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Mosquito control potential of secondary metabolites isolated from *Aspergillus flavus* and *Aspergillus fumigatus*

Kasilingam Balumahendhiran, Perumal Vivekanandhan, Muthugounder Subramanian Shivakumar *

Molecular Entomology Laboratory, Department of Biotechnology, Periyar University, Salem, 636011, Tamil Nadu, India

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ABSTRACT

Repeated use of same or different types of chemical pesticide causes environmental pollution and results in resistance in mosquitoes. Currently vector control programs need alternative tools from biological source. Plant and fungi crude metabolites maybe jointly or independently prove its efficacy on mosquitoes. In the present study, we tested the *Aspergillus flavus* and *Aspergillus fumigatus* crude metabolites on larvae of *Anopheles stephensi*, *Culex quinquefasciatus*, and *Aedes aegypti*, after 24 h and 48 h exposure in laboratory condition. Crude metabolites were characterized using GC-MS analysis. Our results clearly, shows that lower LC_{50} and LC_{90} value were observed in *A. flavus* on *A. aegypti* was 18.018 mg/l, 31.053 mg/l for 24 h exposure whereas, *A. aegypti* had 39.687 mg/l, 82.263 mg/l 48 h exposure respectively. Also lower LC_{50} and LC_{90} values in *A. fumigatus* on *C. quinquefasciatus* were, 11.436 mg/l and 28.309 mg/l, for 24 h exposure, *A. fumigatus* crude metabolites are highly toxic to mosquito larvae than *A. flavus* crude metabolites. Fungi cultures were confirmed as *A. flavus* and *A. fumigatus* by using 18S rDNA sequencing. Present study concluded that fungi derived secondary metabolites is eco-friendly in nature, cheaper, effective, target-specific and lower toxicity to non-target organisms.

1. Introduction

Mosquitoes can transmit several diseases namely, malaria, dengue, filariasis, Japanese encephalitis, chikungunya and yellow fever to humans and other domesticated animals (Budhiraja et al., 2013). Female Anopheles mosquitoes transmitted malarial parasites to humans. According to the latest estimates 214million cases of malaria in 2015 and 438 000 deaths (WHO, 2016). *C. quinquefasciatus* mosquitoes are vectors of diseases like lymphatic filariasis, Japanese encephalitis, Ross River viral and West Nile Virus. About 120 million people are infected in the worldwide (WHO, 2000). *A. Aegypti* is vectors of Dengue and Zika virus which are recently considered as a major health risk to humans (WHO, 2015a,b).

Currently, synthetic pesticides used for mosquito control in worldwide. Organophosphates and pyrethroids are two major classes of insecticides used in mosquito control programs (Yang et al., 2002). Repeated use of chemical insecticides is led to environmental pollution and deleterious effects on non-target organisms. Therefore, alternative ecofriendly sources from plant, fungi and bacteria are being studied (Vivekanandhan et al., 2018a, b, c, d, e, f; Vivekanandhan et al., 2019). Plant secondary metabolites and essential oils have been known to produce significant toxicity in various stages of mosquitoes(Vivekanandhan et al., 2019; Vivekanandhan et al., 2018a,b), botanical derived extracts highly effective on different stage of mosquito larvae (Rahuman et al., 2009), and microbial mosquito larvicides, such as *Bacillus sphaericus*, entomopathogenic fungus such as, *Beauveria bassiana* and *Fusarium oxysporum* and their secondary metabolites are used as alternative for mosquitoes control (Dharumadurai et al., 2014; Vivekanandhan et al., 2018c,d,e). Pathogenic fungi namely *F. oxysporum* and *B. bassiana* derived crude metabolites contain a variety of bioactive principles which are toxic to mosquitoes (Vivekanandhan et al., 2018c, d, e; Vyas et al., 2007). Above the reasons we target entomopathogenic fungi from the soil environment because soil born entomopathogenic fungi derived crude metabolites shows highly toxic to mosquito larvae (Vivekanandhan et al., 2018c).

