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Mosquito larvicidal properties of *Orthosiphon thymiflorus* (Roth) Sleesen. (Family: Labiatae) against mosquito vectors, *Anopheles stephensi*, *Culex quinquefasciatus* and *Aedes aegypti* (Diptera: Culicidae)

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ABSTRACT

Objective: To determine the mosquito larvicidal activities of hexane, chloroform, ethyl acetate, acetone and methanol leaf extract of *Orthosiphon thymiflorus (O. thymiflorus)* against *Anopheles stephensi (An. stephensi)*, *Culex quinquefasciatus (Cx. quinquefasciatus)* and *Aedes aegypti (Ae. aegypti)*. **Methods:** The larvicidal activity was assayed against three mosquito species at various concentrations ranging from (50–450 ppm) under the laboratory conditions. The LC_{50} and LC_{90} value of the *O. thymiflorus* leaf extract was determined by Probit analysis. **Results:** The LC_{50} values of hexane, chloroform, ethyl acetate, acetone and methanol extract of *O. thymiflorus* third instar larvae of *An. stephensi* were LC_{50}= 201.39, 178.76, 158.06, 139.22 and 118.74 ppm; *Cx. quinquefasciatus* were LC_{50}=228.13, 209.72, 183.35, 163.55 and 149.96 ppm and *Ae. aegypti* were LC_{50}=215.65, 197.91, 175.05, 154.80 and 137.26 ppm, respectively. Maximum larvicidal activity was observed in the methanolic extract followed by acetone, ethyl acetate chloroform and hexane extract. The larval mortality was observed after 24 h exposure. No mortality was observed in control. **Conclusions:** The present results suggest that the effective plant crude extracts have potential to be used as an ideal eco-friendly approach for the control of mosquito vectors. This study provides the first report on the larvicidal activity of this plant crude solvent extract of against *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti* mosquitoes.

1. Introduction

Mosquitoes constitute a major public health problem as vectors of serious human diseases like malaria, filariasis, dengue fever, chikunguya and yellow fever[1] cause substantial mortality and morbidity among people living in tropical and subtropical zones. Vector Control is a serious concern in developing countries, every year a large part of the population in the world is affected by one or more vector borne diseases. Mosquitoes alone transmit disease to more than 700 million people annually[2].

Anopheles stephensi (*An. stephensi*) (L.) is the primary vector of malaria in India and other West Asian countries, and improved methods of control are urgently needed[3,4]. Malaria infects more than 500 million humans each year, killing approximately 1.2 to 2.7 million per year. About 90% of all malaria cases occur in Africa, as do approximately 90% of the world's malaria-related deaths[5]. Malaria, caused by *Plasmodium falciparum* (*P. falciparum*), is one of the leading causes of human morbidity and mortality from infectious diseases, predominantly in tropical and subtropical countries[6]. The highest number of malaria, *P. falciparum* cases, and malaria-related deaths are recorded from the state of Orissa located in the eastern part of India[7]. *Aedes aegypti* (*Ae. aegypti*) (L.) a vector of dengue, is widely distributed in the tropical and subtropical zones. About two-thirds of the world's population lives in areas infested with dengue vectors, mainly *Ae. aegypti*. Currently, dengue is endemic in all continents except Europe, and epidemic dengue haemorrhagic fever occurs in Asia, the Americas and some Pacific islands. Dengue viruses, causative agents of dengue fever and more severe dengue hemorrhagic fever/dengue shock syndrome infect

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over 100 million people every year[8]. Chikungunya virus, a member of the alphavirus genus, is of considerable public health concern in Southeast Asian and African countries[9]. *Culex quinquefaciatus* (*Cx. quinquefaciatus*) (S.) is an important vector of *Bancroftian fialriasis* (*B. fialriasis*) in tropical and subtropical regions. According to WHO[10] about 90 million people worldwide are infected with *Wuchereria bancrofti* (*W. bancrofti*), the lymphatic dwelling parasite and ten times more people are at the risk of being infected. In India alone 25 milion people suffer from filarial diseases manifestations (NICD)[11,12]. *Cx. quinquefasciatus* is one of the potential vectors of *W. bancrofti*, the causative agent of human lymphatic filariasis infecting over 120 million people all over the world[13].

