas, the fourth instar larvae were found less susceptible to the metabolites. The LC_{50} 20 ppm, LC_{90} 89.12 ppm, and LC_{99} 144.54 ppm were observed for the fourth 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.

***F. oxysporum* f.sp. pisi (1:9 ratio) metabolites against the *Ae. aegypti* larvae:** The metabolites of *F. oxysporum* f.sp. pisi were effective against the all instars of *Ae. aegypti* larvae. The LC_{50} 17.37 ppm, LC_{90} 120 ppm and LC_{99} 199.52 ppm values were calculated for the first instars. For second instars LC_{50} 20 ppm, LC_{90} 123.02 ppm and LC_{99} 239.88 ppm values were calculated. In third instars LC_{50} 40 ppm, LC_{90} 134.89 ppm, and LC_{99} 309.02 ppm were recorded. Whereas, for fourth instars LC_{50} 60 ppm, LC_{90} 154.88, and LC_{99} 363.07 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 *Ae. aegypti* (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 *Ae. aegypti* in order of first instar > second instar > third instar > fourth instar.

***F. oxysporum* f.sp. pisi (2:8 ratio) metabolites against the *An. stephensi* larvae:** The metabolites of *F. oxysporum* f.sp. pisi 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} 16.21 ppm, LC_{90} 120 ppm and LC_{99} 177.82 ppm were observed for first instar larvae of *An. stephensi.* In second instar larvae LC_{50} 19.05 ppm, LC_{90} 123.02 ppm, and LC_{99} 239.88 ppm were recorded. For third instar larvae LC_{50} 40 ppm, LC_{90} 147.91 and LC_{99} 346.73 ppm were observed. Whereas, for fourth instar larvae LC_{50} 80 ppm, LC_{90} 190.54 and LC_{99} 467.73 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 *An. stephensi* (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 *An. stephensi* in order of first instar > second instar > third instar > fourth instar.

DISCUSSIONS

Unlike other mosquito control agents, the entomopathogenic fungi are unique. 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. *F. oxysporum* f.sp. pisi 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 agent for the mosquito population. Fungal agents have reduced inputs of harmful synthetic chemical pesticide in agriculture, horticultural, and forest system. Laboratory investigation using the plants such as, *Vetiveria zizanioides, Ocimum basilicum* and the microbial pesticide spinosad against the malarial vector *An. stephensi* has been made [25]. The efficacy of the leaf extracts of *Momordica tuberosa* on the larval and pupal period, larval, pupal and adult mortality, and percentage of adult emergence and growth index of the filarial mosquito *Cx. quinquefasciatus* has been carried out [26]. 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) (2×10^{10} conidia m^{-2}) applied on mud panels (simulating walls of traditional Tanzanian houses), black cotton cloth and polyester netting was evaluated against adult *An. gambiae* [27]. They concluded that both fungal isolates reduced mosquito survival on immediate exposure up to 28d after application. A study with the spores of *C. lobatum* also shows 100% mortality to each instar larvae of *An. stephensi* [7]. Formulations of *M. anisopliae* and *B. bassiana* conidia 2×10^{10} conidia m^{-2} has been tested against the *An. gambiae* [12]. They concluded that fungal infection reduced the survival of mosquitoes regardless of their age and blood-feeding status. 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 *B. bassiana* (Balsamo) metabolites for controlling malaria and filaria in tropical countries have been evaluated [28]. They observed that these metabolites were found to be more effective on *An. stephensi* comparatively *Cx. quinquefasciatus* larvae. Again, the efficacy of *C. tropicum* metabolites is effective against mixed population of adult mosquito (*Cx. quinquefasciatus, An. stephensi*, and *Ae. aegypti*) after purification with flash chromatography have been observed [9]. Further, the pathogenicity of *F. oxysporum* against the larvae of *Cx. quinquefasciatus* (Say) and *An. stephensi* (Liston) in laboratory have been tested [19]. 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 *Cx. quinquefasciatus* larvae. In these experiments the metabolites were applied directly to all instars after filtration through Whatman no-1 paper. While in our study, we have purified the metabolites through the column and flash chromatography. The efficacy of entomopathogenic fungi *F. pallidoroseum* has been tested against the female *Cx. quinquefasciatus* [18]. They found that the All the female *Cx. quinquefasciatus* were killed within 4 days of exposure to *F. pallidoroseum* at a concentration of 1.11×10^{10} conidia per m^2. The above experiment was aimed against the adult mosquitoes, while in our experiment the metabolites after purification through flash chromatography were applied against the instars of *Cx. quinquefasciatus, An. stephensi* and *Ae. aegypti* larvae only. Recently, the potential pathogenicity of culture filtrates of *C. clavisporus* has been evaluated against the adults of *Cx. quinquefasciatus, Ae. aegypti* and *An. stephensi* [29]. Later on, the culture filtrates of *F. oxysporum, L. giganteum, T. ajelloi,* and *C. clavisporus* have been tested against adults of *Cx. quinquefasciatus* [30]. The results of above studies were against the adult mosquitoes, while in our studies the extracellular metabolites of *F. oxysporum* f.sp. pisi were tested against the larvae of mosquito.