The present study was isolation and identification of *A. flavus* and *A. fumigatus* from soil environment and evaluation of mosquito control efficacy on *A. stephensi*, *A. aegypti* and *C. quinquefasciatus* larvae and the crude metabolites chemical constituents were characterized using GC-MS analysis.

* Corresponding author.
E-mail address: skentomology@gmail.com (M.S. Shivakumar).

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2. Materials and methods

2.1. Soil sample collection

Soil samples were collected from Yercaud hills, latitude and longitude are, (11.7211°N; 78.1835°E) and Salem district, Tamilnadu, India. Then the sample was brought to the laboratory in sterile polyethylene bags and stored at 4 °C for further use.

2.2. Isolation and identification of fungal culture

Potato dextrose agar (Potatoes-200 g, Dextrose-20 g and Agar-15 g/L) 100 ml medium were prepared and sterilized at 121 °C in 15 min. Subsequently, the sterilized medium was supplemented with chloramphenicol (50 μg) to prevent bacterial contamination (Vivekanandhan et al., 2018a). Then one gram soil sample was mixed with 50 ml of sterile distilled water, finally the soil sample are serially diluted up to 10^{-1} -10^{-10} to dilutions. 100 μl of the aliquots from the concentration of 10^{-5}, 10^{-7}, 10^{-4} and 10^{-3} were spread over the already prepared PDA medium and plates were incubated at (28 ± 2 °C) for 3 days. After the incubation period, the fungi colonies were individually separated and maintained in the fresh PDA medium for further studies.

A. flavus and *A. fumigatus* fungal culture were morphologically identified using slide culture method with lactophenol cotton blue staining (Vivekanandhan et al., 2018a, b). The lactophenol cotton blue stained slides were visualized under light microscope at 40X magnification.

2.3. Mass culturing of Aspergillus spp

Broth was prepared for culturing *A. flavus* and *A. fumigatus*. In Potato dextrose broth (Potatoes-200 g, Dextrose-20 g/L) was prepared in five 250 ml conical flask, each containing 200 ml of potato dextrose broth were autoclaved at 15 psi for 30 min. The fungi broth was supplemented 50μg/ml chloramphenicol as used for preventing the bacterial contamination. Then 1×10^7 conidia/ml for ml was inoculated in culturing media. The inoculated culture media were incubated in an orbital shaker at (27°± 2°C) for 15 days (Logeswaran et al., 2019).

2.4. Extraction of secondary metabolites

After 15 days incubation, the culture medium was centrifuged at 5000 rpm for 30mins, after centrifugation the pellet was discarded and supernatant were taken for secondary metabolites extraction. Then ethyl acetate was added to the supernatant with different ratios (1:2, 1:1, 2:1) and the culture filtrate was transferred into a separating funnel. The culture filtrates were mixed and allowed were soaked ethyl acetate for 4–6days and the aqueous organic phase were collected. Organic phase was kept at room temperature for solvent evaporation at room temperature (28±2°C). The concentrated secondary metabolites were kept at 4°C for further study.

2.5. Mosquito rearing

A. aegypti, A. stephensi and *C. quinquefasciatus* larvae were obtained from the Centre for Research in Medical Entomology (CRME) Madurai, Tamil Nadu, India. Mosquito larvae were maintained in the laboratory condition at (25 ± 2 °C), Relative humidity of (70 ± 5%) and a photoperiod of (14:10) (light: Dark). Different instars of *An. stephensi, C. quinquefasciatus* and *A. aegypti* were maintained in separate enamel containers (25 cm × 15 cm × 5 cm). All larvae were fed with dog biscuit and yeast powder in the ratio of 3:2 (Vivekanandhan et al., 2019; Kalaimurugan et al., 2018). Larvae were reared in double-distilled water at pH 7.0. Fresh water was changed every day.

