RSC Advances

PAPER

View Article Online
View Journal | View Issue

Cite this: *RSC Adv.*, 2017, **7**, 3838

Beauveria bassiana (Clavicipitaceae): a potent fungal agent for controlling mosquito vectors of *Anopheles stephensi*, *Culex quinquefasciatus* and *Aedes aegypti* (Diptera: Culicidae)

Chinnasamy Ragavendran,[a] Nawal Kishore Dubey[b] and Devarajan Natarajan*[a]

Mosquitoes are the carriers of severe and well-known illnesses such as malaria, arboviral encephalitis, dengue, chikungunya and yellow fever, which cause significant morbidity and mortality in humans and domestic animals around the world. Entomopathogenic fungal metabolites act as a mosquito control agent and are potential alternatives to chemical control because they can be innovative and more selective than chemical insecticides. The main aim of the present study was to perform experiments on the larvicidal and pupicidal effects of the entomopathogenic fungus *Beauveria bassiana* (isolated from infected grasshopper) against the first to fourth instar larvae of *Anopheles stephensi*, *Culex quinquefasciatus* and *Aedes aegypti*. The larval and pupal mortality were observed after 24 h of exposure. The efficacy of an ethyl acetate mycelium extract at all the tested concentrations (50, 100, 150, 200, 250 and 300 μg mL^{-1}) exhibited better activity against the 1st to 4th instar larvae of *An. stephensi* (LC_{50} = 42.82, 39.45, 25.72, and 32.66; LC_{90} = 254.67, 367.11, 182.27, and 199.20 μg mL^{-1}), *Cx. quinquefasciatus* (LC_{50} = 72.38, 68.11, 27.06, and 35.495; LC_{90} = 481.68, 254.69, 129.83, and 146.24 μg mL^{-1}) and *Ae. aegypti* (LC_{50} = 62.50, 52.89, 58.60, and 47.12; LC_{90} = 314.82, 236.18, 247.53, and 278.52 μg mL^{-1}), respectively. The pupicidal activity of the fungal mycelium extracts was tested against *An. stephensi*, *Cx. quinquefasciatus* and *Ae. Aegypti*, where the ethyl acetate extracts had different LC_{50} values (LC_{50} = 40.66, 54.06, 44.26, and LC_{90} = 184.02, 225.61, and 263.02 μg mL^{-1}). Based on Fourier transform infrared spectroscopy (FTIR) analysis and gas chromatography-mass spectrometry (GC-MS) analyses, the ethyl acetate mycelium extract contained six major chemical compounds identified as 9,12-octadecadienoic acid (ZZ)– (63.16%), *n*-hexadecanoic acid (21.28%), octadecanoic acid, phenyl methyl ester (10.45%), dehydroegosterol 3,5-dinitrobenzoate (1.86%), squalene (1.66%) and bis[3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)prophyl]maleate (1.56%). The *n*-hexadecanoic acid standard was found to be better larvicidal against *An. stephensi*, *Cx. quinquefasciatus*, followed by *Ae. aegypti*. The HPLC analysis of the ethyl acetate mycelium extract was compared with that of the *n*-hexadecanoic acid standard and it was found to show a similar chromatographic peak (at a retention time of 3.383 and 3.378 min). The outcome of the present study identifies the bioactive compounds obtained from *B. bassiana* that can be used as effective and alternate larvicidal and pupicidal agents against the *An. stephensi Cx. quinquefasciatus* and *Ae. aegypti* mosquito vectors.

Received 26th October 2016
Accepted 6th December 2016

DOI: 10.1039/c6ra25859j

www.rsc.org/advances

Introduction

Vector-borne diseases are illnesses caused by pathogens and parasites in human populations. Globally, every year there are about more than 1 billion cases and over 1 million deaths due to vector-borne diseases, such as malaria, dengue, schistosomiasis, human African trypanosomiasis, leishmaniasis, chagas disease, yellow fever, Japanese encephalitis and onchocerciasis. Vector-borne diseases account for over 17% of all infectious diseases. Malaria is a parasitic disease spread by infected *Anopheles* mosquitoes, which is caused by parasite species namely *Plasmodium falciparum*, *P. vivax*, *P. malariae* and *P. ovale*.[1] Malaria causes symptoms that typically include fever and headache, which in severe cases can lead to coma or death. A recent survey released in December 2014 reported about 198 million cases of malaria in 2013 with an uncertainty range from 124 million to 283 million and an estimated 584000 deaths (with an uncertainty range of 367 000 to 755 000). Malarial mortality rates have fallen globally by 47% since 2000 and 54% reported in the African regions.[2]

[a]*Natural Drug Research Laboratory, Department of Biotechnology, School of Biosciences, Periyar University, Salem – 636 011, Tamil Nadu, India. E-mail: mdnataraj@rediffmail.com; natarajpu@gmail.com*

[b]*Centre of Advanced Study in Botany, Banaras Hindu University, Varanasi-221005, India*

Culex mosquitoes are painful and persistent biters and are responsible for filariasis. Lymphatic filariasis is a neglected tropical disease. Lymphatic filariasis is commonly known as elephantiasis and infection occurs when filarial parasites are transmitted to humans through mosquitoes.[3] When a mosquito with infective stage larvae bites a person, the parasites are deposited on the person's skin from where they enter into the body. The larvae then migrate to the lymphatic vessels where they develop into adult worms in the lymphatic system. Worldwide, more than 1.3 billion people from 72 countries are threatened by lymphatic filariasis, commonly known as elephantiasis.[4] Chikungunya is a viral tropical disease transmitted by *Aedes* mosquitoes. The disease is prevalent in Africa, Asia, the islands in the Caribbean, India and Pacific oceans. Typical symptoms are an acute illness with fever, skin rash and incapacitating joint pain that can last for weeks.[5] The latter distinguishes chikungunya virus from dengue, which otherwise shares the same vectors, symptoms and geographical distribution. There is no cure or commercial vaccine for the disease. Most patients recover fully; however, in some cases, joint pain may persist for several months or even years. As with dengue, the only method to reduce the transmission of the chikungunya virus is to control vector mosquitoes and protect against mosquito bites. Yellow fever is an acute viral hemorrhagic disease transmitted by *Aedes* mosquitoes. The "yellow" in the name refers to the jaundice that affects some patients. There are an estimated 200 000 cases of yellow fever, which cause 30 000 deaths worldwide per year. The virus that causes yellow fever is endemic in densely populated countries, *viz.*, Tropical Africa and Latin America. Small numbers of imported cases occur in countries free of yellow fever.[6]

The common control agents for mosquito larvicides are mainly dependent on chemical methods using synthetic insecticides that are likely to include, organophosphates such as temephos, fenthion, phytochemicals and insect growth regulators such as diflubenzuron, and methoprene.[7] However, most of these synthetics have adverse effects on the environment. Due to their residual nature there are reports on the development of pesticide resistance in mosquitoes[8] rendering them ineffective for further applications. These problems encourage the search for safer and better alternative bioactive larvicidal agents. Although various biocontrol measures are in vogue, to date, their effective control of larval mosquitoes has not been practically highlighted. Microbial control is recommended as an alternative way, and microbial based larvicides are employed for minimizing the mosquito population, which provides an effective, environmentally friendly and sociable approach to bring the mosquito population to the lowest level.[9,10]

Beauveria bassiana (Clavicipitaceae) is a soil borne fungus that feeds on insects and can be used effectively to control thrips, aphids, whitefly, caterpillars, beetles, and subterranean insects like ants and termites. *B. bassiana* is non-toxic to mammals, birds and plants, and its use is not expected to have any deleterious effects on human health or the environment.[11] Conidia of *B. bassiana* has been reported to be effective in killing mosquito larvae when applied as conidia dust in the breeding sites. Besides infecting larvae, the fungus has also proven to be virulent to adult mosquitoes.[12] *B. bassiana* is applied to the target pest as a spore, which is the reproductive and dispersal structure of the fungus. Once the spores have made contact with the insect exoskeleton, they grow hyphae (long, branching vegetative appendages) that secrete enzymes, which in turn dissolve the cuticle (outermost layer of the skeleton). These fungal hyphae grow into the insect, feed on its body tissue, produce toxins, and reproduce. It takes up to seven days for the insect to die. During favorable (moist) conditions (92% humidity or greater), *B. bassiana* will "bloom" and release more spores into the environment to repeat the cycle on other pest insects.[13] The species of *Beauveria* has been reported to produce secondary metabolites, including bassianin, bassiacridin, beauvericin, bassianolide, beauverolides, tenellin and oosporein.[14–16] It also produces proteases, chitinases and lipases, which can degrade the insect cuticle.[17] In this regard, the entomopathogenic fungi, *viz.*, *Aspergillus flavus*, *A. parasiticus*, *Penicillium falicum*, *Fusarium vasinfectum* and *Trichoderma viride* and soil bacteria, *Bacillus thuringiensis* and *B. sphaericus* have been reported to be effective against *Cx. quinquefasciatus*.[18] Hence, the present study was focused on the insecticidal potential of *Beauveria bassiana* mycelial extracts against target mosquitoes.