Many researchers have been reported on plant extracts against the larvae of mosquito vectors. Sivagnanme and Kalyanasundram[14] evaluated the methanolic extracts of the leaves of *Atlanta monophylla* (*A. monophylla*) for mosquitocidal activity against immature stages of three mosquito species, *Cx. quinquefasciatus*, *An. stephensi* and *Ae. aegypti* in the laboratory. The leaves methanol and 95% ethanol extracts of *Musa paradisiaca* (*M. paradisiaca*) were tested against III instar larvae of *An. stephensi* and L4 larvae of *Ae. aegypti*, respectively[15,16].

The acetone, chloroform, ethyl acetate, hexane and methanol leaf extracts of *Leucas aspera* (*L. asepera*) were studied against fourth instar larvae of *Ae. aegypti* and *Cx. quinquefasicatus*[17]. The leaf benzene, chloroform, ethyl acetate and methanol extract of *Acalypha indica* (*A. indica*) were tested for larvicidal activity, ovicdal activity and oviposition attractancy against *An. stephensi*[18]; Hexane extact obtained from leaves of *Eucalytus citriodora* (*E. citriodora*) was tested against larvae of *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti*[19]. Laboratory and field investigations have been made to evaluate the combined effect of *Clerodendron inerme* (*C. inerme*) and *Acanthus ilicifolius* (*A. ilicifolius*) on three species of mosquito vectors, *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus*. Different concentrations of *C. inerme* and *A. ilicifolius* have been tested for larvicidal and pupicidal activities against the mosquito vectors[20]. The leaf extract of *Acalypha alnifolia* (*A. alnifolia*) with different solvents of hexane, chloroform, ethyl acetate, acetone and methanolic extract were tested early fourth instar larvae against three important mosquitoes such as *An. stephensi*, *Ae. aegypti*, *Cx. quinquefasciatus*[21] and we determined the biological activities of methanol extracts of *Acalypha indica (A. indica)* L. (Euphorbiaceae) and *Achyranthes aspera (A. aspera)* L. (Amaranthaceae) leaves individually and in combination as botanical insecticides against *Ae. aegypti*[22].

Orthosiphon thymiflorus (O. thymiflorus) (Roth.) Sleensen (Labiatae) is a medicinal plant native to South East Asia and some parts of tropical Australia. It is an herbaceous shrub which grows to a height of 1.5 meters. It is a popular garden plant because of its unique flower, which is white and bluish with filaments resembling a cat's whiskers. *O. thymiflorus* aqueous extracts have found to be having diuretic[23], anti-inflammatory and acetylcholine antagonistic action 27[24, 25]. Leaf juice has been used by the tribes as a lotion[26]. The flowers are an antidote to poison and the stem, bark and rind of the fruits are diuretic[27]. In evaluation, Methanolic extract of the leaf powder was prepared and screened for *in vitro* antioxidant activities by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity and by reducing power assay method. Both the methods have proven the effectiveness of various extracts compared to the standard antioxidant, ascorbic acid. The methanolic leaf extract of *O. thymiflorus* exhibited a significant dose dependent inhibition of DPPH activity[28].

The present research was carried out to determine the larvicidal properties of *O. thymiflorus* leaf extract against the medically important mosquito vector, *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus*.

2. Material and methods

2.1. Collection of eggs and maintenance of larvae

The eggs of *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti* were collected from National Centre for Disease Control (NCDC) field station of Mettupalayam, Tamil Nadu, India, using an 'O' type brush. These eggs were brought to the laboratory and transferred to 18 cm×13 cm× 4 cm enamel trays containing 500 mL of water for hatching. The mosquito larvae were pedigree dog biscuits and yeast at 3:1 ratio. The feeding was continued until the larvae transformed into the pupal stage.

2.2. Maintenance of pupae and adults

The pupae were collected from the culture trays and transferred to plastic containers (12 cm×12 cm) containing 500 mL of water with the help of a dipper. The plastic jars were kept in a 90 cm×90 cm×90 cm mosquito cage for adult emergence. Mosquito larvae were maintained at (27±2) ℃, 75%-85% RH, under a photoperiod of 14 L: 10 D. A 10% sugar solution was provided for a period of 3 d before blood feeding.

2.3. Blood feeding of adult mosquito vectors

The adult female mosquitoes were allowed to feed on the blood of a rabbit (a rabbit per day, exposed on the dorsal side) for 2 d, to ensure adequate blood feeding for 5 d. After blood feeding, enamel trays with water from the culture trays were placed in the cage as oviposition substrates.