2.6. Larvicidal bioassay

Larvicidal activity of both fungi metabolites on *C. quinquefasciatus*, *A. stephensi* and *A. aegypti* was assessed followed by using the standard method (WHO, 2005; Vivekanandhan et al., 2018a, b; Logeswaran et al., 2019). All mosquito larvae of *C. quinquefasciatus, A. stephensi* and *A. aegypti* were separated and placed in a container with microbe free deionized water. After that, the metabolites are mixed with Dimethyl Sulfoxide (DMSO) of different concentrations in 250 ml deionized water. Bioassays were conducted separately for each instar at three different test concentrations (10, 30, and 50 ppm) of crude metabolites. 25 larvae of each stage were separately exposed to 250 ml of tests of the larvicidal activity of extracellular crude metabolites. Similarly, the control was run to test the natural mortality. After the test mortality and survival were determined after 24, 48, and 72 h of the exposure. Larvae without feeding were maintained at throughout the experimental period. The experiments were done by using three replicates to validate results. All test containers were tightly covered with pierced aluminum foil and placed at room temperature.

2.7. GC-MS analysis and identification of compounds

Secondary metabolites of *A. flavus* and *A. fumigatus* was analyzed by Gas liquid chromatography and mass spectrometry (PerkinElmer Clarus 600 (EI)). Which is used only one type of column (Polaris Q Ion Trap GC/FID). A fused silica column (30 m × 0.25 mm) and coated with an Elite-5ms (DF = 250 μm). The injection temperature was maintained at 250°C. The column temperature program was 60°C for 2 min, ramp 10°C/min to 300°C, hold 6 min, and the total run time is 32 min. Helium was used as the carrier gas with a flow rate of 1 ml/min. The samples (1 μl) were injected neat with a 1:10 split ratio (solvent Delay = 2.00 min, Transfer Temp = 240°C, Source Temp = 240°C). The mass spectrum was obtained at a70eV ionized voltage the fragments from 40 to 600 Da. The identification of individual compounds was done using the Wiley/NBS Registry of mass spectral database, the NIST (version 3.0) database. Furthermore, the retention time (RT) and Kovats Index (KI) values of several authentic reference compounds were compared with isolated compounds for identification followed by Pratheeba et al. (2019).

2.8. Statistical analysis

The average larval mortality data were subjected to probit analysis for calculatingLC_{50}, LC_{90} by using SPSS (version 9.0). Other statistics at 95% fiducial limits of Upper confidence limits (UCL) and lower confidence limits (LCL), and chi-square values were calculated by using (Finney, 1971) method.

3. Results

3.1. Fungi identification

Isolated fungi colony color was light green in color; aerial mycelial structure was green in color with round shape and another one was light blue and conidial structure was round light blue color (Fig. 1a and b). Based on preliminary confirmation isolated fungi were conformed as Aspergillus spp. Further molecular techniques like, genomic DNA isolation, PCR amplification and phylogenetic construction were used for identification (Figs. 2 and 3). Fungal sequences were submitted to the National Center for Biotechnology Information (NCBI) data base. The accession no is MG871558 and MG552677.

3.2. Larval bioassay

Larvicidal activity was conducted by using the fungi crude extract of *A. flavus* and *A. fumigatus* on three mosquito vectors, such as *A. aegypti,*

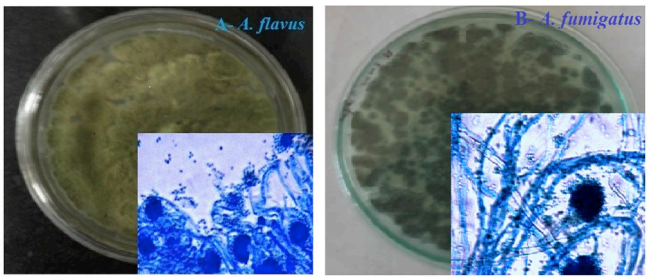

Fig. 1. Morphological identification of fungi species. A-is *A. flavus* and B- is *A. fumigatus* was cultured on potato dextrose agar plate at (28 ± 2 °C).