Materials and methods

Isolation and identification of entomopathogenic fungus

The entomopathogenic fungus *B. bassiana* was isolated from an infected grasshopper (*Melanoplus sanguinipes*) collected from an agricultural field (latitude 11.6500° N, longitude 78.1600° E) in the Salem District, Tamilnadu, India. The cadaver was placed on potato dextrose agar (PDA (Hi-Media)) supplemented with streptomycin (1 mg/100 mL) and incubated for 7 days at 27 °C ± 2 °C.[19] After 7 days of incubation, the pure culture of *B. bassiana* was subcultured into PDA using the streak plate method. The isolated culture was identified using the slide culture method subjected to lactophenol cotton blue staining and observed under a light microscope (Labomed). Mycotaxonomic keys followed by Samson[20] and Samson *et al.*[21] were used to identify the fungus.

Morphological identification of *B. bassiana*

The fungus was primarily identified based on its morphological features, descriptions of species, keys to taxa and additional information from ''Studies in Mycology''.[22] Colonies of *B. bassiana* fungus were cultivated on Sabouraud's dextrose agar at 25 °C for 7 days. The following morphological characteristics were assessed: colony growth (length and width), the presence or absence of aerial mycelium, colony color, presence of wrinkles, furrows and pigment production.[21]

Preparation of Sabouraud's dextrose broth and mass culture of *B. bassiana*

The broths were prepared for the culture of fungus as per the modified method of Gardner and Pillai.[23] *B. bassiana* was grown

in Sabouraud's Dextrose Broth (SDB). Ten 250 mL conical flasks, each containing 100 mL of SDB (dextrose 40 g, peptone 10 g, deionized water 1000 mL), autoclaved at 20 psi for 20 min. The broths were supplemented with 50 μg mL^{-1} chloramphenicol, which acted as a bacteriostatic agent. The *B. bassiana* colonies grown on the Sabouraud's dextrose agar plates were transferred to each flask (using an inoculation needle). The conical flasks inoculated with *B. bassiana* were incubated at 25 °C for 25 days.

Secondary metabolite extraction from *B. bassiana*

Mass cultivation of the fungus was carried out in a 250 mL Erlenmeyer flask containing 100 mL of Sabouraud's dextrose broth medium. The culture flasks were incubated under the optimized culture conditions (pH 7.0, temperature 27 °C) for 25 days. For the liquid culture, the fungal mycelium mat was washed three times with sterile distilled water to remove adherent filtrate and subjected to an extraction of biologically active components using ethyl acetate and methanol solvents. The solvents were mixed to the mycelia mat for cold extraction for 7 days at room temperature. After thorough mixing, the immiscible portion of ethyl acetate (pale yellow colored) was separated from the mycelium. The mycelium was filtered through Whatmann no. 1 filter paper. The separated portions of ethyl acetate and methanol extracts were finally dried using a rotary vacuum evaporator at 45 °C as per the modified method of Belofsky *et al.* (2000).[24]

Larvae collection and rearing

For the laboratory trial, the different (1^{st} to 4^{th}) instar larvae stages of *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti* were obtained from the Institute of Vector Control and Zoonoses, (IVCZ), (latitude 12.7200° N, longitude 77.8200° E), Hosur, Tamilnadu, India. The larvae were kept in plastic enamel trays containing dechlorinated tap water. They were maintained as per the previous report of Patil *et al.*[25] The larvae were fed on dog biscuits and yeast powder in 3 : 1 ratio. Adults were fed with blood through a paraffin membrane and provided with 10% sucrose solution. Mosquitoes were kept at 28 °C ± 2 °C and 70–85% relative humidity with a photoperiod of 12 h light/12 h dark.

Larvicidal bioassay

The larval mortality bioassays were carried out according to the method suggested by the World Health Organization[26] with slight modifications.[27] Sufficient amounts of ethyl acetate and methanol extracts were transferred to a vial, and the residual solvent was removed under high vacuum. Stock solutions of each test mycelium extract in dimethyl sulfoxide (DMSO) were prepared with a concentration of 10% w/v (1 mg of extracts in 1000 μL of DMSO) prepared into five different concentrations *viz.* 50, 100, 150, 250 and 300 μg mL^{-1} with distilled water at pH 7.0. Twenty numbers of late first to early fourth-instar mosquito larvae were placed in a 2% v/v aqueous solution of DMSO (99 mL of distilled water plus 1 mL of DMSO), followed by the addition of the test solutions. Five replicates per dose were maintained, and a treatment with 99 mL of tap water and 1 mL of DMSO was added to each bioassay as the control at pH 7.0. During this experiment, no food was provided to the larvae. The larval mortality was calculated after 24 h of exposure.

$$\text{Corrected mortality} = \frac{\text{observed mortality in treatment} - \text{observed mortality in control}}{100 - \text{control mortality}} \times 100$$

Pupal toxicity tests

The laboratory colony of mosquito pupae was used to test the pupicidal activity of the *B. bassiana* extracts. Twenty freshly emerged pupae were kept in a 100 mL glass beaker containing 99 mL of dechlorinated water and a different concentrations of mycelium extracts (50, 100, 150, 200, 250 and 300 μg mL^{-1}). The experiment consists of five replicates; the control containing 1 mL of DMSO in 99 mL of dechlorinated water at pH 7.0. The mortality in the treatments and control was corrected using Abbott's formula.[28] The LC_{50} and LC_{90} were calculated from toxicity data using probit analysis.[29]

$$\text{Percentage of mortality} = \frac{\text{number of dead larvae/pupae}}{\text{number of larvae/pupae introduced}} \times 100$$

Dose response bioassay

The stock solutions obtained from the mycelia extract at different concentrations (ranging from 50 to 300 μg mL^{-1}) were prepared as per the method of Rahuman *et al.*[30] Based on the preliminary screening results, the mycelium extracts of *B. bassiana* were subjected to a dose-response bioassay for larvicidal and pupicidal activity against first to fourth instar larvae and pupae of *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti*. The number of dead larvae were counted after 24 h of exposure, and the percentage mortality was reported from the average of five replicates.

Preparation of the standard

n-Hexadecanoic acid was procured from Sigma, USA and DMSO was used as the solvent to prepare the stock solution. The stock solution was diluted further to produce the required concentrations to perform the bioassay tests.[31]

Control experiment (*Acremonium* sp. non-pathogenic fungi)

The larval and pupal mortality bioassays of *Acremonium* sp. were carried out according to the method suggested by the World Health Organization with slight modifications.[26,27] A sufficient amount of the *Acremonium* sp. ethyl acetate extracts was transferred to a vial, and the residual solvent was removed under high vacuum. The stock solution of *Acremonium* mycelial ethyl acetate extract was prepared using dimethyl sulfoxide (DMSO) with a concentration of 10% w/v (1 mg of extracts in 1000 μL of DMSO). Then, it was diluted into five different concentrations, *viz.*, 50, 100, 150, 250 and 300 μg mL^{-1}, and used for bioassay.

Fourier transformed infrared spectroscopy (FTIR)

1.0 mg of sample was mixed with 100 mg of KBr (binding agent) using a clean mortar and a pestle to produce a powder. The powder was made into pellets using a hydraulic press. The pellets were then subjected to FTIR analysis on a BRUKER α-T FTIR spectrometer. The precision of the FTIR spectra was better than 0.09 cm^{-1} and the scanning range was from 4000 to 500 cm^{-1}.[32] FTIR analysis was carried out in the Department of Physics, Periyar University, Salem, Tamilnadu, India.

Gas chromatography-mass spectrophotometry (GC-MS) analysis

GC-MS analysis of the samples was carried out on a Perkin Elmer (clarus 680) series GC-MS (Marathon, USA) system equipped with clarus 600 (EI) auto-sampler coupled with an Elite-5 MS capillary column (30 m $\times$ 0.25 mm i.d., and 0.250 μm) (PerkinElmer, Inc, made in USA). Helium was used as the carrier gas at a flow rate of 1 mL min^{-1}; split ratio of 10 : 1; mass scan 50–600 Da; ionization energy, 70 eV; ion source temperature, 240 °C; injector temperature, 250 °C. The oven temperature was programmed as follows: initially at 60 °C for 2 min, rising at 10 °C min^{-1} to 300 °C and then held isothermally (6 min) at 300 °C with a total run time of 32 min. The percentage composition of the crude extract constituents was expressed as a percentage of the peak area. The chemical compounds were identified and characterized based on their retention time (RT). The obtained mass spectral data (GC-MS) was matched with those of standards available in the existing computer library (NIST) data base.[33] The GC-MS analysis was carried out in the Sophisticated Instrument Facility, (SAIF). Vellore Institute of Technology (VIT), Vellore, Tamilnadu, India.

High performance liquid chromatography (HPLC) analysis

The *B. bassiana* mycelium ethyl acetate extract and pure *n*-hexadecanoic acid were diluted and subjected to high performance liquid chromatography (HPLC) analysis. For the chromatographic analysis of ethyl acetate extract and pure *n*-hexadecanoic acid, the samples were detected using an LC-20AD HPLC system (Shimadzu Chromatographic Instruments, Japan) equipped with a C18 reverse phase column (particle size: 5 μm; length: 4.6 $\times$ 250 mm) and a SPD-20A UV/Vis detector at 242 nm absorbance with methanol : water (50 : 50) at a flow rate of 1 mL min^{-1} and head pressure of 300 kgf cm^{-2}. The entire instrument room setup was maintained at room temperature (23 °C) following the method of Junaid Khan *et al.*[34] *n*-Hexadecanoic acid was used as the standard. The amount of specific compounds that resembles the standard was expressed as micrograms per gram.