2.4. Plant collection and preparation of extract

The *O. thymiflorus* were collected from Maruthamalai hills, Coimbatore, Tamil Nadu, India. The plants were authenticated at BSI (Botanical Survey of India), and the voucher specimens were deposited entomology division, Zoology Department, Bharathiar University, Coimbatore, Tamil Nadu, India. *O. thymiflorus* plant was washed with tap water and shade-dried at room temperature (27±2) ℃. The dried leaves (1.0 kg) were powdered mechanically using commercial electrical stainless steel blender and extracted (3.0 L) with hexane, chloroform, ethyl acetate, acetone and methanol in a Soxhlet apparatus[29], boiling point range 60-80 ℃ for 8 h. The extract was concentrated under reduced pressure 22-26 mmHg at 45 ℃ and the residue obtained was stored at 4 ℃. The extracts filtered through a Buchner

funnel with Whatman number 1 filter paper. The yield of extracts was hexane (10.86 g), chloroform (12.48 g), ethyl acetate (9.35 g), acetone (13.10 g), and methanol (14.21 g). One gram of the plant residue was dissolved in 100 mL of acetone (stock solution) considered as 1% stock solution. From the stock solution, 50 to 450 ppm various concentration was prepared with decholorinated tap water, respectively.

2.5. Larval toxicity test

A laboratory colony of *An. stephensi Cx. quinquefasciatus* and *Ae. aegypti* larvae was used for the larvicidal activity. Twenty-five third instar larvae were kept in 500 mL glass beaker containing 249 mL of dechlorinated water and 1 mL of desired concentration of plant extracts were added. Larval food was given for the test larvae. At each tested concentration, two to five trials were made and each trial consists of five replicates. The control was setup by mixing 1 mL of acetone with 249 mL of dechlorinated water. The larvae exposed to dechlorinated water without acetone served as control. Larval mortality was assessed after 24 h of exposure by probing the larvae with needle and moribund larvae were counted as dead. The LC_{50} and LC_{90} value was calculated after 24 by probit analysis[30].

2.6. Statistical analysis

The average larval mortality data were subjected to probit analysis for calculating, LC_{50}, LC_{90} and other statistics at 95% confidence limits of upper confidence limit (UCL) and lower confidence limit (LCL) and chi-square values calculated using the SPSS 9.0 version (software package). The values were expressed as mean ± standard deviation of five replicates. Results with $P<0.05$ were considered to be statistically significant.

Mortality was corrected using the following formula[31].

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

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

3. Results

The result shows that larvicidal activity of the crude hexane, chloroform, ethyl acetate, acetone and methanol

Table 1
Larvicidal activity of different solvent extracts of *O. thymiflorus* against *An. stephensi*.

Name of the extract	Concentration (ppm)	% Mortality ± SD	LC_{50} (ppm) (LCL-UCL)	LC_{90} (ppm) (LCL-UCL)	χ^2
Hexane	Control	0.00±0.00	201.39 (171.67-228.18)	490.86 (441.67-561.49)	1.885
	50	26.20±1.72			
	150	42.40±1.85			
	250	56.00±1.67			
	350	71.00±1.41			
	450	89.60±1.62			
Chloroform	Control	0.00±0.00	178.76 (148.21-205.22)	456.88 (412.18-520.22)	3.614
	50	28.00±1.89			
	150	47.80±1.72			
	250	60.20±1.32			
	350	73.60±1.85			
	450	93.20±1.93			
Ethyl acetate	Control	0.00±0.00	158.06 (126.28-184.71)	427.76 (386.31-485.94)	3.941
	50	31.00±1.41			
	150	51.40±1.35			
	250	63.80±1.72			
	350	77.40±1.74			
	450	95.40±1.85			
Acetone	Control	0.00±0.00	139.22 105.85-166.42	402.85 (363.76-457.41)	4.154
	50	34.20±1.72			
	150	54.40±1.01			
	250	67.60±1.85			
	350	80.40±1.62			
	450	97.00±1.41			
Methanol	Control	0.00±0.00	118.74 (10.51-178.69)	373.13 (9 299.67-540.63)	6.181
	50	38.80±1.16			
	150	57.60±1.85			
	250	70.40±1.01			
	350	83.80±1.72			
	450	99.20±0.74			

Control-Nil mortality, LCL = Lower confidence limit, UCL=Upper confidence limit, χ^2=Chi-square value, Significant at $P < 0.05$ level.

Table 2
Larvicidal activity of different solvent extracts of *O. thymiflorus* against *Cx. quinquefasciatus*.