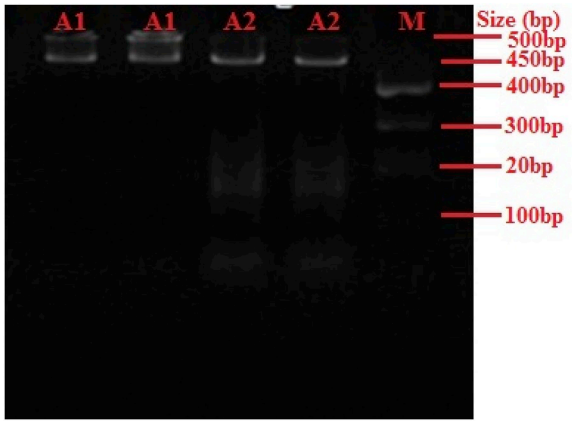

Fig. 2. DNA electrophoresis pattern of fungi genomic DNA. A1 is *A. flavus* genomic DNA; A2 is *A. fumigatus* genomic DNA and M-DNA marker.

A. stephensi and *C. quinquefasciatus* followed by (WHO, 2005), 24 h and 48 h post treatment.

Larval mortality rate was observed in the *A. flavus* crude extract on *A. stephensi*, *C. quinquefasciatus*, and *A. aegypti*. Highest mortality was observed in the extract of *A. flavus* on in *A.aegypti* with LC_{50} and LC_{90} values were, 18.018 mg/l and 31.053 mg/l (Table 1). Isolated secondary metabolites from *A. flavus* produced significant mortality after 48 h of exposure with LC_{50} and LC_{90} values of 39.687 mg/l and 82.263 mg/l (Table 2).

A. fumigatus shows remarkable toxicity on *C. quinquefasciatus* with low LC_{50} and LC_{90} values, 11.436 mg/l and 28.309 mg/l, after 24 h exposure (Table 3). After 48 h exposure of *A. fumigatus* resulted in high mortality in *A.stephensi* with LC_{50} and LC_{90} values of 29.357 mg/l and 59.276 mg/l respectively (Table 4).

3.3. Chemical constitution from *A. flavus* and *A. fumigatus* metabolites

GC-MS analysis results of *A. flavus* metabolites shows 13 compounds namely, 4H-Pyran-4-One, 5-Hydroxy-2-(Hydroxymethyl)-(13.813%), Nonane, 2-Bromo-5-Ethyl (4.751), 4-Hexenoic Acid, 5-Hydroxy-3-Oxo-2-Propionyl, D-Lactone (3.095), 3-(Prop-2-Enoyloxy) Tridecane (4.449), 1-Docosene (2.489), Di-N-Octyl Phthalate (1.678), Sulfurous Acid, 2-Propyl Tetradecyl Ester(1.396) (Fig. 4, Table 5).

GC-MS analysis results of *A. fumigatus* clearly showed 13 compounds (Fig. 5, Table 6). Among the compounds three chemical are major namely, Di-N-Octyl Phthalate (9.029), (1H-Benzoimidazol-2-Yl)-[4-(4-Methyl-Piperazin-1-Yl)-Phenyl]-Amine (25.658), 6,8-Dimethyl-5-Oxo-2,3,5,8-Tetrahydroimidazo[1,2-A]Pyrimidine (40.048) maybe involved in mosquito larvicidal activity.

4. Discussion

Secondary metabolites from plant and microbes are known to be effective on a wide range of medical and agricultural pest control programme (Sriwattanarungsee et al., 2008; Vivekanandhan et al., 2018a, c, d). The plant and entomopathogenic fungi secondary metabolites may jointly or independently show its efficacy on the mosquitoes as, ovicidal, larvicidal, pupicidal, adulticidal and by inhibition of their growth (Vivekanandhan et al., 2018a, 2019). Fungi secondary metabolites are considered to be a potential alternative approach in mosquito control