Statistical analysis

The percentage of larval mortality was calculated using the Abbott formula.[28] The dose-response data were subjected to probit regression analysis[29] for calculating the LC_{50}, LC_{90}, 95% fiducial limits of upper confidence limit and lower confidence limit, and the chi-square values were calculated using the IBM SPSS (Statistical Package of Social Sciences) software version 20.0 developed by Reddy *et al.*[35] Results with $P < 0.05$ were considered to be statistically significant.

Results

The fungal strain was isolated from an infected grasshopper, *Melanoplus sanguinipes*. The SDA plates showed (after incubation) a fungus with white fluffy cottony growth with pale yellow edges. The piece of mycelium was stained with lactophenol cotton blue and observed under a microscope (Lobomed, 40×) showing abundant conidiospores arising from the vegetative hyphae, bearing groups of clustered conidiogenous cells with the apical zig-zag appearance, branched to give rise to further conidiogenous cells; globose to flask-shaped, one-celled spherical conidia were recorded. Previously Hermanides[36] and Seyed Ali Safari[37] identified *B. bassiana* using fungal key manual 'Studies in Mycology'.

The larvicidal activity of mycelium ethyl acetate and methanol extracts obtained from *B. bassiana* was investigated. The ethyl acetate mycelium extract had a promising larvicidal activity (Table 1) against the 1st to 4th instar larvae (after 24 h of exposure period) on *An. stephensi* (LC_{50} = 42.82, 39.45, 25.72, and 32.66; LC_{90} = 254.67, 367.11, 182.27 and 199.20 μg mL^{-1}) *Cx. quinquefasciatus* (LC_{50} = 72.38, 68.11, 27.06, and 35.495; LC_{90} = 481.68, 254.69, 129.83, and 146.24 μg mL^{-1}) and *Ae. aegypti* (LC_{50} = 62.50, 52.89, 58.60, and 47.12; LC_{90} = 314.82, 236.18, 247.53, and 278.52 μg mL^{-1}). The methanol mycelium extract (Table 2) showed considerable mortality against the vector mosquitoes *i.e. An. stephensi*, which had the better LC_{50} and LC_{90} values (LC_{50} = 65.22, 68.96, 67.64 and 52.95; LC_{90} = 317.77, 431.59, 345.35 and 687.70 μg mL^{-1}) followed by *Cx. quinquefasciatus* (LC_{50} = 98.56, 80.85, 61.72 and 41.16; LC_{90} = 678.66, 399.97, 336.85 and 470.47 μg mL^{-1}) and *Ae. aegypti* (LC_{50} = 64.94, 72.61, 61.90 and 57.65; LC_{90} = 961.97, 901.21, 439.32 and 916.04 μg mL^{-1}). At a concentration of less than 50 μg mL^{-1} from *B. bassiana*, the mortality rates were slower, but the larvae became very slow-moving when compared with the control. The sub-lethal effects on the first and second larval instars were correlated with the minimum survival of the third and fourth instar larvae. The third and fourth instars larvae were also susceptible in the bioassay at the lowest lethal concentration. The dose dependent assay results showed that maximum (100%) mortality was obtained at a higher concentration (300 μg mL^{-1}) against the different stages of instar larvae of the *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti* mosquitoes. At a higher concentration of extracts, the mortality rate was exhibited within 5 h of exposure. More than 50% mortality was observed within the first 10 h. The control showed a nil mortality in the concurrent assay. The χ^2 value was significant at the $P < 0.05$ level.

The results of the pupal mortality of mosquitoes (Table 3) were tested with six different concentrations (50 to 300 μg mL^{-1}) of the fungus extracts. The fungal ethyl acetate mycelium extracts show better results against *An. stephensi* (LC_{50} = 40.66; LC_{90} = 184.02 μg mL^{-1}) followed by *Cx. quinquefasciatus* (LC_{50} = 54.06; LC_{90} = 225.61 μg mL^{-1}) and *Ae. aegypti* (LC_{50} = 44.26;

Table 1 The larvicidal activity of *B. bassiana* fungal mycelium extract (ethyl acetate) against the larvae of *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti* (after 24 h of exposure)[a]

Mosquito species	Larvae stage	Concentration ($\mu g\ mL^{-1}$)	Percentage[b] mortality ± SE	LC_{50} (LCL–UCL) ($\mu g\ mL^{-1}$)	LC_{90} (LCL–UCL) ($\mu g\ mL^{-1}$)	χ^2 (df = 3)
An. stephensi	I	50	62.66 ± 2.5	42.826 (22.661–59.994)	254.679 (196.072–400.697)	14.266
		100	68.00 ± 1.0			
		150	71.66 ± 2.0			
		200	81.66 ± 1.5			
		250	93.66 ± 5.1			
		300	99.33 ± 1.1			
	II	50	63.33 ± 3.5	39.459 (15.560–60.018)	367.114 (253.777–811.269)	14.442
		100	66.66 ± 1.5			
		150	68.66 ± 1.5			
		200	74.66 ± 2.0			
		250	87.33 ± 2.0			
		300	98.33 ± 2.0			
	III	50	71.00 ± 1.0	25.727 (8.271–42.558)	182.275 (140.331–278.069)	9.289
		100	79.00 ± 5.5			
		150	83.33 ± 3.0			
		200	86.33 ± 3.0			
		250	93.66 ± 5.0			
		300	100 ± 0.0			
	IV	50	67.66 ± 3.2	32.664 (14.232–49.187)	199.206 (155.735–297.324)	8.545
		100	73.00 ± 2.0			
		150	84.00 ± 1.0			
		200	85.33 ± 1.5			
		250	93.00 ± 3.6			
		300	100 ± 0.0			
Cx. quinquefasciatus	I	50	48.33 ± 1.5	72.385 (47.687–92.674)	481.686 (334.801–960.417)	17.270
		100	54.00 ± 2.0			
		150	62.33 ± 1.5			
		200	65.00 ± 1.7			
		250	77.33 ± 1.1			
		300	98.33 ± 0.5			
	II	50	48.33 ± 3.2	68.117 (51.556–82.429)	254.698 (208.256–343.894)	13.911
		100	52.00 ± 2.6			
		150	73.33 ± 3.0			
		200	83.00 ± 3.0			
		250	90.00 ± 3.6			
		300	100 ± 0.0			
	III	50	74.66 ± 1.5	27.063 (11.301–41.487)	129.836 (103.262–172.038)	8.658*
		100	81.66 ± 1.5			
		150	85.33 ± 2.0			
		200	95.33 ± 2.5			
		250	98.66 ± 1.5			
		300	100 ± 0.0			
	IV	50	70.33 ± 1.5	35.495 (19.588–49.247)	146.249 (119.821–190.880)	15.145
		100	75.00 ± 2.0			
		150	82.33 ± 3.2			
		200	95.00 ± 5.5			
		250	99.66 ± 0.5			
		300	100 ± 0.0			
Ae. aegypti	I	50	53.00 ± 1.0	62.506 (42.337–79.404)	314.823 (242.389–487.932)	14.334
		100	55.33 ± 2.0			
		150	66.66 ± 1.5			
		200	80.00 ± 2.0			
		250	85.66 ± 2.5			
		300	99.33 ± 1.1			
	II	50	56.00 ± 3.0	52.896 (34.846–68.158)	236.183 (189.696–332.423)	13.939
		100	65.00 ± 3.0			
		150	73.66 ± 4.1			
		200	82.33 ± 5.8			
		250	93.66 ± 4.0			
		300	100 ± 0.0			
	III	50	52.00 ± 2.0	58.603 (40.851–73.647)	247.535 (199.550–345.351)	16.537
		100	65.33 ± 1.5			

Table 1 (*Contd.*)

Mosquito species	Larvae stage	Concentration (μg mL^{-1})	Percentage[b] mortality ± SE	LC_{50} (LCL–UCL) (μg mL^{-1})	LC_{90} (LCL–UCL) (μg mL^{-1})	χ^2 (df = 3)
		150	68.33 ± 2.0			
		200	81.00 ± 7.0			
		250	94.00 ± 4.5			
		300	100 ± 0.0			
	IV	50	57.00 ± 1.0	47.125 (26.419–64.574)	278.528 (212.833–445.541)	15.999
		100	69.00 ± 1.3			
		150	74.00 ± 1.0			
		200	78.66 ± 0.5			
		250	87.66 ± 1.5			
		300	100 ± 0.0			

[a] Control (deionized water) – nil mortality. LC_{50} – lethal concentration that kills 50% of the exposed larvae, LC_{90} – lethal concentration that kills 90% of the exposed larvae, LCL = lower confidence limit, UCL = upper confidence limit, df degree of freedom, * χ^2 – chi-square values are significant at $P < 0.05$ levels. [b] The mean value of five replicates (±SE).

LC_{90} = 263.02 μg mL^{-1}) (Fig. 1), whereas the methanol extract revealed moderate pupicidal effects against *An. stephensi* (LC_{50} = 51.92; LC_{90} = 1196 μg mL^{-1}), *Cx. quinquefasciatus* (LC_{50} = 69.29; LC_{90} = 862.25 μg mL^{-1}) and *Ae. aegypti* (LC_{50} = 76.34; 1178.15 μg mL^{-1}), (Table 4). At the concentrations of 300 μg mL^{-1} for the *B. bassiana* ethyl acetate constituents, about 90% of the mortality was observed within 18 h for *An. stephensi* and *Cx. quinquefasciatus*, followed by *Ae. Aegypti*, and a 100% pupal mortality was observed at the higher concentration of the extracts. The pupal toxicity revealed a dose-dependent mortality in the treatment against the *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti*. Based on the results, the ethyl acetate extract obtained from the fungal species was found to be an excellent pupicidal agent against the targeted mosquitoes *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti*.