Name of the extract	Concentration (ppm)	% Mortality ± SD	LC_{50} (ppm) (LCL–UCL)	LC_{90} (ppm) (LCL–UCL)	χ^2
Hexane	Control	0.00±0.00	215.65 (186.73–242.53)	507.82 (456.60–581.66)	2.066
	50	24.80±1.32			
	150	40.00±1.41			
	250	52.60±1.85			
	350	69.00±1.78			
	450	88.20±1.16			
Chloroform	Control	0.00±0.00	197.91 (167.68–224.93)	489.44 (440.06–560.53)	2.545
	50	26.00±1.89			
	150	44.40±1.85			
	250	56.80±1.72			
	350	70.40±1.49			
	450	90.00±1.41			
Ethyl acetate	Control	0.00±0.00	175.05 (144.64– 201.29)	448.83 (405.34–510.14)	3.528
	50	28.60±1.85			
	150	48.20±1.72			
	250	60.40±1.62			
	350	75.00±1.41			
	450	93.80±1.32			
Acetone	Control	0.00±0.00	154.80 (121.80–182.19)	431.08 (388.57–491.14)	2.417
	50	31.80±1.60			
	150	52.00±1.41			
	250	63.40±1.85			
	350	79.00±1.89			
	450	94.20±1.72			
Methanol	Control	0.00±0.00	137.26 (102.27–165.47)	410.79 (369.95–468.38)	3.124
	50	35.20±1.72			
	150	54.60±1.85			
	250	66.60±1.62			
	350	80.80±1.93			
	450	96.00±1.41			

Control – Nil mortality, LCL = Lower confidence limit, UCL=Upper confidence limit, χ^2=Chi-square value, Significant at $P < 0.05$ level.

extracts of leaf against *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti*. The *O. thymiflorus* were studied for use as eco-friendly insecticides instead. Results on the larvicidal activities of leaf extracts obtained in this study (Tables 1, 2 and 3) confirm their potential for the control of larval population of mosquito vectors. Hexane, chloroform, ethyl acetate, acetone resulted in moderate mortality; however, the highest larval mortality was methanolic extract observed in three mosquito vectors. The third-instar larvae of *An. stephensi* had values of LC_{50}=201.39, 178.76, 158.06, 139.22 and 118.74 ppm and LC_{90}=490.86, 456.88, 427.76, 402.85and 373.13 ppm, respectively. The *Cx. quinquefasciatus* had values of LC_{50}=215.65, 197.91, 175.05, 154.80 and 137.26 ppm and LC_{90}=507.82, 489.44, 448.83, 431.08 and 410.79 ppm, respectively. The *Ae. aegypti* had values of LC_{50}=228.13, 209.72, 183.35, 163.55 and 149.96 ppm and LC_{90}=526.12, 502.84, 463.35, 442.32 and 426.16 ppm, respectively. The χ^2 values are significant at P<0.05 level. The 95% confidence limits LC_{50} (LCL-UCL) and LC_{90} (LCL-UCL) were also calculated. Larval mortality was observed after 24 h exposure. No mortality was observed in the control group. The results of larvicidal activity clearly indicate that the percentage of mortality being directly proportional to the concentration of the extract. Solvents of the plant extract of *O. thymiflorus* were used at different concentrations, ranging from 50 to 450 ppm, respectively.

4. Discussion

Mosquito-borne diseases, such as filariasis, malaria, dengue, yellow fever, and Japanese encephalitis, contribute significantly to disease burden, death, poverty, and social debility in tropical countries[32]. David *et al*[33] found that phytochemicals primarily affect the midgut epithelium and secondarily affect the gastric caeca and the malpighian tubules in mosquito larvae. Furthermore, the crude extracts may be more effective compared to the individual active compounds, due to natural synergism that discourages the development of resistance in the vectors[34]. Development of resistance to commercial acaricides by parasites has stimulated the search for new control strategies. In recent times, many plant extracts have been tested against endo-

Table 3
Larvicidal activity of different solvent extracts of *O. thymiflorus* against *Ae. aegypti*.