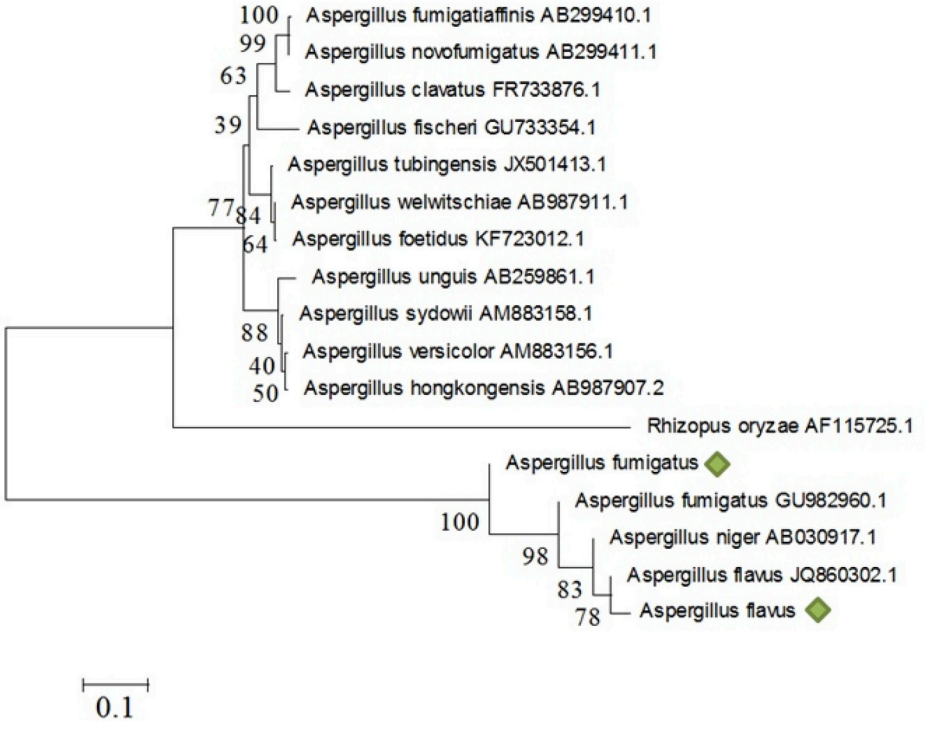

Fig. 3. Phylogenetic tree of *A. flavus* and *A. fumigatus*.

Table 1
Efficacy of *Aspergillus flavus* crude extract on *A. stephensi*, *C. quinquefasciatus*, and *A.aegypti* after 24 h treatment.

Mosquito species	Concentration (mg/L)	LC50 (LCL–UCL) (mg/L)	LC90 (LCL–UCL) (mg/L)	χ^2 (df = 12)
C. quinquefasciatus	10 30 50	18.217 (16.287–20.427)	50.044 (48.817–53.002)	9.168
A. stephensi	10 30 50	21.459 (19.921–23.432)	49.704 (47.009–50.764)	8.672
A. aegypti	10 30 50	18.018 (16.207–20.298)	31.053 (27.155–38.010)	1.572

Table 2
Efficacy of *Aspergillus flavus* crude extract on *A. stephensi, C. quinquefasciatus*, and *A. aegypti* after 48 h treatment.

Mosquito species	Concentration (mg/L)	LC50 (LCL–UCL) (mg/L)	LC90 (LCL–UCL) (mg/L)	χ^2 (df = 12)
C. quinquefasciatus,	10 30 50	42.817 (40.007–44.826)	87.204 (85.014–88.984)	2.419
A. stephensi,	10 30 50	42.009 (40.823–44.206)	89.271 (87.075–90.989)	7.242
A. aegypti	10 30 50	39.687 (37.825–41.620)	82.263 (80.271–85.726)	6.667

Table 3
Efficacy of *Aspergillus fumigatus* crude extract on *A. stephensi*, *C. quinquefasciatus*, and *A. Aegypti* after 24 h treatment.

Mosquito species	Concentration (mg/L)	LC50 (LCL–UCL) (mg/L)	LC90 (LCL–UCL) (mg/L)	χ^2 (df = 12)
C. quinquefasciatus	10 30 50	11.436 (9.589–13.428)	28.309 (26.291–29.986)	4.572
A. stephensi	10 30 50	15.290 (13.975–17.075)	36.966 (35.019–37.987)	10.290
A. aegypti	10 30 50	14.796 (12.321–15.921)	35.401 (33.721–37.017)	5.397

Table 4
Efficacy of *Aspergillus fumigatus* crude extract on *A. stephensi*, *C. quinquefasciatus*, and *A. aegypti* after 48 h treatment.