In addition, the toxicity of the *n*-hexadecanoic acid standard was tested against *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti*. The LC_{50} values of *n*-hexadecanoic acid against the first, second, third and fourth instar larvae of *An. stephensi* (LC_{50} = 50.22, 58.72, 2.27 and 38.61; LC_{90} = 105.09, 148.19, 15.910 and 81.98) and *Cx. quinquefasciatus* (LC_{50} = 10.64, 23.23, 12.75 and 0.72; 39.82, 55.53, 38.47 and 5.18) followed by *Ae. aegypti* (LC_{50} = 5.53, 12.46, 8.13 and 9.41; 21.25, 33.75, 30.57 and 27.36 μg mL^{-1}) were recorded from present investigation. Similar observations were made for the pupicidal activity against *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti*; the LC_{50} and LC_{90} values were represented as follows: 8.66, 0.69, 3.05; 28.86, 4.38 and 11.43 μg mL^{-1}, respectively. *n*-Hexadecanoic acid was found to show effective insecticidal activity against *An. stephensi* and *Cx. quinquefasciatus*, followed by *Ae. aegypti*.

Simultaneously, the *Acremonium* mycelium ethyl acetate extract showed larvicidal effects after 24 h of exposure. Considerable mortality was evident after the treatment of *Acremonium* for 1–4th instar larvae of three important mosquitoes. The LC_{50} and LC_{90} values of the first, second, third and fourth instars of *An. stephensi* (LC_{50} = 11.38, 8.18, 8.56 and 5.30; LC_{90} = 22.42, 17.19, 17.23 and 11.84 μg mL^{-1}); *Cx. quinquefasciatus* (LC_{50} = 10.11, 13.35, 4.01 and 8.06; LC_{90} = 20.23, 25.13, 9.83 and 17.83 μg mL^{-1}) and *Ae. aegypti* (LC_{50} = 8.50, 9.58, 15.26 and 10.35; LC_{90} = 18.02, 20.00, 28.88 and 21.51 μg mL^{-1}) and the LC_{50} and LC_{90} values of the pupae (LC_{50} = 5.48, 9.60 and 3.99; LC_{90} = 14.46, 20.56 and 11.10 μg mL^{-1}) were obtained from the present study.

FTIR spectroscopy was used to identify the functional groups of the active compounds based on the peak value in the infrared region. FTIR analysis of the ethyl acetate mycelium extract showed the presence of prominent bands due to the O–H group of hydrogen-bonded alcohols or phenols (3420.94), =C–H aromatics (3002.58), C–H alkanes (2916.88), –C≡C– nitriles (2122.99), –C=C– alkanes (1654.84), C–H alkanes (1436.22), C–C aromatics (1409.89), C–O carboxylic acids (1315.64), C–N aliphatic amines (1021.42), =C–H alkenes (953.59), N–H primary amines (901.85) and C=O ketones (706.10) cm^{-1} (Fig. 2 and Table 5).

The GC-MS results obtained from the ethyl acetate extract of *B. bassiana* indicated the presence of six major compounds *viz.* 9,12-octadecadienoic acid (ZZ)– (63.16%), *n*-hexadecanoic acid (21.28%), octadecanoic acid, phenyl methyl ester (10.45%), dehydroegosterol 3,5-dinitrobenzoate (1.86%), squalene (1.66%), and bis[3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)prophyl] maleate (1.56%) (Fig. 3 and Table 6). Hence, the isolated bioactive compounds obtained from the *B. bassiana* derived products, with proven potential as an insecticide, can play an important role in the interruption of the transmission of mosquito-borne diseases. The larvicidal and pupicidal activity of the ethyl acetate extract may be due to the presence of major bioinsecticide constituents such as 9,12-octadecadienoic acid (ZZ)– and *n*-hexadecanoic acid.

HPLC analysis of the ethyl acetate mycelium extract of *B. bassiana* and the *n*-hexadecanoic acid standard showed a similar chromatographic peak (at the retention time 3.383 and 3.378 min) (Fig. 4a and b).

Discussion

Microbial sources serve as a guide for the isolation of several bioactive compounds particularly mosquito control agents. The entomopathogenic fungi have the ability to directly infect the

Table 2 The larvicidal activity of *B. bassiana* fungal mycelium extract (methanol) against the larvae of *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti* (after 24 h of exposure)[a]

Mosquito species	Larvae stage	Concentration ($\mu g\ mL^{-1}$)	Percentage[b] mortality ± SE	LC_{50} (LCL–UCL) ($\mu g\ mL^{-1}$)	LC_{90} (LCL–UCL) ($\mu g\ mL^{-1}$)	χ^2 (df = 3)
An. stephensi	I	50	44.33 ± 1.5	65.224 (45.224–82.072)	317.772 (246.041–484.853)	5.135
		100	64.33 ± 3.0			
		150	69.00 ± 1.0			
		200	79.00 ± 1.0			
		250	83.33 ± 4.1			
		300	96.00 ± 5.2			
	II	50	43.33 ± 2.8	68.964 (45.281–88.493)	431.598 (308.932–799.046)	7.405
		100	64.00 ± 2.0			
		150	66.00 ± 0.5			
		200	68.33 ± 1.5			
		250	81.00 ± 1.0			
		300	92.66 ± 2.5			
	III	50	49.00 ± 4.3	67.647 (46.999–85.017)	345.357 (262.909–547.261)	8.495
		100	52.66 ± 4.1			
		150	68.00 ± 1.0			
		200	73.00 ± 1.0			
		250	85.00 ± 2.0			
		300	92.33 ± 6.6			
	IV	50	51.66 ± 2.5	52.954 (21.812–77.823)	687.709 (398.781–2673.123)	3.488
		100	62.66 ± 2.5			
		150	67.66 ± 0.5			
		200	71.66 ± 2.0			
		250	75.33 ± 1.1			
		300	88.33 ± 1.5			
Cx. quinquefasciatus	I	50	40.00 ± 7.2	98.565 (72.255–121.752)	678.665 (441.025–1565.144)	16.361
		100	45.00 ± 2.0			
		150	54.33 ± 1.5			
		200	60.00 ± 3.6			
		250	67.00 ± 2.6			
		300	94.00 ± 6.9			
	II	50	45.00 ± 5.5	80.851 (59.967–98.793)	399.970 (300.347–648.342)	7.986
		100	45.6 ± 3.0			
		150	66.00 ± 1.0			
		200	73.33 ± 1.5			
		250	81.33 ± 1.5			
		300	92.33 ± 6.8			
	III	50	48.33 ± 3.2	61.721 (40.554–79.435)	336.852 (255.152–542.614)	1.783
		100	62.66 ± 2.5			
		150	69.00 ± 1.0			
		200	84.33 ± 1.5			
		250	88.33 ± 1.5			
		300	88.33 ± 1.5			
	IV	50	59.66 ± 4.7	41.165 (14.889–63.584)	470.474 (302.367–1328.572)	3.475
		100	66.66 ± 2.0			
		150	72.33 ± 2.5			
		200	74.33 ± 1.5			
		250	83.66 ± 4.0			
		300	90.00 ± 1.0			
Ae. aegypti	I	50	50.66 ± 4.1	64.944 (29.362–92.251)	961.973 (501.659–5352.134)	4.495
		100	51.66 ± 1.5			
		150	60.33 ± 1.5			
		200	67.33 ± 2.5			
		250	72.00 ± 1.7			
		300	85.00 ± 4.5			
	II	50	43.33 ± 0.5	72.613 (38.530–99.148)	901.215 (494.937–3877.842)	0.352
		100	55.00 ± 1.0			
		150	62.66 ± 4.9			
		200	72.00 ± 2.6			
		250	74.33 ± 1.5			
		300	76.33 ± 2.8			
	III	50	46.33 ± 6.6	61.909 (37.187–82.136)	439.325 (307.906–869.960)	2.492

Table 2 (*Contd.*)

Mosquito species	Larvae stage	Concentration (μg mL^{-1})	Percentage[b] mortality ± SE	LC_{50} (LCL–UCL) (μg mL^{-1})	LC_{90} (LCL–UCL) (μg mL^{-1})	χ^2 (df = 3)
		100	63.00 ± 2.0			
		150	66.66 ± 3.5			
		200	71.00 ± 1.0			
		250	84.00 ± 1.0			
		300	85.00 ± 1.7			
	IV	50	51.00 ± 3.6	57.651 (22.651–84.807)	916.043 (478.320–5338.628)	0.937
		100	55.00 ± 2.6			
		150	67.60 ± 2.0			
		200	71.00 ± 1.0			
		250	76.66 ± 3.0			
		300	79.66 ± 4.7			

[a] Control (deionized water) – nil mortality. LC_{50} – lethal concentration that kills 50% of the exposed larvae, LC_{90} – lethal concentration that kills 90% of the exposed larvae, LCL = lower confidence limit, UCL = upper confidence limit, df degree of freedom, χ^2 – chi-square values are significant at $P < 0.05$ levels. [b] The mean value of five replicates (±SE).