In comparison with the results mentioned above, it was perceptible that ethanol and metabolite mixed (5:5, 1:9 and 7:3) filtrates, thrice filtered by flash chromatography, tested in this study exerted promising mosquito larvicidal 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 *F. oxysporum* f.sp. pisi 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.

CONCLUSIONS

The metabolites of *F. oxysporum* f.sp. pisi was when tested against the major mosquito larvae of *An. stephensi*, *Cx. quinquefasciatus and Ae. aegypti* after flash chromatography for the first time were found successful. The purification made metabolites more effective than the crude. Now concluded here that the use of extracellular metabolites of this fungus may provide better technology alternatives for controlling large population of mosquito larvae and also in managing the development of resistance in mosquitoes.

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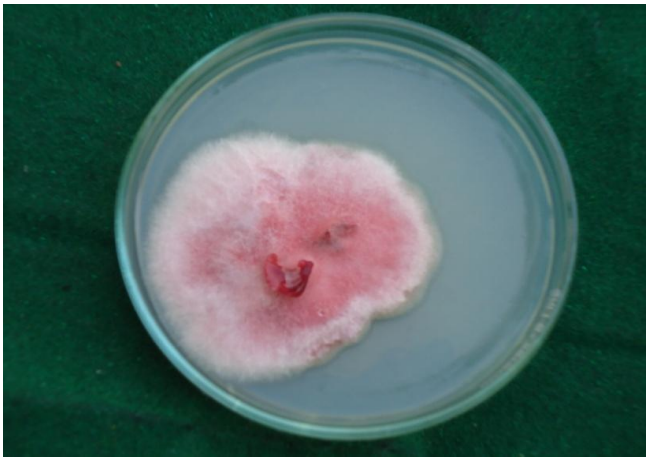

Figure 1: Culture of fungal colonies of *F. oxysporum* f.sp. pisi on Potato dextrose agar after seven days of growth at 25^{o}C in the laboratory.

Figure 2: Culture of fungal colonies of *F. oxysporum* f.sp. pisi on Potato dextrose broth after fifteen days of growth at 25^{o}C in the laboratory.

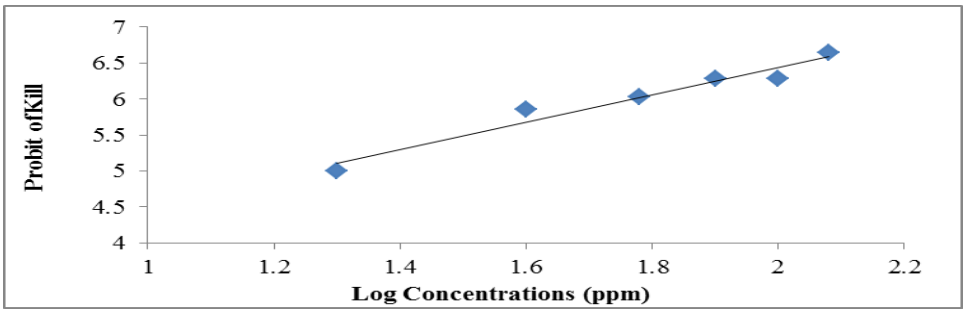

Figure 3: Relationship between probit of kill and log concentrations of *F. oxysporum* f.sp. pisi filtrate metabolites (5:5) showing probit regression lines in fourth instar larvae of *Cx. quinquefasciatus* after 72 h in the laboratory after flash chromatography.