Mosquito species	Concentration (mg/L)	LC50 (LCL–UCL) (mg/L)	LC90 (LCL–UCL) (mg/L)	χ^2 (df = 1)
C. quinquefasciatus	10 30 50	32.078 (30.901–35.072)	65.843 (63.987–65.928)	6.777
A. stephensi	10 30 50	29.357 (27.031–32.024)	59.276 (57.193–61.078)	4.472
A. aegypti	10 30 50	35.374 (33.751–37.860)	73.301 (72.014–37.860)	5.989

and also have several advantages like, cheaper, effective, environmental safety, target specific, pollution free, and lower toxic to non-target organisms (Vivekanandhan et al., 2018a,c; Ehrlich et al., 2010; Kalaimurugan et al., 2018).

Our results, clearly shows that *A. flavus* derived crude metabolites produced remarkable toxicity on three major mosquito species namely, *A. aegypti*, *A. stephensi* and *C. quinquefasciatus* among the mosquito species the *A. aegypti* is highly susceptible to *A. flavus* crude metabolites at 24 h post treatment (Tables 1 and 2). *B. bassiana* derived crude metabolites produced remarkable toxic effect to *A. aegypti*, *A. stephensi* and *C. quinquefasciatus* mosquito larvae at 24 h post treatment (Vivekanandhan et al., 2018c). Similarly the *Ganoderma applantum* (Per.) derived secondary metabolites show highly toxic to three major mosquito species such as *A. aegypti*, *A. stephensi* and *C. quinquefasciatus* under laboratory condition and their histopathological studies show gut tissues highly damaged by the fungi metabolites (Logeswaran et al., 2019). *F. oxysporum* derived silver nanoparticles are highly effective for control the larvae of major mosquito species at (Vivekanandhan et al., 2018e). The Aspergillus fungi species produced verity of secondary metabolite such as, Aflatoxins, Butyl 9-hexadecenoate, pyrazolin- 5-one, bis(Hex-5-en-1-yloxy) (dimethyl)silane shown chemical constituents are used in several biological activities namely, anti-bacterial, anti-fungal,

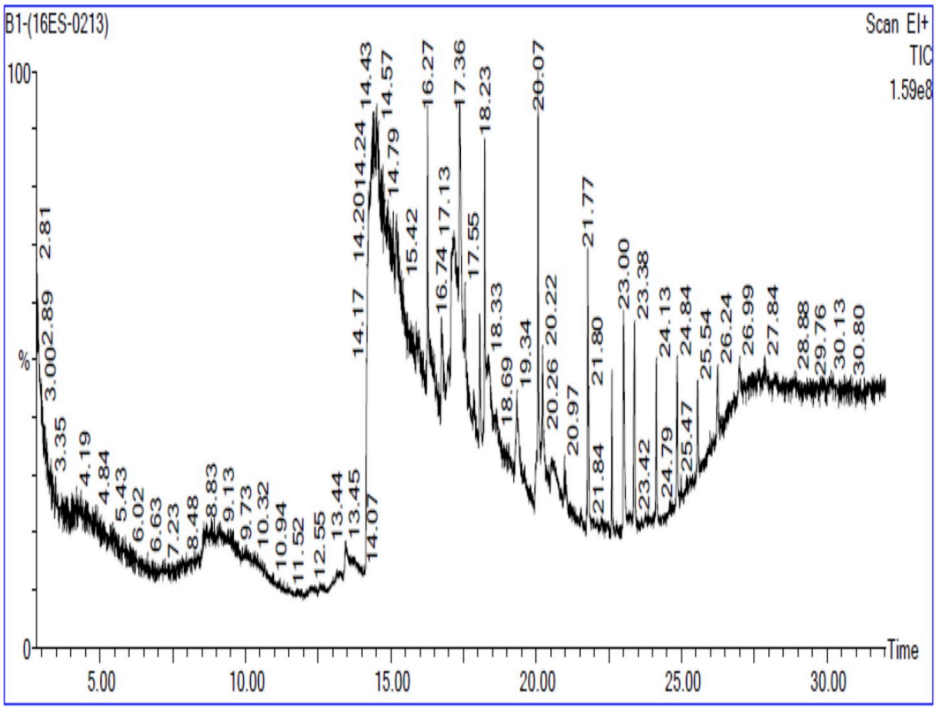

Fig. 4. *A. flavus* crude metabolites chemical constituents were identified by using GC-MS analysis.

