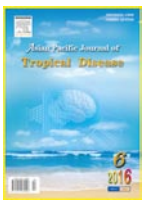
HOSTED BY

ELSEVIER

Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Disease

journal homepage: www.elsevier.com/locate/apjtd



Entomological research doi: 10.1016/S2222-1808(16)61069-1

Isolation and characterization of dipropyl-, S-propyl ester from *Exiguobacterium mexicanum* (MSSRF-S9) against larvae of malaria and dengue vectors

Shanmugam Perumal Shanthakumar[1], Kalimuthu Kovendan[2], Savariar Vincent[3], Vasantharaj David[4], Eliningaya J. Kweka[5,6*]

[1]*Department of Zoology, St. Joseph's College, Lang Ford Road, Bangalore, Karnataka, India*

[2]*Division of Entomology, Department of Zoology, School of Life Sciences, Bharathiar University, Coimbatore-641 046, Tamil Nadu, India*

[3]*Centre for Environmental Research and Development, P.G. Research & Department of Advanced Zoology and Biotechnology, Loyola College, Nungambakkam, Chennai-600 034, Tamil Nadu, India*

[4]*Centre for Toxicology and Developmental Research, Sri Ramachandra University, Chennai-600 116, Tamil Nadu, India*

[5]*Tropical Pesticides Research Institute, Division of Livestock and Human Diseases Vector Control, Mosquito Section, P.O. Box 3024, Arusha, Tanzania*

[6]*Department of Medical Parasitology and Entomology, Catholic University of Health and Allied Sciences, P.O. Box 1464, Mwanza, Tanzania*

ARTICLE INFO

Article history:
Received 13 Oct 2015
Received in revised form 29 Feb, 2nd revised form 2 Mar 2016
Accepted 25 Apr 2016
Available online 14 Jun 2016

Keywords:
Dipropyl-, S-propyl ester
Exiguobacterium mexicanum
Anopheles culicifacies
Aedes albopictus
Larvicidal activity

ABSTRACT

Objective: To evaluate the insecticidal property of the compound dipropyl-, S-propyl ester extracted from the Gram-positive rhizosphere bacterium against the fourth instars larvae of malaria vector, *Anopheles culicifacies* (*An. culicifacies*) and dengue vector, *Aedes albopictus* (*Ae. albopictus*).

Methods: Bacteria were taxonomically identified as *Exiguobacterium mexicanum* (*E. mexicanum*) and different crude solvent extracts were examined for its larvicidal potential against mosquito vectors. The crude extract was eluted in silica gel column chromatography and the pure compound was identified.

Results: The highest larvicidal activity against *An. culicifacies* was exhibited by ethyl acetate extract. The bacterium of *E. mexicanum* extract at 150, 300, 450, 600, 750 mg/L caused against both vector mosquito species. Hexane, chloroform, acetone, methanol and ethyl acetate caused moderate considerable mortality. The highest larval mortality was observed in ethyl acetate extract.

Conclusions: The results of the bacterial extract of *E. mexicanum* are promising as potential larvicidal compound against the mosquito vectors (*An. culicifacies* and *Ae. albopictus*). Therefore, this study provides first report on the larvicidal activity with dipropyl-, S-propyl ester compound from India.

1. Introduction

Mosquito-borne diseases including yellow fever, dengue fever and chikungunya and Zika virus are transmitted by *Aedes aegypti*, the malaria parasite carried by the vector belonging to the genus *Anopheles*[1,2]. Mosquitoes transmit diseases to more than 700 million people each year and are responsible for 655 000 deaths every year for malaria alone from World Health Organization (WHO) report of 2013[3]. *Anopheles stephensi* and *Anopheles culicifacies* (*An. culicifacies*) are the two primary malarial vectors in India[4]. At the moment, bendiocarb (carbamate), malathion (organophosphate), and deltamethrin, cyfluthrin, α-cypermethrin and lambda-cyhalothrin (synthetic pyrethroids) are the most commonly used as insecticides for vector control in the public health for indoor residual spray and insecticides treated bed nets[5]. Susceptibility status of vectors from several districts in India was carried out in 2009 using WHO treated papers of dichloro-diphenyl-trichloroethane, deltamethrin and malathion according to standard WHO protocol[6]. *An. culicifacies* was found to have developed tolerance against all evaluated insecticides[7]. Malaria control mostly in India relies on the use of malathion and pyrethroids. The evolution of insecticides tolerance among vectors is jeopardising the efficacy of the current