Table 3 The pupicidal activity of *B. bassiana* mycelium extract (ethyl acetate) against *An. stephensi, Cx. quinquefasciatus* and *Ae. aegypti*[a]

Mosquito species	Concentration (μg mL^{-1})	Percentage[b] mortality ± SE	LC_{50} (LCL–UCL) (μg mL^{-1})	LC_{90} (LCL–UCL) (μg mL^{-1})	χ^2 (df = 3)
An. stephensi	50	64.66 ± 1.0	40.661 (23.465–55.408)	184.022 (149.315–250.834)	14.510
	100	73.66 ± 1.1			
	150	74.33 ± 2.5			
	200	91.33 ± 1.5			
	250	97.33 ± 1.5			
	300	100 ± 0.0			
Cx. quinquefasciatus	50	54.33 ± 1.5	54.064 (36.734–68.769)	225.619 (183.306–309.150)	10.558
	100	66.66 ± 1.5			
	150	73.00 ± 1.0			
	200	85.00 ± 1.0			
	250	93.66 ± 3.2			
	300	100 ± 0.0			
Ae. aegypti	50	62.00 ± 2.6	44.263 (23.883–61.530)	263.002 (201.843–417.120)	14.921
	100	67.00 ± 1.0			
	150	71.00 ± 2.6			
	200	81.33 ± 4.1			
	250	91.66 ± 3.7			
	300	100 ± 0.0			

[a] Control (deionized water) – nil mortality. LC_{50} – lethal concentration that kills 50% of the exposed larvae, LC_{90} – lethal concentration that kills 90% of the exposed larvae, LCL = lower confidence limit, UCL = upper confidence limit, df degree of freedom, * χ^2 – chi-square values are significant at $P < 0.05$ levels. [b] The mean value of five replicates (±SE).

host insect by penetrating into the cuticle and do not require ingesting by the insect to cause diseases. The fungi have a very narrow range and significant progress has been made in recent years towards the improvement of environmentally benign spores and mycelium-based biocontrol agents for mosquito populations. Fungal biocontrol agents have cheap inputs of unsafe synthetic chemical pesticides in agriculture, horticultural and forest systems.[14] The results of fungal identification showed conidiogenous cells of *B. bassiana* densely clustered in whorls, globose or flask-like base, hyaline, smooth and short. The new conidium, giving a distinct zig-zag appearance in its colonies on PDA were round and flat, like a hyaline film from the radial growing mycelium. Similar results from *B. bassiana* were reported by Draganova *et al.*[38] *B. bassiana* (Balsamo) is considered a very important and promising fungal agent for use in the control of insects.[39] The fungus causes high mortalities in mosquito populations, as tested in numerous laboratories; Neetu Vyas *et al.*[40] reported that *Lagenidium giganteum* fungus metabolites showed 100% mortality in first instar larvae against *An. stephensi*, *Ae. aegypti*, and *Cx. quinquefasciatus*. Mohanty and Prakash[41] have described that the filtrate metabolites of *Trichophyton ajelloi* are effective on the larvae of two mosquito species, *Cx. quinquefasciatus* and *An. stephensi*. The culture filtrate metabolites of *Chrysosporium tropicum* were also found to be toxic and showed an LC_{50} and LC_{90} toxicity for all larval instars of *An. stephensi* tested at different concentrations.

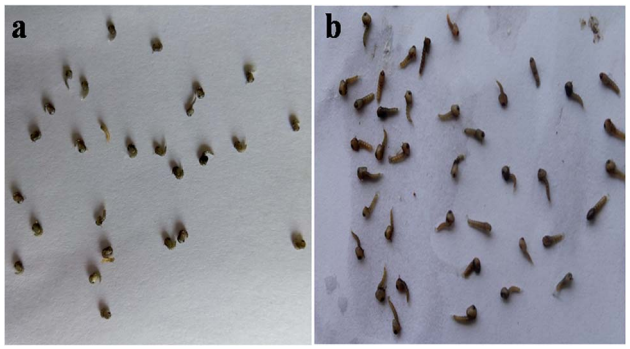

Fig. 1 The pupicidal efficacy of the ethyl acetate extracts of *B. bassiana* against *Cx. quinquefasciatus* after 24 h of exposure: (a) control pupa, (b) pupa treated at a concentration of 300 μg mL^{-1}.

The present study exhibited that the bioactive metabolites of *B. bassiana* have larvicidal and pupicidal activity against *Anopheles*, *Culex* and *Aedes* mosquitoes. These metabolites may destroy the cuticle layer of the larvae and pupae, which leads to the death of the larvae and pupa. A similar study has been reported by Ababutain *et al.*,[42] which identified *Streptomyces* sp. having better mosquitocidal properties. The use of fungus and their products are virulent and are a promising alternative insecticidal control agent.[43] The efficacy of the insecticidal activity of *B. bassiana* products against the larvae of *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti* larvae showed that the LC_{50} and LC_{90} values for *Cx. quinquefasciatus* and *Ae. aegypti* were higher than *An. stephensi*. The LC_{50} values for the 1st to 4th instar larvae values were observed to be as follows: 65.22, 68.96, 67.64 and 52.95; LC_{90} = 317.77, 431.59, 345.35 and 687.70 μg mL^{-1}, respectively. In the present study, after the treatment of the various larval stages of *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti* with the *B. bassiana* mycelia extracts at different concentrations, 100% mortality was observed based on the dose-dependent manner. Recently, Kovendan *et al.*,[44] studied *B. thuringiensis* var. *israelensis* against the larvae of *Cx. quinquefasciatus* at different concentrations. The LC_{50} and LC_{90} values were reported as follows: the LC_{50} value of I instar was 9.332%, II instar was 9.832%, III instar was 10.212%, and IV instar was 10.622%, whereas the LC_{90} value of I instar was 15.225%, II instar was 15.508%, III instar was 15.887% and IV instar was 15.986%. Similar studies have been carried out by several researchers using bacteria *Bacillus thuringiensis*,[45,46] *Bacillus sphaericus*[47] and fungus *Trichoderma viride*[48] and *Actinobacteria*,[49] entomopathogenic fungi *Metarhizium*,[50] *Trichophyton*,[41] *Tolypocladium*,[51] *Chrysosporium*[52] and *Lagenidium*[53] were reported as potential insecticidal agents.

The outcome of present study proved that mycelium extract of *B. bassiana* had a broad spectrum larval mortality against *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti* and the values were found to be as follows: for *An. stephensi*, LC_{50} = 65.22, 68.96, 67.64, and 52.95; LC_{90} = 317.77, 431.59, 345.35 and 687.70 μg mL^{-1}; for *Cx. quinquefasciatus*, LC_{50} = 98.56, 80.85, 61.72, and 41.16; LC_{90} = 678.66, 399.97, 336.85 and 470.47 μg mL^{-1} and for *Ae. aegypti*, LC_{50} = 64.94, 72.61, 61.90 and 57.65; LC_{90} = 961.97, 901.21, 439.32 and 916.04 μg mL^{-1}. Similarly, Vijayan and Balaraman[54] isolated 94 actinomycetes from marine soil samples collected at different locations, out of which 35 samples exhibited improved larvicidal activity against *Cx. quinquefasciatus*, *An. stephensi* and *Ae. aegypti* with LC_{50} values in the range of 1–3 μL mL^{-1}.

The larval and pupal mortality of *Cx. quinquefasciatus* after 24 h of treatment with the *n*-hexadecanoic acid standard

Table 4 The pupicidal activity of *B. bassiana* mycelium extract (methanol) against *An. stephensi, Cx. quinquefasciatus* and *Ae. aegypti*[a]

Mosquito species	Concentration (μg mL^{-1})	Percentage[b] mortality ± SE	LC_{50} (LCL–UCL) (μg mL^{-1})	LC_{90} (LCL–UCL) (μg mL^{-1})	χ^2 (df = 3)
An. stephensi	50	51.00 ± 3.6	51.925 (14.109–81.604)	1196.224 (541.648–15 498.889)	1.285
	100	61.33 ± 1.5			
	150	65.00 ± 1.0			
	200	68.33 ± 1.5			
	250	71.33 ± 2.0			
	300	81.33 ± 1.5			
Cx. quinquefasciatus	50	48.66 ± 3.7	69.299 (35.648–95.455)	862.253 (477.816–3641.619)	1.827
	100	54.33 ± 1.5			
	150	62.00 ± 2.0			
	200	68.33 ± 1.5			
	250	77.66 ± 2.5			
	300	80.33 ± 1.5			
Ae. aegypti	50	48.66 ± 3.2	76.346 (38.351–105.396)	1178.151 (578.043–7953.579)	4.314
	100	51.33 ± 2.5			
	150	58.33 ± 1.5			
	200	62.00 ± 4.3			
	250	70.66 ± 2.0			
	300	82.66 ± 2.5			

[a] Control (deionized water) – nil mortality. LC_{50} – lethal concentration that kills 50% of the exposed larvae, LC_{90} – lethal concentration that kills 90% of the exposed larvae, LCL = lower confidence limit, UCL = upper confidence limit, df degree of freedom, χ^2 – chi-square values are significant at $P < 0.05$ levels. [b] The mean value of five replicates (±SE).

