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Isolation of mosquito larvicidal molecule from the leaves of *Clausena anisata*

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ABSTRACT

The vector-borne diseases caused by mosquitoes are one of the major health problems in many countries, especially in tropical and sub-tropical countries. The resistance of mosquitoes to synthetic chemicals and environmental toxicity created by the chemicals raised the demand for a finding of alternate natural molecules that control mosquito. In the present study, the compound 1, 2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester was isolated from the ethyl acetate extract of leaves of *Clausena anisata* and it was identified by various spectral studies. The larvicidal potential of the isolated compound was evaluated against early 4th instar larvae of *Culex quinquefasciatus*, *Aedes aegypti*, and *Anopheles stephensi.* The compound exhibited 100% larval mortality against *A. aegypti* and *A. stephensi* at 40 ppm with LC_{50} values of 8.944 and 9.230 ppm, respectively. The molecule also showed the LC_{50} value of 12.067 ppm against *C. quinquefasciatus.* The molecule isolated from *C. anisata* can be better explored for the control of mosquito population after toxicological evaluation.

KEY WORDS: *Aedes aegypti*, *Anopheles stephensi*, *Clausena anisata*, *Culex quinquefasciatus*, larvicidal activity

INTRODUCTION

Mosquito-borne diseases, such as dengue, chikungunya, filariasis, and malaria are the major public health problems in the tropical and subtropical countries due to their climatic conditions. In recent years, climate change is likely to expand the geographical distribution of vector and vector-borne diseases, and these have a significant social and economic impact. *Culex quinquefasciatus*, the vector of lymphatic filariasis is widely distributed in tropical and subtropical countries, with around 120 million people infected worldwide and 44 million people having common chronic manifestation (Bernhard *et al.,* 2003). India alone contributes around 40% of global filariasis burden, and the estimated annual economic loss is about 720 crores (Hotez *et al.,* 2004). The *Aedes aegypti* mosquito (Diptera: Culicidae) is the vector for the etiologic agents of yellow fever, chikungunya and dengue fever (Chhabra *et al.,* 2008). Dengue fever is currently considered as the most important viral vector-borne disease. It is estimated that more than 2.5 billion people live in transmission risk areas (World Health Organization [WHO], 2013). Dengue fever was initially described during an epidemic in Philadelphia in 1780, and since then, intermittent pandemics have affected Asia, Africa and the Americas at 10-30 years intervals (Omena *et al.,* 2007). Outbreaks of dengue fever have repeatedly occurred in Brazil since 1980s, after the resurgence of the dengue virus in the country (Garcez *et al.,* 2009). *Anopheles stephensi* is the primary vector of malaria in India and other West Asian countries and improved methods of control are urgently needed (Burfield and Reekie 2005; Mittal *et al.,* 2005).

Conventional pesticides such as malathian, DDT and pyrethroides that are generally used for mosquito control are known to cause the problems such as environmental pollution, residual effects and resistance of mosquito species. Development of resistance in *C. quinquefasciatus A. aegypti* and *A. stephensi* have been noted by WHO (1989) and by other studies (Polson *et al.,* 2011; Ocampo *et al.,* 2011; Raghavendra *et al.,* 2011). These problems forced to search for new, alternative and safer control measures, especially from a plant source. Because, plant-derived molecules are eco-friendly, biodegradable, and target specific (Nathan and Kalaivani, 2005). Moreover, the development of resistance by vectors against plant-derived molecules has not been reported so far. Botanical and microbial insecticides have been increasingly used

for mosquito control because of their efficacy and documented non-toxic effects on non-target organisms (Ascher *et al.,* 1995).

The genus *Clausena* (Rutaceae) is represented by 20 species in India, and they are traditionally used for various diseases (Kirtikar and Basu, 1991). Various extracts, their isolated compounds and essential oil of *Clausena anisata* were already investigated for mosquito larvicidal activity (Govindarajan, 2010; Mavundza *et al.,* 2013; Mukandiwa *et al.,* 2015; Jayaraman *et al.,* 2015). Based on our previous study (Jayaraman *et al.,* 2015), the ethyl acetate extract of *C. anisata* was studied to isolate larvicidal molecule responsible for the activity.