*Corresponding author: Eliningaya J. Kweka, Tropical Pesticides Research Institute, Division of Livestock and Human Diseases Vector Control, Mosquito Section, P.O. Box 3024, Arusha, Tanzania.
Tel: 255754368748
E-mail: pat.kweka@gmail.com
The journal implements double-blind peer review practiced by specially invited international editorial board members.

tools. New innovative and eco-friendly compounds to supplement the existing tool are priority at the moment.

There are molecules reported from plant extracts which are found to compromise the strength, vitality and physiology of the insect pests. Bioresmethrin, styryl-lactones and acetogenins are among the insecticides known to affect both growth and developmental hormone and are grouped as insect growth regulators[8]. Chalcone oxides have been found to be the inhibitors of the juvenile hormone hydrolysis by supressing *Manduca sexta* tissue to reduce the production of epoxide hydrolase[9].

The objective of the current study was to isolate and characterize dipropyl-, S-propyl ester from *Exiguobacterium mexicanum* (MSSRF-S9) (*E. mexicanum*) and evaluate its insecticidal property against larvae of malaria vector, *An. culicifacies* and dengue vector, *Aedes albopictus* (*Ae. albopictus*).

2. Materials and methods

2.1. Bacteria and mosquito culture

E. mexicanum (Gen Bank Ac No: KF471138) bacterium with insecticidal activity was obtained from entomology laboratory of M.S. Swaminathan Research Foundation, India. The larvicidal action of the bacterial culture filtrate against the mosquito larvae was confirmed in preliminary screening studies. The eggs of *An. culicifacies* and *Ae. albopictus* were collected from different habitats (Kallar River and stagnant water). These eggs were brought to the molecular entomology laboratory and transferred to enamel trays (18 cm × 13 cm × 4 cm) containing 500 mL of distilled water for hatching. The mosquito larvae were allowed to feed with pedigree dog biscuits and yeast at 3:1 ratio. In insectary, larvae were reared at a temperature of (27 ± 2) ℃ and relative humidity of 75%–85% in a room having a photophase of 14:10 (light/dark). Pupae were collected from the culture trays and transferred to plastic containers (12 cm × 12 cm) containing 500 mL of water. The plastic jars were kept in a 90 cm × 90 cm × 90 cm mosquito cage for adult emergence. A 10% sugar solution was provided for a period of 3 days before blood feeding.

2.2. Preparation of the crude extract

After 48 h, culture filtrate of *E. mexicanum* was prepared and separated with the hexane, chloroform, acetone, methanol and ethyl acetate in the ratio of 1:1 (v/v) followed by shaking for 30 min in a separating funnel. The extraction was repeated thrice and the fractions were pooled and evaporated under vacuum using a rotary evaporator (Buchi Type, Switzerland). The crude extracts thus obtained were evaluated for the larvicidal activity with different concentrations *viz*., 150, 300, 450, 600, 750 mg/L. Ethyl acetate was selected for identifying compound based on the highest mosquito larvicidal activity.

2.3. Purification of active compound

The concentrated crude extract was mixed with chloroform-silica gel slurry and loaded onto a silica gel 60–120 mesh (E-Merck, Darmstadt, Germany) column, packed in ethyl acetate: chloroform: acetic acid (5:4:1) as the solvent system (the dimension of the column was 450 mm × 30 mm). A total of 100 tubes of 10 mL of the fractions were collected and analyzed by thin layer chromatography (TLC). Fractions showing similar spots with same R_f values were pooled and concentrated by a SpeedVac under low pressure with evaporating temperature of 40 ℃.

2.4. TLC profile of the secondary metabolites

TLC of the solvent extract was performed on a pre-coated silica gel TLC plate grade (60.20 × 20.0.5 mm, Merck) with ethyl acetate: chloroform: acetic acid (5:4:1) as the solvent system. Ethyl acetate extract was spotted on the silica plate using a capillary tube and pointed in the TLC chamber containing the solvent and was appropriated to operate for about 15 cm. The chromatogram was developed in closed tanks, in which the air had been saturated with eluent vapor by wetting a filter paper lining. The chromatogram was visualized under UV light (365 nm).