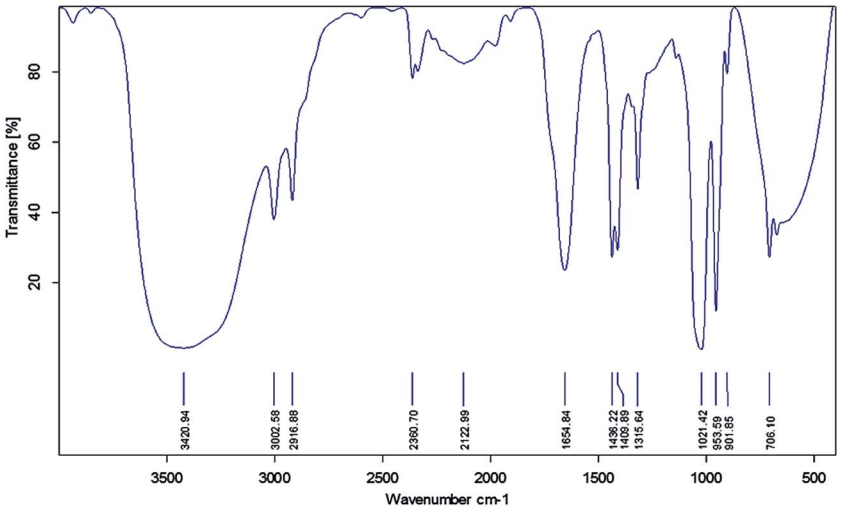

Fig. 2 FTIR analysis of the ethyl acetate mycelia extract obtained from *B. bassiana*.

Table 5 The FTIR spectrum of the ethyl acetate mycelium extract obtained from *B. bassiana*

Observed wavenumber (cm^{-1})	Functional group	Bonding pattern
3420.94	O–H stretch alcohols or phenols	Strong, broad
3002.58	=C–H stretch aromatics	Sharp
2916.88	C–H alkanes	Medium
2122.99	–C≡C– stretch nitriles	
1654.84	–C=C– stretch alkanes	Medium
1436.22	C–H bend alkanes	Medium
1409.89	C–C stretch aromatics	Medium
1315.64	C–O stretch alcohols, carboxylic acids, esters, ethers	Sharp
1021.42	C–N stretch aliphatic amines	Medium
953.59	=C–H bending alkenes	Sharp
901.85	N–H wagging primary amines	Strong, broad
706.10	C=O ketone	Sharp

showed the highest larvicidal (LC_{50} = 2.27 and LC_{90} = 15.91 μg mL^{-1}) and pupal toxicity (LC_{50} = 0.69 and LC_{90} = 4.38 μg mL^{-1}) than *An. stephensi* and *Ae. aegypti*. Similarly, Rahuman *et al.*[55] reported a bioassay-guided fractionation of the acetone extract of *Feronia limonia*, which was shown as a potent mosquito larvicide, identified as *n*-hexadecanoic acid and found to be effective against fourth instar larvae of *Ae. aegypti*, *Cx. quinquefasciatus* and *An. stephensi*. Similarly, Sivakumar *et al.*[31] found the larvicidal and repellent activity of pure tetradecanoic acid against *Ae. aegypti* and *Cx. quinquefasciatus*. The LC_{50} values were 14.08 and 25.10 μg mL^{-1}. More recently, Srinivasan *et al.*[56] reported the larvicidal potential of isolated thujone against the 4th instar larvae of *Ae. aegypti* (LC_{50} = 4.23 mg L^{-1}) and *An. stephensi* (LC_{50} = 3.30 mg L^{-1}). Fungal secondary metabolites have play an important roles in pathogenesis and the larvicidal activity, which can help in controlling mosquito populations and reduce the spread of vector borne diseases. *Acremonium* ethyl acetate metabolites were found to be more effective against *Ae. aegypti* and *Cx. quinquefasciatus*, followed by *An. stephensi* larvae. Furthermore, the pathogenicity of *Acremonium* sp. was also reported to possess good parasitic properties.[57] Similarly, Stanly Pradeep *et al.*[58] proved that *F. oxysporum* metabolites are more effective against *An. stephensi* than *Cx. quinquefasciatus* larvae.

The FTIR results indicated that the ethyl acetate mycelium extract showed the presence of chemical bands due to O–H group hydrogen-bonded alcohols or phenols (3420.94), =C–H aromatics (3002.58), C–H alkanes (2916.88), –C≡C– nitriles (2122.99), –C=C– alkanes (1654.84), C–C aromatics (1409.89), C–O carboxylic acids or alcohols (1315.64), C–N aliphatic amines (1021.42), N–H primary amines (901.85) and C=O ketones (706.10) cm^{-1}. Similar functional groups were obtained by Nagajyothi *et al.*[59] The GC-MS analysis results revealed that the larvicidal and pupicidal activity of mycelium ethyl acetate extracts from *B. bassiana* were exhibited due to six major compounds, namely 9,12-octadecadienoic acid (ZZ)– (63.16%), n-hexadecanoic acid (21.28%), octadecanoic acid, phenyl methyl ester (10.45%), dehydroegosterol 3,5-dinitrobenzoate (1.86%), squalene (1.66%), bis[3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)prophyl]maleate (1.56%). Earlier, Ragavendran and Natarajan[60] reported that the *Aspergillus terreus* ethyl acetate extract contains six bioactive compounds and its constituents

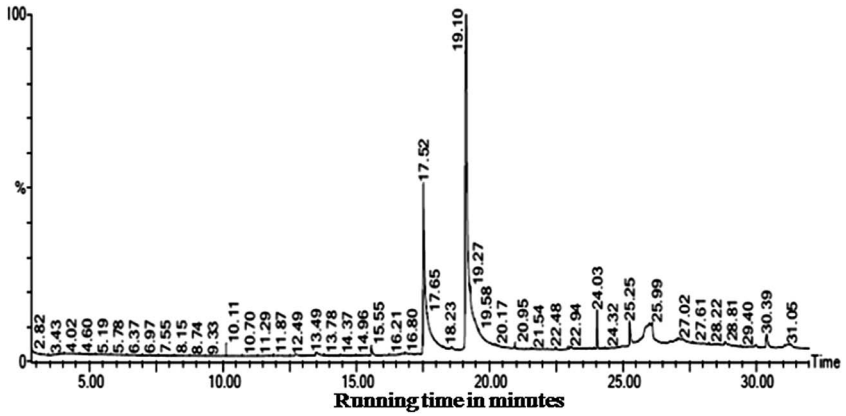

Fig. 3 The insecticidal compounds identified in the ethyl acetate mycelium extracts obtained from *B. bassiana*.

Table 6 The major bioactive compounds identified in the ethyl acetate mycelium extracts of *B. bassiana* using GC-MS analysis[a]

Rt	Area	Area%	Molecular weight/formula	Compound name	Biological activity	References
17.519	80 887 080.0	21.286	256, $C_{16}H_{32}O_2$	*n*-Hexadecanoic acid	Nematicide, pesticide	Ragavendran and Natarajan 2015,[60] Rajeswari *et al.* 2012,[73] Zahir Hussain *et al.* 2010 (ref. 74)
19.120	240 006 224.0	63.160	280, $C_{18}H_{32}O_2$	9,12-Octadecadienoic acid (ZZ)–	Larvicide	Velu *et al.* 2014 (ref. 75)
24.032	6 320 307.5	1.663	410, C_3CH_{50}	Squalene	Pesticide, antioxidant and antitumor	Rajeswari *et al.* 2012,[73] WHO 1997 ref. 76
25.253	7 088 480.0	1.865	588, $C_{35}H_{44}O_6N_2$	Dehydroegosterol 3,5-dinitrobenzoate	Not known	Nil
26.098	39 740 176.0	10.458	374, $C_{25}H_{42}O_2$	Octadecanoic acid, phenyl methyl ester	Hypocholesterolemic and nematicide	Dr Duke's Phytochemical and Ethnobotanical Database[77]
30.390	5 952 307.5	1.566	608, $C_{38}H_{56}O_6$	Bis[3-(3,5-di-*tert*-butyl-4-hydroxyphenyl) prophyl]maleate	Not known	Nil

[a] Components identified based on computer matching of the mass peaks with the NIST-2008 Library.