MATERIALS AND METHODS

Plant Material and Preparation of Crude Extract

Healthy and well grown leaves of *C. anisata* were collected from their natural habitats and leaves were immediately brought to the laboratory using polythene bags. The leaves were first washed with tap water, then surface sterilized in 10% sodium hypochlorite to prevent the contamination of any microbes. They were thoroughly rinsed with sterile distilled water and shade dried followed by oven drying (60°C) and milled in an electrical blender. Air-dried and powdered leaf material of *C. anisata* (2.25 kg-750 g × 3) was extracted in a soxhlet apparatus with threefold of ethyl acetate (w/v) until the complete extraction. The extracts were pooled, and the solvent was evaporated using a rotary evaporator(Heidolf-Germany) under reduced pressure at 40°C. The voucher specimen (Herbarium No. AUBOT#267) was deposited at the Herbarium, Department of Botany, Annamalai University.

Isolation of Larvicidal Molecule

50 g of ethyl acetate residue of *C. anisata* was taken, and slurry was prepared with the equal amount of silica gel (100-200 mesh, Hi-Media, Mumbai) and it was loaded on the glass column (5 cm × 60 cm) using hexane. The column was eluted with solvents of increasing polarity from hexane to chloroform (9:5-0:5). In all, 27 fractions were collected. Each fraction contained 100 ml. The fractions showing similar R_f values in thin layer chromatography (TLC) were combined, and the fractions were dried under vacuum. In total, 16 fractions were obtained in hexane and chloroform combination with the ratio of 8.0:2.0. 16 combined fractions were tested for larvicidal activity. Among the combined fractions tested, F2, F3, F4, and F5 showed the larvicidal activities. Based on the highest activity of F2 fraction, it was further subjected to separation and purification of the active compound. The active fraction F2 (5 g) was packed with 50 g of silica gel (100-200 mesh) in a glass column (2.0 cm × 50 cm)) using hexane. This was eluted with solvents of increasing polarity from hexane to ethyl acetate (7.0:3.0). In all, 40 fractions, each 50 ml were collected. The fractions showing similar R_f values in TLC were combined, and 6 fractions were obtained in hexane and ethyl acetate combination with the ratio of 7.0:3.0. Again 6 combined fractions were tested against larvicidal activity. Among the combined fractions tested f_2 showed the larvicidal activities. Due to the highest activity of f_2 fraction, from that further subjected to separation and purification of the active compound. The active fraction f_2 (1.0 g) was further subjected to glass column (2.0 × 50 cm) packed with 40 g of silica gel (100-200 mesh) using hexane. This was eluted with solvents of increasing polarity from benzene to chloroform (9.90-0.10). In all, 6 fractions, each 20 ml were collected. The fractions showing similar R_f values in TLC were combined. The combined fractions, 3-4 of benzene and chloroform (8.0:2.0) formed white powder substance. The compound was identified by Fourier transform-infrared (FT-IR), ^{1}H, ^{13}C NMR and GC-MS.

Larvicidal Assay

The eggs of *A. stephensi* and *A. aegypti* were received from the Field Station, Centre for Research in Medical Entomology (ICMR-Government of India), Viruthachalam, and the egg rafts of *C. quinquefasciatus* were collected from drainage of local residential area of Annamalai Nagar (11°23′17 N, 79°42′57 E) and reared in laboratory (29±3°C, 75-85% RH). The larvae were fed with Brewer's yeast:dog biscuit (1:3). The larvae at early fourth instar stage were used for the larvicidal assay. The larvicidal activity was analyzed as per the standard procedures recommended by WHO (1981). The compound 1, 2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester was dissolved in 2 ml of dimethyl sulfoxide and the concentrations of 40, 20, 10, 5, and 2.5 ppm were prepared with distilled water. 20 larvae were taken in a glass beaker (250 ml) containing 199 ml of tap water and 1 ml of respective concentrations of the compound. Five replicates were maintained for each concentration and the dead larvae were counted after 24 h.