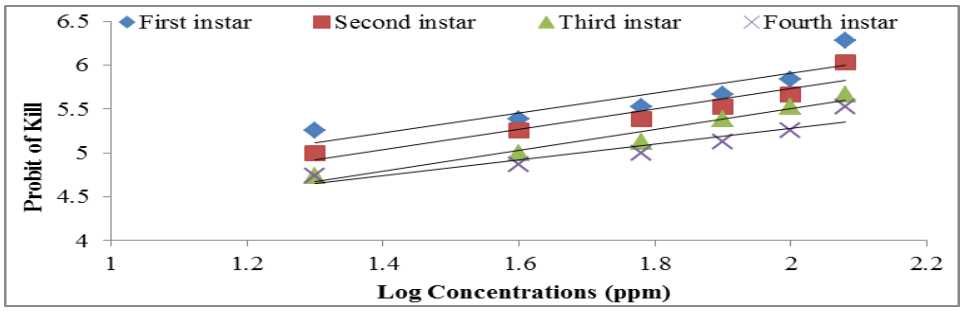

Figure 4: Relationship between probit of kill and log concentrations of *F. oxysporum* f.sp. pisi filtrate metabolites (1:9) showing probit regression lines in fourth instar larvae of *Ae. aegypti* after 72h in the laboratory after flash chromatography.

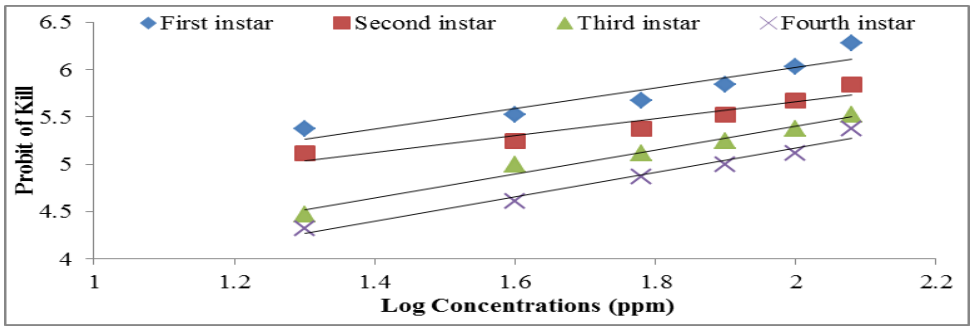

Figure 5: Relationship between probit of kill and log concentrations of *F. oxysporum* f.sp. pisi filtrate metabolites (7:3) showing probit regression lines in fourth instar larvae of *An. stephensii* after 72h in the laboratory after flash chromatography.

Table 1. Probit equations and susceptibility of *Cx. quinquefasciatus* larvae against extracellular metabolites of *F. oxysporum* f.sp. pisi with 95% confidential limits (C L) after 72h after flash chromatography