2.5. Characterization of the metabolite

The purified fractions were subjected to high performance liquid chromatography (on the Bondapak column with a flow rate 1.5 mL/min and pressure up to 300 psi) using acetonitrile (9:1) as elutant to obtain the pure sample. The eluted single fraction was evaporated to dryness and subjected to Fourier transform infra-red (FTIR). Infrared spectral data were measured using Perkin-Elmer 1600 series FTIR spectrometer (Nujol, KBr disks). To determine the molecular weight, the samples were subjected to electrospray ionization quadrupole mass spectrometry with Finnigan LCQ MS detector. Source conditions were set (voltage 5 kV: nitrogen sheath gas pressure of 60 psi: heated capillary temperature 200 ℃: full scan 50 to 2000 *m/z*). ^{13}C-nuclear magnetic resonance (NMR) and ^{1}H-NMR spectra were recorded on a Bruker 300 MHz instrument using tetra methyl silane as the internal standard. Optical density was estimated using UV spectrometry (Shimadzu). Using the spectral data of FTIR, electrospray ionization quadrupole mass spectrometry, ^{1}H-NMR, ^{13}C-NMR and UV spectrum, the structure of the active principles was determined.

2.6. Larvicidal bioassay

The larvicidal activity was evaluated using the methodology recommended by WHO[10]. A total of 25 third-instar larvae were transferred to a paper cup, containing 200 mL of water. The appropriate volume of dilution was added to 200 mL water in the cups to obtain the desired target dosage, starting with the lowest concentration (150 to 750 mg/L). Four replicates were set up for each concentration, and an equal number of controls was set up simultaneously using tap water. To this, 1 mL of ethanol was added. The control mortalities were corrected by using Abbott's formula[11].

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

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

2.7. Statistical analysis

The LC_{50} and LC_{90} were calculated from toxicity data by using probit analysis[12]. The average larval mortality data were subjected to probit analysis for calculating LC_{50} and LC_{90}, and other statistics at a 95% upper fiducial limit (UFL) and lower fiducial limit (LFL), and *Chi*-square values were calculated using SPSS 16.0 version (USA). Results at $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. Purified compound with insecticidal activity

Based on the polyphasic chemical characterization, the chemical structure of dipropyl-, S-propyl ester was derived. The retention time value of the compound was 3.0 at a single peak in high performance liquid chromatography and the UV spectrometer reading for the compound 4 was 231 nm. FTIR values of compound 4 showed the following clear peaks between the range of 1 700–1 750 which implied the presence of carbamic acid, ester and carbonyl (C = O) stretch [1 050–1 200 confirmed the presence of dithiocarbamic group with (C = S) stretch, 1 600–1 680 denoting (C – N) stretch, peaked between 2 800–3 000 range depicting alkane (C – H) stretch, 1 350–1 480 gave alkane (C – H) bending and the H^1NMR (200 MHz $CDCl_3$) spectrum of 0.9–1.5 depicted the presence of H in propyl group]. The C^{13} NMR spectrum shown a peak at range between 170–185 confirmed the presence of C in acid and ester (C = O), 10–15 confirmed C in propyl (R CH_3) group and 16–25 indicated the presence of C in alkane (R_2 CH_3) and the molecular mass was estimated at 203 *m/z* by mass spectroscopy (Figure 1) which confirmed the composition $C_{10}H_{21}NOS$ for dipropyl-, S-propyl ester.