Statistical Analysis

All the data were analyzed using SPSS version 21.0. The LC_{50} and LC_{90} values of mosquito larvicidal activity were calculated by Probit analysis and their lower, and upper confidence levels were determined. The level of significance for the assay was $P < 0.05$.

RESULTS

Identification of Active Molecule

The FT-IR spectrum of the larvicidal molecule was recorded in the spectral range of 4000-450/cm and the bands of compound indicated at 3348, 2931, 2900, 2065, 1649, 1431, 1382, 1340, 1258, 1018, 777, 670, 631, 568, 551, and 537/cm, respectively. It shows two strong absorption at 3348/cm which belongs to OH stretching frequency. Another one strong band absorption at 3000/cm is due to aromatic stretching frequency.

The ^{1}H and ^{13}C NMR spectral results are as follows. White powder substances; molecular formula: $C_{16}H_{22}O_4$; molecular weight: 279.16; m.p. 116-120°C; ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 0.96 (s, 4H), 1.260 (s, 8H), 2.007 (s, 2H), 4.009 (s, 2H), 4.342 (s, 1H), 7.914 (s, 2H), 8.24 (s, 2H), 11.00 (s,1H),; ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 169.4, 166.0, 133.9, 133.5, 132.3, 133.0, 130.2, 129.8, 69.7, 39.6, 30.8, 29.4, 24.8, 23.1, 14.1 and 11.6. The GC-MS spectrum calculated mass of m/z =279.16, and retention time at 17.67. Based on the spectral studies, the isolated molecule was identified as 1, 2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester (Figure 1).

Larvicidal Activity of the Isolated Molecule

The larvicidal activity of 1, 2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester at different concentrations (2.5, 5.0, 10.0, 20.0, and 40 ppm) were evaluated against the early 4th instar larvae of *C. quinquefasciatus*, *A. aegypti*, and *A. stephensi* and the results are given in Table 1. The compound 1, 2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester exhibited cent per cent mortality at 40 ppm against *A. aegypti* and *A. stephensi*. The molecule showed the highest larvicidal activity against *A. aegypti* (LC_{50} = 8.944; LC_{90} = 20.604) follwed by *A. stephensi* (LC_{50} = 9.230; LC_{90} = 24.829) and *C. quinquefasciatus* (LC_{50} = 12.067; LC_{90} = 25.618).

DISCUSSION

Vector control is facing a threat due to the emergence of resistance to synthetic insecticides. Molecules from the botanical origin are suitable and alternatives to reduce or control the resistance problem. In the present study, the isolated compound exhibited 100% mortality of *A. aegypti* and *A. stephensi* at 40 ppm. The larvicidal activity of essential oil from *C. anisata* was already reported by Govindarajan (2010). The essential oil showed significant larvicidal activity against *C. quinquefasciatus*, *A. aegypti*, and *A. stephensi* with the LC_{50} values of 140.96, 130.19, and 119.59 ppm, respectively after 24 h of exposure period. The larvicidal molecule isolated and identified from the ethyl acetate extract of the leaves of *C. anisata* is a phenolic derivative.

Mukandiwa *et al.* (2015) reported larvicidal activity of leaf extracts and seselin from *C. anisata* against *A. aegypti*. The molecule isolated from the present study, 1,2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester exhibited cent per cent mortality at 40 ppm against

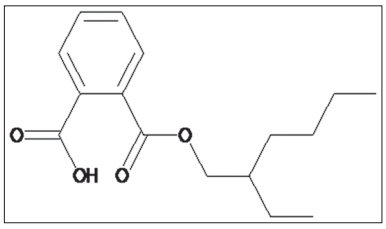

Figure 1: Structure of 1, 2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester

Table 1: Mosquito larvicidal activity of 1, 2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester against three species of vector mosquitoes