First instar				Second instar			Third instar			Fourth instar		
Ethanol: metabolite	LC_{50} (ppm)	LC_{90} (ppm)	LC_{99} (ppm)	LC_{50} (ppm)	LC_{90} (ppm)	LC_{99} (ppm)	LC_{50} (ppm)	LC_{90} (ppm)	LC_{99} (ppm)	LC_{50} (ppm)	LC_{90} (ppm)	LC_{99} (ppm)
9:1	**	**	**	Y=0.08+3.30x 15.13 (13.9-16.36)	100 (98.8-101.2)	154.88 (`153.68-156.08)	Y=0.09+3.10x 20 (18.86-21.14)	120 (118.83-121.17)	213.79 (212.54-215.04)	**	**	**
8:2	Y=0.1+3.20x 16.59 (15.36-17.82)	120 (118.83-121.17)	181.97 (180.77-183.17)	**	**	**	**	**	**	Y=0.07+2.85x 60 (58.86-61.14)	151.35 (150.12-152.58)	346.73 (345.39-348.07)
7:3	Y=0.1+3.20x 16.59 (15.36-17.82)	120 (118.83-121.17)	181.97 (180.77-183.17)	Y=0.08+3.10x 40 (38.86-41.14)	120 (118.83-121.17)	218.77 (217.52-220.02)	Y=0.08+2.98x40 (38.86-41.14)	123.02 (121.82-124.22)	269.15 (267.87-270.43)	Y=0.1+3.19x 16.59 (15.36-17.82)	120 (118.83-121.17)	181.97 (180.77-183.17)
6:4	Y=0.08+3.30x 15.13 (13.9-16.36)	100 (98.86-101.44)	544.88 (543.68-546.08)	Y=0.11+3.10x 17.78 (16.55-19.01)	123.02 (121.85-124.19)	213.79 (212.54-215.04)	Y=0.12+3.02x 19.05 (17.82-20.28)	131.82 (130.65-132.99)	245.47 (244.22-246.72)	Y=0.1+2.92x 40 (38.81-41.14)	LC90-128.82 (127.62-130.02)	295.12 (293.81-296.43)
5:5	****	****	****	****	****	****	****	****	****	**Y=0.09+3.35x 20 (18.8-21.20)**	**89.12 (88-90.24)**	**144.54 (143.37-145.71)**
4:6	**	**	**	**	**	**	Y=0.09+3.01x 40 (38.86-41.14)	120 (118.83-121.17)	251.18 (249.93-252.43)	Y=0.08+3.01x 40 (38.86-41.14)	123.02 (121.82-124.22)	257.03 (255.75-258.31)
3:7	Y=0.09+3.28x 14.45 (13.2-15.7)	100 (98.86-101.14)	158.48 (157.28-159.68)	Y=0.08+2.97x 40 (38.86-41.14)	128.82 (127.62-130.02)	295.12 (293.81-296.43)	Y=0.12+2.96x 20 (18.77-21.23)	123.02 (121.82-124.22)	269.15 (267.87-270.43)	Y=0.12+2.87x 40 (38.86-41.14)	138.03 (136.83-139.23)	323.59 (322.25-324.93)
2:8	Y=0.07+3.31x 20 (18.83-21.17)	89.12 (87.98-90.26)	154.88 (153.68-156.08)	**	**	**	**	**	**	Y=0.07+2.85x 60 (58.86-61.14)	151.35 (150.12-152.58)	346.73 (345.39-348.07)
1:9	Y=0.07+3.31x 20 (18.8-21.20)	100 (98.86-101.14)	181.97 (180.77-183.17)	Y=0.08+3x 40 (38.36-41.14)	123.02 (121.82-124.22)	257.03 (255.75-258.31)	**	**	**	Y=0.09+2.95x 40 (38.86-41.14)	125.89 (124.69-127.09)	281.83 (280.55-283.11)

** 100% mortality was observed

Table 2. Probit equations and susceptibility of *Ae. aegypti* larvae against extracellular metabolites of *F. oxysporum* f.sp. pisi with 95% confidential limits (C L) after 72h after flash chromatography