Dipropyl-, S-propyl ester extracted and purified thus exhibited dose dependent larvicidal activity against the mosquito *An. culicifacies* and *Ae. albopictus*. Different concentrations of the *E. mexicanum* solvent extracts at 150, 300, 450, 600, 750 mg/L were tested against both mosquito species. Hexane, chloroform, acetone, methanol and ethyl acetate solvents were evaluated, however, the highest larval mortality was observed in the ethyl acetate extract in both the mosquito vectors. *Ae. albopictus* was found sensitive to the following orderly LC_{50} values of 448.95, 412.18, 376.95, 332.51 and 290.45 mg/L and LC_{90} values of 928.37, 858.90, 808.77, 716.77 and 640.58 mg/L. The *An. culicifacies* had LC_{50} values of 421.33, 384.26, 341.16, 306.82 and 260.83 mg/L and LC_{90} values of 911.42, 831.29, 754.90, 669.38 and 574.01 mg/L respectively. Among different solvent extracts of *E. mexicanum* bacterial culture filtrate, the highest larvicidal activity against *An. culicifacies* was observed in ethyl acetate extract with mortality ranging between 34.30% ± 0.66% at 150 mg/L to 96.30% ± 0.69% at 750 mg/L (Table 1) for which the LC_{50} and LC_{90} doses were estimated as 260.83 mg/L and 574.01 mg/L (Table 2). Also in case of *Ae. albopictus,* the ethyl acetate extract showed higher mortality ranging from 40.20% ± 0.18% at 150 mg/L to 99.70% ± 0.50% at 750 mg/L for which the LC_{50} and LC_{90} doses were calculated as 290.45 mg/L and 640.58 mg/L (Table 3). The purified compound tested against *An. culicifacies* showed the range of mortality between 41.80% ± 1.70% and 98.50% ± 1.52% and for *Ae. albopictus*, the mortality range was between 37.30% ± 1.37% and 89.30% ± 0.62% (Table 4). The relative efficacy of compound dipropyl-, S-propyl ester against both the mosquito vectors was drawn and found prominent to contain both the vectors LC_{50} value of 127.73 mg/L (LFL-UFL values of 11.20–182.13 mg/L) and LC_{90} value of 337.98 mg/L (LFL-UFL values of 270.18–534.05 mg/L) for *An. culicifacies* [$Y = -0.779 + 0.006x$, $x^2 = 7.973$ ($df = 4$), $P < 0.05$], and LC_{50} value of 155.94 mg/L (LFL-UFL