Mosquito species	Concentration (ppm)	% of mortality±SE	LC_{50} (ppm) (LCL-UCL)	LC_{90} (ppm) (LCL-UCL)	χ^2 (df=3)
Culex quinquefasciatus	2.5	8.00±1.7	12.067 (4.931-23.636)	25.618 (17.604-72.671)	19.930
	5	26.0±1.7			
	10	55.0±2.6			
	20	78.0±2.3			
	40	98±0.0			
Aedes aegypti	2.5	14.0±1.5	8.944 (3.217-15.095)	20.604 (14.663-48.163)	14.006
	5	38.0±2.0			
	10	66.0±2.0			
	20	84.0±3.0			
	40	100±0.00			
Anophels stephensi	2.5	18.0±2.3	9.230 (17.322-16.251)	24.829 (17.322-58.793)	13.661
	5	40.0±1.0			
	10	62.0±2.8			
	20	82.0±2.8			
	40	100±0.00			

SE: Standard error, [a]95% Confidence interval, LCL: Lower confidence limits, UCL: Upper confidence limits, χ^2: Chi-square, df: Degrees of freedom

A. aegypti and *A. stephensi* which is similar to neotenone, an isoflavonoid isolated from *Neorautaenia mitis* tubers that resulted in 100% mortality of *Anopheles gambia* larva (Joseph *et al*. 2004). Pushpalatha and Muthukrishnan (1995) reported that the petroleum ether: ethyl acetate (3:1) fraction of *V. negundo* leaf extract showed LC_{50} value of 8.21 ppm against the 2nd instar larvae of *C. quinquefasicatus.* But the 2nd instar larvae are more susceptible to larvicidal principles than the 4th instar larvae. A new triterpene was isolated from the methanol extract of *Coccinia indica* and identified as an oleanolic acid derivative and the compound showed prominent larvicidal activity against *C. quinquefasciatus, A. aegypti* and *A. stephensi* with the LC_{50} values of 5.6, 5.0, and 4.8 mg/L, respectively (Senthilkumar *et al.,* 2012). A saponin isolated from *Achyranthus aspera* recorded the LC_{50} value of 18.20 and 27.24 ppm against *A. aegypti* and *C. quinquefasciatus*, respectively (Bagavan *et al.,* 2008).

The benzoic acid derivatives with a general structure C6–C1 are widely used as industrial chemicals and agrochemicals, and some of them are reported to have biological activity (Tomás-Barberánand Clifford, 2000). Methyl-2-hydroxybenzoate, which is known as methyl-o-hydroxybenzoate or methyl salicylate was found to exhibit high toxicity on adults and eggs of *Pediculus humanus* (Yang *et al.,* 2003). It also possessed acaricide activity on *Varroa jacobsoni* (Lindberg *et al.,* 2000). Unelius *et al.* (2006) reported that methyl-4-hydroxybenzoate (methyl-p-hydroxybenzoate) was better in the antifeedant activity against pine weevil, *Hylobius abietis* than methyl-2-hydroxybenzoate (methyl-o-hydroxybenzoate). p-Hydroxybenzoic acid is one of the wall-bound phenolic acids that play a major role in plant defense responses against pathogen attack (Dixon and Paiva, 1995). This supports the toxicity of the 1, 2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester towards mosquito larvae. Foliar polyphenols exhibited biocidal effects against mosquito larvae (David *et al.,* 2000). Rey *et al.* (1999) reported that the toxicity of polyphenols is exerted against midgut epithelium of larvae. Kannadasan *et al.* (2011) reported that methyl-p-hydroxybenzoate was evaluated against early 4th instar larvae of *C. quinquefasciatus* and *A. aegypti*. The compound exhibited 100% larval mortality of both the mosquitoes at 20 ppm with LC_{50} values of 5.77 and 4.74 ppm against *C. quinquefasciatus* and *A. aegypti*, respectively. Although, several plants have been reported for mosquito larvicidal activity, only a few botanicals have been taken from the laboratory to field trials, because they are poorly characterized and in most cases active principles are not determined. Thus, the 1, 2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester could be used as a potential mosquito larvicidal compound.

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