First instar				Second instar			Third instar			Fourth instar		
Ethanol: metabolite	LC_{50} (ppm)	LC_{90} (ppm)	LC_{99} (ppm)	LC_{50} (ppm)	LC_{90} (ppm)	LC_{99} (ppm)	LC_{50} (ppm)	LC_{90} (ppm)	LC_{99} (ppm)	LC_{50} (ppm)	LC_{90} (ppm)	LC_{99} (ppm)
9:1	Y=0.10+2.86x 40 (38.86-41.14)	144.54 (143.31-145.79)	331.13 (329.79-332.47)	Y=0.10+2.73x 80 (78.83-81.17)	181.97 (180.72-183.22)	436.51 (435.1-437.92)	Y=0.10+2.73x 80 (78.83-81.17)	181.97 (180.72-183.22)	436.51 (435.1-437.92)	Y=0.08+2.66x 80 (78.83-81.17)	213.79 (212.51-215.07)	524.80 (523.33-526.27)
8:2	Y=0.12+2.96x 20 (18.86-21.14)	123.02 (121.82-124.22)	269.15 (267.87-270.43)	Y=0.10+2.87x 40 (38.86-41.14)	141.25 (140.05-142.45)	331.13 (329.79-332.47)	Y=0.09+2.82x 60 (58.86-61.14)	154.88 (153.65-156.11)	363.07 (361.73-364.41)	Y=0.09+2.78x 60 (58.86-61.14)	165.95 (164.72-167.18)	398.10 (396.72-399.48)
7:3	Y=0.09+3.04x 20 (18.86-21.14)	123.02 (121.82-124.22)	239.88 (238.63-241.13)	Y=0.10+2.87x 40 (38.86-41.14)	141.25 (140.05-142.45)	331.13 (329.79-332.47)	Y=0.04+2.74x 80 (78.83-81.17)	190.54 (189.29-191.79)	457.08 (455.67-458.49)	Y=0.11+2.80x 60 (58.86-61.14)	158.48 (157.25-159.71)	380.18 (378.8-381.56)
6:4	Y=0.07+3.03x 40 (38.86-41.14)	120 (118.77-121.23)	245.47 (244.22-246.72)	Y=0.10+2.88x 60 (58.86-61.14)	123.02 (121.82-124.22)	323.59 (322.28-324.9)	Y=0.07+2.72x 80 (78.83-81.17)	190.54 (189.29-191.79)	467.73 (466.29-469.17)	Y=0.09+2.71x 80 (78.83-81.17)	190.54 (189.29-191.79)	467.73 (466.29-469.17)
5:5	Y=0.11+3.01x 20 (18.86-41.14)	123.02 (121.82-124.22)	251.18 (249.93-252.43)	Y=0.10+2.87x 40 (38.86-41.14)	141.25 (140.05-142.45)	331.13 (329.79-332.47)	Y=0.07+2.69x 80 (78.83-81.17)	199.52 (198.24-200.8)	489.77 (488.33-491.21)	Y=0.09+2.82x 60 (58.86-61.14)	154.88 (153.65-156.11)	363.07 (361.73-364.41)
4:6	Y=0.09+3.16x 16.98 (15.75-18.21)	109.64 (108.5-110.78)	194.98 (193.75-196.21)	Y=0.09+2.91x 40 (38.86-41.14)	131.82 (130.62-133.02)	301.99 (300.68-303.3)	Y=0.09+2.76x 80 (78.83-81.17)	173.78 (172.53-175.03)	416.86 (415.45-418.27)	Y=0.08+2.91x 40 (38.86-41.14)	134.89 (133.69-135.09)	309.02 (307.71-310.33)
3:7	Y=0.11+3.04x 18.62 (17.39-19.85)	123.02 (121.82-124.22)	234.42 (233.17-235.67)	Y=0.12+2.96x 20 (18.86-41.14)	128.82 (127.62-130.02)	269.15 (267.87-270.43)	Y=0.09+2.77x 80 (78.83-81.17)	169.82 (168.59-171.05)	407.38 (406-408.76)	Y=0.09+2.71x 80 (78.83-81.17)	190.54 (189.29-191.79)	467.73 (466.29-469.17)
2:8	Y=0.10+3.14x 17.37 (16.14-18.6)	120 (118.77-121.23)	199.52 (198.29-200.75)	Y=0.07+2.99x 40 (38.86-41.14)	123.02 (121.82-124.22)	263.02 (261.74-264.3)	Y=0.13+2.84x 60 (58.86-61.14)	144.54 (143.31-145.77)	338.84 (337.5-340.18)	Y=0.09+2.82x 60 (58.86-61.14)	154.88 (153.65-156.11)	363.07 (361.73-364.41)
1:9	**Y=0.10+3.14x 17.37 (16.14-18.6)**	**120 (118.77-121.23)**	**199.52 (198.29-200.75)**	**Y=0.09+3.04x 20 (18.86-21.14)**	**123.02 (121.82-124.22)**	**239.88 (238.63-241.13)**	**Y=0.08+2.91x 40 (38.86-41.14)**	**134.89 (133.69-135.09)**	**309.02 (307.71-310.33)**	**Y=0.09+2.82x 60 (58.86-61.14)**	**154.88 (153.65-156.11)**	**363.07 (361.73-364.41)**

Table 3. Probit equations and susceptibility of *An. stephensi* larvae against extracellular metabolites of *F. oxysporum* f.sp. pisi with 95% confidential limits (C L) after 72h after flash chromatography