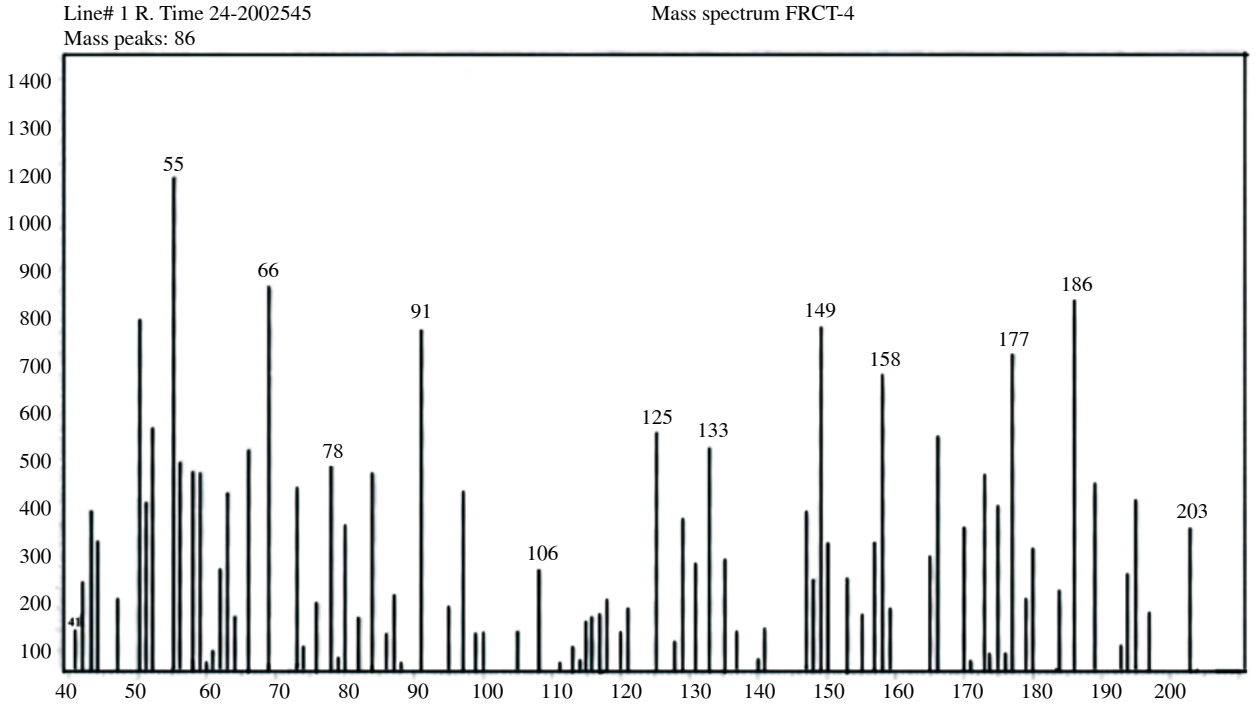


Figure 1. Mass spectroscopy peaks of dipropyl-, S-propyl ester.

values of 123.78–181.31 mg/L) and LC_{90} value of 422.12 mg/L (LFL-UFL values of 375.86–494.02 mg/L) for *Ae. albopictus* [$Y = -0.751 + 0.005x$, $x^2 = 2.577$ ($df = 4$), $P < 0.05$] respectively.

Table 1

Percentage of mortality effects of *E. mexicanum* extract in different concentrations and different solvents against fourth instars larvae of *An. culicifacies* and *Ae. albopictus*.

Solvents	Concentrations (mg/L)	*An. culicifacies*	*Ae. albopictus*
Hexane	Control	0.00 ± 0.00	0.00 ± 0.00
	150	22.50 ± 1.63	24.70 ± 1.19
	300	31.20 ± 1.69	35.40 ± 1.23
	450	52.70 ± 0.88	54.90 ± 0.55
	600	65.10 ± 1.24	67.30 ± 1.11
	750	78.50 ± 1.41	80.60 ± 1.30
Chloroform	Control	0.00 ± 0.00	0.00 ± 0.00
	150	23.90 ± 1.24	26.20 ± 1.10
	300	34.60 ± 0.18	38.30 ± 0.25
	450	56.00 ± 1.63	59.00 ± 1.36
	600	70.00 ± 0.85	72.00 ± 0.28
	750	83.60 ± 0.57	85.90 ± 0.70
Acetone	Control	0.00 ± 0.00	0.00 ± 0.00
	150	26.70 ± 0.90	30.80 ± 0.49
	300	37.20 ± 1.21	40.30 ± 1.18
	450	60.50 ± 1.30	63.50 ± 1.36
	600	75.00 ± 0.18	79.10 ± 0.52
	750	86.30 ± 1.96	90.40 ± 1.10
Methanol	Control	0.00 ± 0.00	0.00 ± 0.00
	150	30.60 ± 1.52	33.80 ± 1.20
	300	40.00 ± 0.45	43.50 ± 0.33
	450	66.30 ± 0.39	67.60 ± 0.48
	600	81.70 ± 1.88	85.10 ± 1.32
	750	92.30 ± 1.14	95.80 ± 1.23
Ethyl acetate	Control	0.00 ± 0.00	0.00 ± 0.00
	150	34.30 ± 0.66	40.20 ± 0.18
	300	47.50 ± 1.22	47.90 ± 1.31
	450	69.00 ± 1.30	74.60 ± 1.36
	600	88.60 ± 0.13	92.80 ± 0.17
	750	96.30 ± 0.69	99.70 ± 0.50

All values were expressed as mean ± SD of five replicates.

Table 2

LC_{50} and LC_{90} values, regression equation and *Chi*-square analysis of larvicidal activity against *Ae. albopictus*.

Solvents	LC_{50} (LFL-UFL) (mg/L)	LC_{90} (LFL-UFL) (mg/L)	Regression equation	x^2 ($df = 4$)
Hexane	448.95 (400.80–509.02)	928.37 (798.35–1 160.12)	$Y = -1.200 + 0.003x$	0.872*
Chloroform	412.18 (368.75–453.20)	858.90 (778.94–974.75)	$Y = -1.182 + 0.003x$	0.556*
Acetone	376.95 (332.53–416.99)	808.77 (736.02–912.69)	$Y = -1.119 + 0.003x$	0.903*
Methanol	332.51 (289.48–369.93)	716.77 (657.70–798.07)	$Y = -1.109 + 0.003x$	1.996*
Ethyl acetate	290.45 (247.37–326.81)	640.58 (589.61–709.34)	$Y = -1.063 + 0.004x$	2.185*

*: $P < 0.05$.