showed better larvicidal and the pupicidal effects on selected mosquito vectors, namely *An. stephensi* (LC_{50} = 97.410, 102.551, 29.802 and 8.907; LC_{90} = 767.957, 552.546, 535.474 and 195.677 μg mL^{-1}), *Cx. quinquefasciatus* (LC_{50} = 89.584, 74.689, 68.265 and 67.40; LC_{90} = 449.091, 337.355, 518.793 and 237.347 μg mL^{-1}) and *Ae. aegypti* (LC_{50} = 83.541, 84.418, 80.407 and 95.926; LC_{90} = 515.464, 443.167, 387.910 and 473.998 μg mL^{-1}). Pupicidal activity was also reported against *An. stephensi* (LC_{50} = 25.228; LC_{90} = 140.487 μg mL^{-1}), *Cx. quinquefasciatus* (LC_{50} = 54.525; LC_{90} = 145.366 μg mL^{-1}) and *Ae. aegypti* (LC_{50} = 10.536; LC_{90} = 63.762 μg mL^{-1}). Squalene is considered as an important substance for practical and clinical use with huge potential in the nutraceutical and pharmaceutical industries.[61] Similarly, Thimiri *et al.*[62] reported that the *Streptomyces* sp. produced the isolated compound (2*S*,5*R*,6*R*)-2-hydroxy-3,5,6-trimethyloctan-4-one observed against the larvae of *R. microplus* (LC_{50} = 88.74 ppm; r^2 = 0.865), *An. subpictus* (LC_{50} = 162.59 ppm; r^2 = 0.817) and *Cx. quinquefasciatus* (LC_{50} = 120.15 ppm; r^2 = 0.782). Kumar Saurav *et al.*[63] reported that *Streptomyces* VITSVK5 sp. yielded the bioactive/isolated compound 5-(2,4-dimethylbenzyl) pyrrolidin-2-one, which had larvicidal activity against the larvae of *R. microplus* (LC_{50} = 210.39 ppm, r^2 = 0.873), *An. stephensi* (LC_{50} = 169.38 ppm, r^2 = 0.840) and *Cx. tritaeniorhynchus* (LC_{50} = 198.75 ppm, r^2 = 0.887). Previously, some researchers have reported the insecticidal activity of isolated compounds obtained from the species of *Streptomyces*, namely tetranectin,[64] avermectins,[65] faeriefungin[66] and macrotetrolides.[67]

The HPLC analysis of the ethyl acetate mycelium extract was compared with the *n*-hexadecanoic acid standard and they showed a similar chromatographic peak (at a retention time of 3.383 and 3.378 min). The HPLC results were in agreement with the earlier reports of Ragavendran and Natarajan,[60] and Manilal *et al.*[68] who obtained (15.31 and 42%) *n*-hexadeconoic acid using different extracts. Previously, several researchers have isolated *n*-hexadecanoic acid from different plants and microbes *i.e. Vitex altissima*, *V. negundo* and *V. trifolia*,[69] *Aspergillus fumigatus*,[70] *A. versicolor*[71] and *Pestalotiopsis* sp.[72] The use of fungus

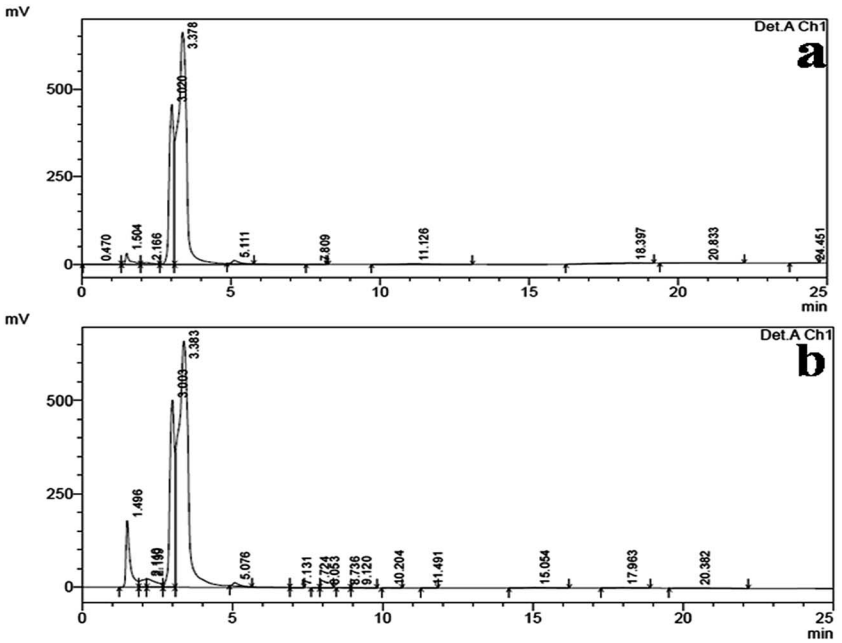

Fig. 4 (a) The HPLC chromatogram of the *n*-hexadecanoic acid standard and (b) the HPLC chromatogram of the ethyl acetate mycelium extract obtained from *B. bassiana*.

based products would be cheaper, target-specific, self-sustained and highly toxic to mosquitoes, even at low doses.

Conclusion

Our findings confirm a promising as well as a novel biological based strategy to be integrated with additional control measures to reduce the global rate of vector-borne disease transmission. At a concentration of 300 μg mL^{-1} of *B. bassiana* ethyl acetate extract, 90% mortality was observed within 18 h against *An. stephensi* and *Cx. quinquefasciatus*, followed by *Ae. aegypti* and 100% pupal mortality was observed at higher concentrations. The pupal toxicity of the mosquitoes was mainly based on the dose-dependent effect against *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti*. GC-MS analysis of the ethyl acetate extract of *B. bassiana* identified six major components, *i.e.* 9,12-octadecadienoic acid (ZZ)– (63.16%), *n*-hexadecanoic acid (21.28%), octadecanoic acid, phenyl methyl ester (10.45%), dehydroegosterol 3,5-dinitrobenzoate (1.86%), squalene (1.66%) and bis[3-(3,5-di-*tert*-butyl-4-hydroxyphenyl) prophyl]maleate (1.56%). The bioactive compounds may be responsible for the larvicidal and pupicidal activity against *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti* mosquitoes. In addition, HPLC analysis of the ethyl acetate mycelium extract of *B. bassiana* and the *n*-hexadecanoic acid standard show a similar chromatographic peak (at a retention time of 3.383 and 3.378 min). Moreover, these metabolites can be used for the development of new insecticidal formulations to control vector borne diseases because they constitute a rich source of bioactive compounds that are more effective, eco-friendly, non-toxic, and potentially suitable for use in the management of target insects/pests. Further studies are ongoing for the isolation of pure active compounds and determination of the mode of action so as to recommend an eco-friendly measure for the control of mosquitoes.

Authors' contributions

Conceived and designed the experiments: CR and DN Performed the experiments and analyzed the data: CR. CR and DN analyzed and interpreted the data and wrote the manuscript. Revision of the manuscript: DN and NKD Finally, all authors have read and approved the final manuscript.

Compliance with ethical standards

All applicable international and national guidelines for the care and use of animals were followed. All procedures performed in the studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Conflict of interest

The authors declare that they have no conflict of interest in this research article.

Acknowledgements

The first author acknowledged to the Periyar University for providing financial support under University Fellowship Scheme (Ref No. PU/A&A-3/URF/2014). We would like to thank the Department of Biotechnology, School of Biosciences, Periyar University for providing necessary infrastructural facility for carrying out this study successfully. We also thank the Institute of Vector Control and Zoonoses (IVCZ) Hosur for supplying mosquitoes and we thank the Vellore Institute of Technology (VIT) for GC-MS analysis of our bio-samples. The authors would like to express their sincere thanks to Prof. P. Perumal, Marine

Biotechnology Laboratory, Department of Biotechnology, Periyar University, Salem, Tamilnadu, India for providing the HPLC instrumental facility for analysis of the samples.