First instar				Second instar			Third instar			Fourth instar		
Ethanol: metabolite	LC_{50} (ppm)	LC_{90} (ppm)	LC_{99} (ppm)	LC_{50} (ppm)	LC_{90} (ppm)	LC_{99} (ppm)	LC_{50} (ppm)	LC_{90} (ppm)	LC_{99} (ppm)	LC_{50} (ppm)	LC_{90} (ppm)	LC_{99} (ppm)
9:1	Y=0.11+3.11x 17.78 (16.55-19.01)	123.02 (121.82-124.22)	208.92 (207.69-210.15)	Y=0.04+2.86x 36.30 (35.13-37.47)	151.35 (150.12-152.58)	346.73 (345.39-348.07)	Y=0.09+2.76x80 (78.83-81.17)	173.78 (172.53-175.03)	416.86 (415.45-418.27)	Y=0.08+2.65x100 (98.8-101.2)	213.79 (212.51-215.07)	537.03 (535.56-538.5)
8:2	Y=0.11+3.11x 17.78 (16.55-19.01)	123.02 (121.82-124.22)	208.92 (207.69-210.15)	Y=0.12+2.87x 40 (38.86-41.14)	138.03 (136.83-137.23)	323.59 (322.25-324.93)	Y=0.08+2.75x60 (58.86-61.14)	177.82 (176.57-179.07)	426.57 (425.16-427.98)	Y=0.09+2.72x80 (78.83-81.17)	186.20 (184.95-187.45)	457.08 (455.67-458.49)
7:3	**Y=0.1+3.21x 16.21 (14.98-17.44)**	**120 (118.77-121.23)**	**177.82 (176.62-179.02)**	**Y=0.12+3.02x 19.05 (17.82-20.28)**	**123.02 (121.82-124.22)**	**239.88 (238.63-241.13)**	**Y=0.08+2.85x40 (38.86-41.14)**	**147.91 (146.68-149.14)**	**346.73 (345.39-348.07)**	**Y=0.09+2.71x80 (78.83-81.17)**	**190.54 (189.29-191.79)**	**467.73 (466.32-469.14)**
6:4	Y=0.12+2.96x 20 (18.86-21.14)	123.02 (121.82-124.22)	269.15 (267.87-270.43)	Y=0.09+2.85x 60 (58.86-61.14)	147.91 (146.68-149.14)	346.73 (345.39-348.07)	Y=0.09+2.76x80 (78.83-81.17)	173.78 (172.53-175.03)	416.86 (415.45-418.27)	Y=0.07+2.73x80 (87.86-81.17)	186.20 (184.95-187.45)	457.08 (455.67-458.49)
5:5	Y=0.1+2.92x 40 (38.86-41.14)	128.82 (127.62-130.02)	295.12 (293.81-296.43)	Y=0.08+2.79x 60 (58.86-61.14)	165.95 (164.72-167.18)	398.10 (396.72-399.48)	Y=0.11+2.80x60 (58.86-61.14)	158.48 (157.25-159.71)	380.18 (378.8-381.56)	Y=0.1+2.73x 80 (78.83-81.17)	181.97 (180.72-183.22)	436.51 (435.1-437.92)
4:6	Y=0.09+2.89x 40 (38.86-41.14)	138.03 (136.83-139.23)	316.22 (314.91-317.53)	Y=0.07+2.73x 80 (78.83-81.17)	186.20 (184.95-187.45)	457.08 (455.67-458.49)	Y=0.07+2.68x80 (78.83-81.17)	204.17 (202.89-205.45)	512.86 (511.42-514.3)	Y=0.06+2.60x100 (98.8-101.2)	245.47 (244.16-246.78)	616.59 (615.08-618.1)
3:7	Y=0.1+2.93x 40 (38.86-41.14)	128.82 (127.62-130.02)	288.40 (287.02-289.78)	Y=0.11+3.11x 17.78 (16.55-19.01)	123.02 (121.82-124.22)	208.92 (207.69-210.15)	Y=0.11+2.80x60 (58.86-61.14)	158.48 (157.25-159.71)	380.18 (378.8-381.56)	Y=0.11+2.80x60 (58.86-61.14)	158.48 (157.25-159.71)	380.18 (378.8-381.56)
2:8	Y=0.11+3.4x 13.48 (12.25-14.71)	80 (78.83-81.17)	125.89 (124.75-127.03)	Y=0.10+2.83x 53.70 (52.56-54.84)	151.35 (150.12-152.58)	354.81 (353.47-356.15)	Y=0.09+2.77x60 (58.86-61.14)	169.82 (168.59-171.05)	407.38 (406-408.76)	Y=0.09+2.70x80 (78.83-81.17)	194.98 (193.73-196.23)	478.63 (477.19-480.07)
1:9	Y=0.11+3.04x 18.62 (17.39-19.85)	123.02 (121.82-124.22)	234.42 (233.17-235.67)	Y=0.1+2.88x 40 (38.86-41.14)	138.03 (136.83-139.23)	323.59 (322.25-324.93)	Y=0.1+2.79x 60 (58.86-61.14)	162.18 (160.95-163.41)	389.04 (387.66-390.42)	Y=0.09+2.70x80 (78.83-81.17)	194.98 (193.73-196.23)	478.63 (477.19-480.07)

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