Table 3

LC_{50} and LC_{90} values, regression equation and *Chi*-square analysis of larvicidal activity against *An. culicifacies*.

Solvents	LC_{50} (LFL-UFL) (mg/L)	LC_{90} (LFL-UFL) (mg/L)	Regression equation	x^2 ($df = 4$)
Hexane	421.33 (374.42–465.99)	911.42 (818.48–1 050.99)	$Y = -1.102 + 0.003x$	0.401*
Chloroform	384.26 (338.88–425.39)	831.29 (754.00–943.17)	$Y = -1.102 + 0.003x$	0.456*
Acetone	341.16 (295.60–380.66)	754.90 (689.08–847.57)	$Y = -1.057 + 0.003x$	1.405*
Methanol	306.82 (263.95–343.43)	669.38 (615.52–742.59)	$Y = -1.085 + 0.004x$	3.009*
Ethyl acetate	260.83 (110.34–348.10)	574.01 (470.99–814.74)	$Y = -1.067 + 0.004x$	8.202*

*: $P < 0.05$.

Table 4

Percentage of mortality effects of compound 4 (dipropyl-, S-propylester) in concentrations against fourth instars larvae of *An. culicifacies* and *Ae. albopictus*.

Species	Concentrations (mg/L)	Mortality (%)
An. culicifacies	Control	0.00 ± 0.00
	75	41.80 ± 1.70
	150	53.40 ± 1.80
	225	69.70 ± 0.77
	300	79.80 ± 1.29
	375	98.50 ± 1.52
Ae. albopictus	Control	0.00 ± 0.00
	75	37.30 ± 1.37
	150	48.60 ± 0.55
	225	60.50 ± 1.49
	300	71.70 ± 0.83
	375	89.30 ± 0.62

All values were expressed as mean ± SD of five replicates.

4. Discussion

The findings of current study revealed that S-propyl ester is an effective mosquitocidal molecule with potential mosquito population intervention property. Other mosquitocidal compounds were reported with mosquitocidal property against *Culex pipiens* larvae such as dihydrocitronellyl acetate, linalyl acetate, citronellyl acetate, neryl acetate, geranyl acetate, dihydrocitronellal, citronellol, dihydrolinalyl acetate, citronellic acid and tetrahydrolinalyl[13]. Also the crude extract of *Solanum nigram* leaves showed significant larvicidal activity against *An. culcifacies*, *Culex quinquefasciatus* and *Aedes aegypti* at a dose equivalent to the LC_{90}, ranging from 0.18% to 0.21%[14]. Besides the crude extracts, the compound styryl-pyrone, goniothalamin, was found to be effective natural plant based insecticide against *Spodoptera exigua*[15].

The present investigation proved dipropyl-, S-propyl ester extracted from bacteria to have potential larvicidal activity. The biological activity of the experimental bacterial metabolites of different solvent extracts is varied, which may be ascribable to the presence of various active compounds produced by the bacteria. These active compounds may have collectively or independently influenced or contributed to produce larvicidal effects against *An. culicifacies* (Table 1).

Secondary metabolite produced by bacteria for their insecticidal activity was not much explored and only a few studies were taken up in the recent past, with detailed the analysis of liquid cultures of the entomopathogenic bacteria and the bacteria are in fact very important secondary metabolite producers that can produce several structurally diverse compounds[16,17]. Therefore, entomopathogenic bacteria can be considered as a novel origin of potential insecticides which have been studied only recently. Other entomopathogenic bacteria also have been identified which have not been analyzed for secondary metabolites and/or genes encoding the corresponding biosynthesis genes, but are members of well known secondary metabolite producing genera[18]. Examples are *Serratia entomophila* (*S. entomophila*) and *Erwinia carotovora*[19]. Whereas, the entomopathogenic activity of *Erwinia carotovora* has been shown only under laboratory conditions, and *S. entomophila* has been isolated from the New Zealand grass grub and *Costelytra zealandica* in the field. *S. entomophila* causes the so-called amber disease in *Costelytra zealandica*, a major pasture pest in New Zealand[20]. *Photorhabdus* produced a small-molecule antibiotic (E)-1, 3-dihydroxy-2-(isopropyl) -5-(2 phenylethenyl) benzene (ST) that acted as an inhibitor of phenoloxidase in the insect host *Manduca sexta* as found by Eleftherianos and others[21].