Table 5
The *Aspergillus flavus* secondary metabolites chemical constituents were identified using GC-MS analysis.

S. No	RT	Area%	Compound Name	Activity	Reference
1	14.388	13.813	4H-Pyran-4-One, 5-Hydroxy-2-(Hydroxymethyl)-	Pesticidalactivity and Antioxidant activity.	None
2	16.269	4.751	Nonane, 2-Bromo-5-Ethyl	None	None
3	17.364	3.095	4-Hexenoic Acid, 5-Hydroxy-3-Oxo-2-Propionyl-, D-Lactone	None	None
4	18.230	4.499	3-(Prop-2-Enoyloxy)Tridecane	None	None
5	20.065	2.489	1-Docosene	Antibacterial activity	Hameedha Beevi et al., 2014
6	23.002	1.678	Di-N-Octyl Phthalate	PesticidalActivity, and Antimicrobial activity	ATSDR.Agency for Toxic Substances and Disease Registry, 1998, KaariaP.K2009
7	23.377	1.396	Sulfurous Acid, 2-Propyl Tetradecyl Ester	None	None

anti-cancer and antioxidant activity.

A. fumigatus crude metabolites were evaluated on *A. stephensi*, *C. quinquefasciatus*, and *A. Aegypti* among the mosquito species the fungi metabolites highly toxic to *C. quinquefasciatus* and *A. stephensi* mosquito larvae (Tables 3 and 4). Similarly the *C. lobatum* derived secondary metabolites have remarkable toxicity on *A. stephensi* and *C. quinquefasciatus* under laboratory condition at 24 h post treatment (Mohanty and Prakash, 2004). Significantly higher mortality was recorded in *F. oxysporum* derived metabolites combined with Temephos on three mosquito larvae of *A. stephensi*, *A. aegypti* and *C. quinquefasciatus* (Vivekanandhan et al., 2018d). *F. oxysporum* secondary metabolites studied against the larvae of *C. quinquefasciatus* (Say) and *A. stephensi* (Prakash et al., 2010). *C. keratinophium* derived metabolites evaluated for larvicidal activity under laboratory condition against *C. quinquefasciatus* have been studied (Soni and Prakash, 2010). Efficacy of *B. bassiana* metabolites has been evaluated as microbial larvicide on major mosquito species (Singh and Prakash, 2010; Vivekanandhan et al., 2018c).

GC-MS analysis results of *A. flavus* crude metabolites shows 13 compounds (Fig. 4, Table 6). Among these constituents three chemicals namely, Di-N-Octyl Phthalate (9.029%), (1H-Benzoimidazol-2-Yl)-[4-(4-Methyl-Piperazin-1-Yl)-Phenyl]-Amine (25.658%) and 6,8-Dimethyl-5-Oxo-2,3,5,8-Tetrahydroimidazo [1,2-A] Pyrimidine(40.048%) maybe involved in mosquito larvicidal activity. Similar chemical constituents were reported from entomopathogenic fungi *Beauveria bassiana* and *Fusarium oxysporum* secondary metabolites (Vivekanandhan et al., 2018a,b; Vivekanandhan et al., 2019). Logeswaran et al. (2019) reported that *Ganoderma applantum* (Per.) secondary metabolites contain similar kind of chemical constituents involved in mosquito larvicidal activity.