References

1 V. Corby-Harris, A. Drexler, L. Watkins de Jong, Y. N. Antonova and N. Pakpour, *PLoS Pathog.*, 2010, **6**, 1–10, e1001003.
2 WHO, *Larval source management: a supplementary measure for malaria vector controls an operational manual*, 2013, pp. 1–116.
3 M. Govindarajan, T. Mathivanan, K. Elumalai, K. Krishnappa and A. Anandan, *Asian Pac. J. Trop. Biomed.*, 2011, **1**, 43–48.
4 WHO, http://www.who.int/media centre/factsheets/fs102/en/, 2012.
5 Z. Peng, J. Yang, H. Wang and F. E. R. Simons, *Insect Biochem. Mol. Biol.*, 1999, **29**, 909–914.
6 WHO, http://www.who.int/features/factfiles/malaria/en/index.html, 2013.
7 M. Z. Hossam El-Din and S. A. M. Abdelgaleil, *J. Agric Res.*, 2010, **36**, 385–401.
8 T. Su and M. S. Mulla, *J. Am. Mosq. Control Assoc.*, 1998, **14**, 204–209.
9 S. Poopathi, *Journal of Biofertilizers & Biopesticides*, 2012, **3**, 1–14.
10 M. Maheu-Giroux and M. C. Castro, *PLoS One*, 2013, **8**, 1–11.
11 EPA, *Biopesticide Fact Sheet: Beauveria bassiana strain ATCC. 74040*, 2000 128818.
12 F. V. A. Howard, R. Guessan, C. J. M. Koenraadt, A. Asidi, F. Marit, M. Akoqbeto, M. B. Thomas, B. G. H. Knols and W. Takken, *Malar J.*, 2010, **3**, 1–11.
13 EPA, *Biopesticide Registration Action Document: Beauveria bassiana HF23*, 2006.
14 H. Strasser, A. Vey and T. Butt, *Biocontrol Science and Technology*, 2000, **10**, 717–735.
15 A. Vey, R. E. Hoagland and T. M. Butt, *Toxic metabolites of fungal biocontrol agents. Fungi as biocontrol agents. progress, problems and potential*, ed. T. M. Butt, C. Jackson and N. Magan, CABI Publishing, Oxford, UK, 2001, pp. 311–346.
16 E. Quesada-Moraga and A. Vey, *Mycol. Res.*, 2004, **108**, 441–452.
17 A. K. Charnley and R. J. S. Leger, The role of cuticle-degrading enzymes in fungal pathogenesis in insects, p. 267–287, in, *Fungal Spore Disease Initiation in Plants and Animals*, ed. E. T. Cole and H. C. Hoch, Plenum Press, New York, 1991, p. 555.
18 M. Govindarajan, A. Jebanesan and D. Reetha, *Trop. Biomed.*, 2005, **22**, 1–3.
19 N. Haraprasad, S. R. Niranjans, H. S. Prakash, H. S. Shetty and W. Seema, *Ind. Biocontrol Sci. Tech.*, 2001, **11**, 251–260.
20 R. A. Samson, Identification: Entomopathogenic Deuteromycetes, in, *Microbial Control of Pests and Plant Diseases*, ed. H. D. Burges, Academic press, London, 1981, pp. 93–106.
21 R. A. Samson, H. C. Evans and J. P. Latgé, *Atlas of Entomopathogenic Fungi*, Springer-Verlag, Berlin, 1988.
22 G. S. Hoog de, *Stud. Mycol.*, 1972, **1**, 1–41.
23 J. M. Gardner and J. S. Pillai, *Mycopathologia*, 1987, **97**, 77–82.
24 G. N. Belofsky, M. Anguera, P. R. Jensen, W. Fenical and M. Kock, *Chem.–Eur. J.*, 2000, **6**, 1355–1360.
25 S. V. Patil, C. D. Patil, B. K. Salunke and R. B. Salunkhe, *Trop. Biomed.*, 2010, **27**, 360–365.
26 WHO, 2005, WHO/CDS/WHOPES/GCDPP/2005:13.
27 M. Govindarajan and G. Benelli, *J. Parasitol. Res.*, 2016, **115**, 925–935.
28 W. S. Abbott, *J. Econ. Entomol.*, 1925, **18**, 265–266.
29 D. J. Finney, *Probit analysis*, Cambridge University Press, Cambridge, 1971, pp. 76–80.
30 R. R. Rahuman and P. Venketesan, *J. Parasitol. Res.*, 2008, **103**, 133–139.
31 R. Sivakumar, A. Jebanesan, M. Govindarajan and P. Rajasekar, *Asian Pac. J. Trop. Med.*, 2011, **4**, 706–710.
32 K. V. Bhaskara Rao, K. S. Hemath Naveen, G. Kumar and L. Karthik, *Arch. Appl. Sci. Res.*, 2010, **2**, 161–167.
33 M. A. Hossain and M. D. M. Shah Sakari, *Asian Pac. J. Trop. Med.*, 2011, **4**, 637–641.
34 M. Junaid Khan, S. Swarnlata and S. Shailendra, *J. Ethnopharmacol.*, 2016, DOI: 10.1016/j.jep.2016.08.021.
35 P. J. Reddy, D. Krishna, U. S. Murthy and K. Jamil, *CABIOS, Comput. Appl. Biosci.*, 1992, **8**, 209–213.
36 E. J. Hermanides-Nijihof, *(Stud. Mycol.15) Centraalbureau voor Schimecultures*, Baarn, 1977, vol. 1, pp. 141–177.
37 S. Seyed Ali, *J. Plant Prot. Res.*, 2010, **50**, 159–163.
38 S. A. Draganova, I. Danail, D. Takov and D. Doychev, *Pestic. Phytomed.*, 2010, **25**, 59–63.
39 W. B. Shi and M. G. Feng, *Biol. Control*, 2004, **30**, 165–173.
40 N. Vyas, K. K. Dua and S. Prakash, *J. Parasitol. Res.*, 2007, **101**, 385–390.
41 S. S. Mohanty and S. Prakash, *Curr. Sci.*, 2004, **86**, 323–325.
42 M. Ababutain, K. Zeinab, A. Abdul Azizand Nijla and A. L. Meshhen, *Can. J. Pure Appl. Sci.*, 2012, **6**, 1739–1748.
43 E. J. Scholte, K. Ng'habi, J. Kihonda, W. Takken, K. Paaijmans, S. Abdulla and G. F. Killeen, *B. G. J. Knols Science*, 2005, **10**, 1641–1642.
44 K. Kovendan, K. Murugan, S. Vincent and S. Kamalakannan, *J. Parasitol. Res.*, 2011, **109**, 1251–1257.
45 K. Balaraman, *ICMR Bulletin*, 1995, **25**, 45–51.
46 G. Prabhakaran, V. Padmanaban and K. Balaraman, *J. Biol. Contr.*, 2000, **14**, 63–66.
47 G. Rajendran, S. Sabesan, K. Kuppusamy and K. Balaraman, *Entomon*, 1991, **16**, 213.
48 I. Geetha and K. Balaraman, *J Biol Contr*, 2001, **15**, 93.
49 D. Dhanasekaran, V. Sakthi, N. Thajuddin and A. Panneerselvam, *Int. J. Appl. Biol. Pharm. Technol.*, 2010, **1**, 374–381.
50 D. W. Roberts, Some effects of *Metarhizium anisopliae* and its toxins on mosquito larvae, in *Insect pathology and microbial control*, ed. Laan PA van der, North-Holland Publishing Company, 1967, pp. 243–246.
51 V. Matha, J. Weiser and J. Olejnicek, *Parasitology*, 1988, **35**, 379–381.
52 A. Priyanka and S. Prakash, *J. Am. Mosq. Control Assoc.*, 2003, **19**, 404–407.

53 N. Vyas, K. K. Dua and S. Prakash, *Bull. Bio. Sci.*, 2006, **4**, 65–69.
54 V. Vijayan and K. Balaraman, *Indian J. Med. Res.*, 1991, **93**, 115–117.
55 A. A. Rahuman, G. Gopalakrishnan, B. S. Ghouse, S. Arumugam and B. Himalayan, *Fitoterapia*, 2000, **71**, 553–555.
56 R. Srinivasan, D. Natarajan, M. S. Shivakumar, T. Vinuchakkaravarthy and D. Velmurugan, *Ind. Crops Prod.*, 2015, **76**, 394–401.
57 G. F. Atkinson, Some diseases of cotton, *Albama Polytechnical Inst. Expt. Sta.*, 1892, **41**, 61–65.
58 F. Stanly Pradeep, M. Palaniswamya, S. Ravi, A. Thangamani and B. V. Pradeep, *Process Biochem.*, 2015, **50**, 1479–1486.
59 P. C. Nagajyothi, T. V. M. Sreekanth, J. L. Lee and K. D. Lee, *J. Photochem. Photobiol., B*, 2014, **130**, 299–304.
60 C. Ragavendran and D. Natarajan, *Environ. Sci. Pollut. Res.*, 2015, **22**, 17224–17237.
61 S. K. Kim and F. Karadeniz, *Adv. Food Nutr. Res.*, 2012, **65**, 223–233.
62 L. D. Thimiri, K. Kannabiran, V. Gopiesh Khanna, G. Rajakumar, C. Jayaseelan, T. Santhoshkumar and A. A. Rahuman, *J. Parasitol. Res.*, 2012, **111**, 1151–1163.
63 S. Kumar, G. Rajakumar, K. Kannabiran, A. A. Rahuman, K. Velayutham, G. Elango, C. Kamaraj and A. A. Zahir, *J. Parasitol. Res.*, 2013, **112**, 215–226.
64 K. Ando, How to discover new antibiotics for insecticidal use, in *Pesticide chemistry: human welfare and the environment. Natural products*, ed. T. Takahashi, H. Yoshioka, T. Misato and S. Matusunaka, Pergamon, New York, 1983, pp. 253–259.
65 S. Pampiglione, G. Majori, G. Petrangeli and R. Romi, *Trans. R. Soc. Trop. Med. Hyg.*, 1985, **79**, 797–799.
66 Anonymous, *Biotechnol Abstr.*, 1990, **9**, 58.
67 Z. Zizka, J. Weiser, M. Blumauerova and J. Jizba, *Cytobios*, 1989, **58**, 85–91.
68 A. Manilal, S. Sujitha, J. Selvin, C. Shakir and G. Seghal Kiran, *Indian J. Exp. Biol.*, 2009, **78**, 161–166.
69 K. Kannathasan, A. Senthilkumar, V. Venkatesalu and M. Chandrasekaran, *J. Parasitol. Res.*, 2008, **103**, 999–1001.
70 J. Xu, L. Xianqun, Z. J. Z. Wenhua and T. Renxiang, *J. Chem. Pharm. Res.*, 2014, **6**, 893–897.
71 G. Senthilkumar, P. Madhanraj and S. Panneerselvam, *Asian J. Pharm. Sci.*, 2011, **1**, 19–21.
72 D. Li, Y. Shihong, P. Proksch, Z. Liang, Q. Li and J. Xu, *Afr. J. Biotechnol.*, 2013, **12**, 3802–3806.
73 G. Rajeswari, M. Murugan and V. R. Mohan, *Res. J. Pharm., Biol. Chem. Sci.*, 2012, **3**, 301–307.
74 A. Zahir Hussain, I. Aruna and J. Asian, *J. Chem.*, 2010, **22**, 3591–3595.
75 K. Velu, D. Elumalai, P. Hemalatha, M. Babu, A. Janaki and P. K. Kaleena, *Int. J. Mosq. Res.*, 2015, **2**, 1–08.
76 WHO, *Squalene-based adjuvants in vaccines*, Global Advisory Committee on Vaccine Safety, 1997.
77 Dr. Duke's Phytochemical and Ethnobotanical Databases compiled by Dr. Jim Duke of the Agricultural Research Service/USDA.