Insecticidal compounds produced by bacteria are less described in the literature and mostly represent small molecules like benzylideneacetone, iodine, phenethylamines and indole derivatives, and also more complex compounds like the *Xenorhabdus* and xenorxides and xenocoumacin[22-25]. Despite the fact that, few compounds with insecticidal activity were reported from entomopathogenic bacteria, several of them were structurally unique *e.g.* zwittermicin from *Bacillus thuringiensis*, isopropylstilbene from *Photorhabdus luminescence*, which were complementary sources of natural products compared to much more well established natural product producers like *Streptomycetes*. Toxin complex has been demonstrated in insect pathogens, including the closely related *Xenorhabdus* and the free living *S. entomophila*[26,27]. In the present results, the purified compound tested against *An. culicifacies* showed the range of mortality between 41.8% and 98.5% and for *Ae. albopictus,* the range of mortality was between 37.3% and 89.3%. The comparative efficacy of compound dipropyl-, S-propyl ester against both the mosquito vectors was drawn and found prominent to control both the vectors, *An. culicifacies* and *Ae albopictus*.

The outcomes of the present study showed that the bacterial compound dipropyl-, S-propyl ester isolated exhibited good insecticidal activity against *An. culicifacies* and *Ae. albopictus*. The possible utilization of these naturally available microbes for effective control of insect pest is recommended from this study. The insecticidal compound that was identified from the bacterium *E. mexicanum* could be used against *An. culicifacies* and *Ae. albopictus* by subjecting them to further biosafety evaluation.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

The laboratory facilities provided by M. S. Swaminathan Research Foundation to carry out this task are highly recognized. The authors are grateful to Dr. Mark S. Goettel for reviewing and shaping this paper. The intellectual support of Dr. Joseph for training and deciphering the chemical construction of the compound was gratefully acknowledged.

References

[1] Choumet V, Desprès P. Dengue and other flavivirus infections. *Rev Sci Tech* 2015; **34**: 473-8.

[2] Valero N. [Zika virus: another emerging arbovirus in Venezuela?] *Invest Clin* 2015; **56**: 241-2. Spanish.

[3] World Health Organization. Factsheet on the world malaria report 2013. Geneva: World Health Organization; 2013. [Online] Available from: http://www.who.int/malaria/media/world_malaria_report_2013/en/ [Accessed on 15th September, 2015]

[4] Surendran SN, Ramasamy MS, De Silva BG, Ramasamy R. *Anopheles culicifacies* sibling species B and E in Sri Lanka differ in longevity and in their susceptibility to malaria parasite infection and common insecticides. *Med Vet Entomol* 2006; **20**: 153-6.

[5] Amerasinghe PH, Amerasinghe FP, Konradsen F, Fonseka KT, Wirtz RA. Malaria vectors in a traditional dry zone village in Sri Lanka. *Am J Trop Med Hyg* 1999; **60**: 421-9.

[6] World Health Organization. Test procedures for insecticide resistance monitoring in malaria vector mosquitoes. Geneva: World Health Organization; 2013. [Online] Available from: http://apps.who.int/iris/bitstream/10665/8013 [Accessed on 15th September, 2015]

[7] Raghavendra K, Barik TK, Sharma SK, Das MK, Dua VK, Pandey A, et al. A note on the insecticide susceptibility status of principal malaria vector *Anopheles culicifacies* in four states of India. *J Vector Borne Dis* 2014; **51**: 230-4.

[8] Mishra AK, Chand SK, Barik TK, Dua VK, Raghavendra K. Insecticide resistance status in *Anopheles culicifacies* in Madhya Pradesh, central India. *J Vector Borne Dis* 2012; **49**: 39-41.

[9] Xu J, Morisseau C, Yang J, Mamatha DM, Hammock BD. Epoxide hydrolase activities and epoxy fatty acids in the mosquito *Culex quinquefasciatus*. *Insect Biochem Mol Biol* 2015; **59**: 41-9.