5. Conclusion

Our results clearly show that *A. flavus* and *A. fumigatus* derived crude metabolites has potential for controlling the *A. stephensi*, *A. aegypti* and *C. quinquefasciatus* mosquito vectors. *A. flavus* and *A. fumigatus* metabolites show several biological activity and pest control program. The upcoming work connecting the bioassay guided fractionation can be useful in separation of active insecticidal molecules, which could have commercial importance. In the present study *A. flavus* and *A. fumigatus* derived crude metabolites are effective mosquito larvicides. These fungal metabolites can be used as an alternative for chemical insecticides in mosquito control.

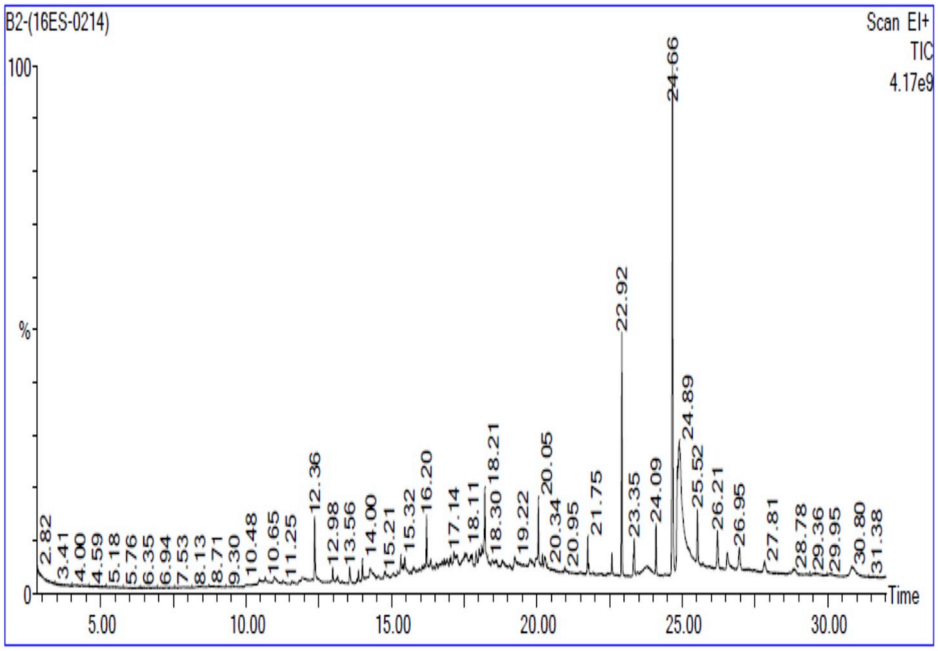

Fig. 5. *A. fumigatus* crude metabolites chemical constituents were identified by using GC-MS analysis.

Table 6
The *Aspergillus fumigatus* secondary metabolites chemical constituents were identified using GC-MS analysis.

S. No	RT	Area%	Compound Name	Biological Activity	Reference
1	12.362	2.985	3-Ethylidenecycloheptene	None	None
2	16.204	1.998	1-Hexadecene	None	None
3	18.210	3.554	8-Heptadecene	None	None
4	20.050	3.669	5-Eicosene	None	None
5	22.916	9.026	Di-N-Octyl Phthalate	Pesticidal activity	ATSDR.Agency for Toxic Substances and Disease Registry, 1998
6	23.347	2.262	Octacosane	Antimicrobial activity	Kaaria P·K 2009
7	24.092	1.961	Tetratetracontane	None	None
8	24.662	25.658	(1H-Benzoimidazol-2-Yl)-[4-(4-Methyl-Piperazin-1-Yl)-Phenyl]-Amine	None	None
9	24.892	40.048	6,8-Dimethyl-5-Oxo-2,3,5,8-Tetrahydroimidazo[1,2-A] Pyrimidine	None	None
10	25.518	2.594	Heptacosane	None	None
11	26.543	1.488	3-Nitrophthalhydrazide	None	None
12	26.968	2.137	Dotracontane	Larvicital activity	Thangavel et al., 2015

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101334.

LC50; lethal concentration that kills 50% of the exposed larvae, LC90: lethal concentration that kills 90% of exposed larvae, UCL; upper confidence limit (95% fiducial limit), LCL: lower confidence limit (95% fiducial limit), χ^2:chi-square.

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