[10] World Health Organization. Guidelines for laboratory and field testing of mosquito larvicides. Geneva: World Health Organization; 2005. [Online] Available from: http://apps.who.int/iris/bitstream/10665/69101/1/WHO_CDS_WHOPES_GCDPP_2005.13.pdf [Accessed on 15th September, 2015]

[11] Abbott WS. A method of computing the effectiveness of an insecticide. 1925. *J Am Mosq Control Assoc* 1987; **3**: 302-3.

[12] Finney DJ. *Probit analysis*. Cambridge: Cambridge University Press; 1971.

[13] Vera SS, Zambrano DF, Méndez-Sanchez SC, Rodríguez-Sanabria F, Stashenko EE, Duque Luna JE. Essential oils with insecticidal activity against larvae of *Aedes aegypti* (Diptera: Culicidae). *Parasitol Res* 2014; **113**: 2647-54.

[14] Maia MF, Moore SJ. Plant-based insect repellents: a review of their efficacy, development and testing. *Malar J* 2011; **10**: S11.

[15] Senthil Nathan S, Choi MY, Paik CH, Seo HY, Kim JD, Kang SM. The toxic effects of neem extract and azadirachtin on the brown planthopper, *Nilaparvata lugens* (Stål) (BPH) (Homoptera: Delphacidae).*Chemosphere* 2007; **67**: 80-8.

[16] Brachmann AO, Forst S, Furgani GM, Fodor A, Bode HB. Xenofuranones A and B: phenylpyruvate dimers from *Xenorhabdus szentirmaii*. *J Nat Prod* 2006; **69**: 1830-2.

[17] Lang G, Kalvelage T, Peters A, Wiese J, Imhoff JF. Linear and cyclic peptides from the entomopathogenic bacterium *Xenorhabdus nematophilus*. *J Nat Prod* 2008; **71**: 1074-7.

[18] Vallet-Gely I, Lemaitre B, Boccard F. Bacterial strategies to overcome insect defences. *Nat Rev Microbiol* 2008; **6**: 302-13.

[19] Stock SP, Glazer I, Boemare N, Vandenberg J. *Insect pathogens: molecular approaches and techniques*. Wallingford: CABI; 2009.

[20] Basset A, Khush RS, Braun A, Gardan L, Boccard F, Hoffmann JA, et al. The phytopathogenic bacteria *Erwinia carotovora* infects *Drosophila* and activates an immune response. *Proc Natl Acad Sci U S A* 2000; **97**: 3376-81.

[21] Eleftherianos I, Boundy S, Joyce SA, Aslam S, Marshall JW, Cox RJ, et al. An antibiotic produced by an insect-pathogenic bacterium suppresses host defenses through phenoloxidase inhibition. *Proc Natl Acad Sci* 2007; **104**: 2419-24.

[22] Penesyan A, Tebben J, Lee M, Thomas T, Kjelleberg S, Harder T, et al. Identification of the antibacterial compound produced by the marine epiphytic bacterium *Pseudovibrio* sp. D323 and related sponge-associated bacteria. *Mar Drugs* 2011; **9**: 1391-402.

[23] Sordillo LM. New concepts in the causes and control of mastitis. *J Mammary Gland Biol Neoplasia* 2011; **16**: 271-3.

[24] Li J, Chen G, Webster JM, Czyzewska E. Antimicrobial metabolites from a bacterial symbiont. *J Nat Prod* 1995; **58**: 1081-6.

[25] McInerney BV, Gregson RP, Lacey MJ, Akhurst RJ, Lyons GR, Rhodes SH, et al. Biologically active metabolites from *Xenorhabdus* spp., Part 1. Dithiolopyrrolone derivatives with antibiotic activity. *J Nat Prod* 1991; **54**: 774-84.

[26] Morgan KK, Hicks J, Spitze K, Latta L, Pfrender ME, Weaver CS, et al. Patterns of genetic architecture for life-history traits and molecular markers in a subdivided species. *Evolution* 2001; **55**: 1753-61.

[27] Dodd SJ, Hurst MR, Glare TR, O'Callaghan M, Ronson CW. Occurrence of sep insecticidal toxin complex genes in *Serratia* spp. and *Yersinia frederiksenii*. *Appl Environ Microbiol* 2006; **72**: 6584-92.