Name of the extract	Concentration (ppm)	% Mortality ± SD	LC_{50} (ppm) (LCL–UCL)	LC_{90} (ppm) (LCL–UCL)	χ^2
Hexane	Control	0.00±0.00	228.13 (199.40–255.53)	526.12 (472.19–604.44)	1.304
	50	23.40±1.85			
	150	37.80±1.72			
	250	51.00±1.41			
	350	67.40±1.85			
	450	85.80±1.32			
Chloroform	Control	0.00±0.00	209.72 (180.25–236.70)	502.84 (451.99–576.19)	1.997
	50	24.40±1.35			
	150	42.20±1.72			
	250	55.00±1.67			
	350	69.00±1.78			
	450	88.40±1.85			
Ethyl acetate	Control	0.00±0.00	183.35 (153.06–209.81)	463.35 (417.88–527.91)	2.781
	50	27.80±1.60			
	150	46.80±1.93			
	250	58.00±1.41			
	350	74.40±1.85			
	450	92.20±1.72			
Acetone	Control	0.00±0.00	163.55 (131.28–190.70)	442.32 (398.68–504.14)	2.333
	50	30.40±1.49			
	150	50.20±1.16			
	250	62.60±1.85			
	350	77.20±1.72			
	450	93.40±1.62			
Methanol	Control	0.00±0.00	149.96 (116.35–177.60)	426.16 (383.96–485.83)	3.291
	50	33.00±1.41			
	150	52.40±1.85			
	250	64.80±1.72			
	350	78.40±1.49			
	450	95.20±1.16			

Control – Nil mortality, LCL = Lower confidence limit, UCL=Upper confidence limit, χ^2 = Chi-square value, Significant at $P < 0.05$ level.

or ectoparasites and pests, which may contaminate food and/or rooms with agents of diseases[35,36].

Karunamoorthi and Ilango[37] have reported that the LC_{50} and LC_{90} values of methanol leaf extracts of *Croton macrostachyus (C. macrostachyus)* were 89.25 and 224.98 ppm, respectively against late third instar larvae of malaria vector, *Anopheles arabiensis (An. arabiensis)*. In their study, Sharma *et al*[38] reported that the acetone extract of *Nerium indicum (N. indicum)* and *Thuja orientelis (T. orientelis)* had LC_{50} values of 200.87, 127.53, 209.00, and 155.97 ppm against III instar larvae of *An. stephensi* and *Cx. quinquefasciatus*, respectively. Authors of previous studies have reported that the methanol leaf extracts of *Vitex negundo (V. negundo), V. trifolia*, *V. peduncularis*, and *V. altissima* were used for larvicidal assay with LC_{50} values of 212.57, 41.41, 76.28, and 128.04 ppm, respectively, against the early fourth-instar larvae of *Cx. quinquefasciatus*[39-46]. The leaf methanol extract of *Cassia fistula (C. fistula)* was tested for larvicidal and ovicidal activity of against *Cx. quinquefasciatus* and *An. stephensi*, with the LC_{50} values of 17.97 and 20.57 mg/L, respectively[47]. The isolated compound saponin from ethyl acetate extract of *Acanthus aspera (A. aspera)* was effective against the larvae of *Ae. aegypti* and *Cx. quinquefasciatus* with LC_{50} value of 18.20 and 27.24 ppm, respectively[48].

The crude leaf extract of *Azadirachta indica* with different solvents, *viz.* benzene, chloroform, ethyl acetate and methanol were tested for larvicidal activity against *An. stephensi*. The LC_{50} values were 19.25, 27.26, 23.26 and 15.03, respectively. Kamaraj *et al*[49] have reported that the peel methanol extract *C. sinensis*, leaf and flower ethyl acetate extracts of *O. canum* against larvae of *An. stephensi* (LC_{50} =95.74, 101.53, 28.96; LC_{90}=303.20, 492.43 and 168.05 ppm) respectively. The highest larval mortality was found in methanol extract of *O. canum* against the larvae of *Ae. aegypti* (LC_{50}=99.42, 94.43 and 81.56 ppm) and against *Cx. quinquefasciatus* (LC_{50}=44.54, 73.40 and 38.30 ppm), respectively[50].

Zahir *et al*[5] have also reported that the highest parasite mortality was found in leaf acetone and methanol extracts of *Anisomeles malabarica (A. malabarica)* seed methanol of *Gloriosa superba (G. superba)* and leaf methanol of *Ricinus communis (R. communis)* against *H. bispinosa* (LC_{50}=466.15,

719.78, 476.06, and 243.87 ppm, and LC_{90}=1 837.96, 2 014.47, 1 904.36, and 2 692.15 ppm, respectively. Kovendan *et al*[21] reported that the leaf hexane, chloroform, ethyl acetate, acetone, and methanol extracts of *Acalypha alnifolia* (*A. alnifolia*) tested against the early fourth instar larvae of *An. stephensi* had values of LC_{50}=197.37, 178.75, 164.34, 149.90 and 125.73 ppm, and LC_{90}=477.60, 459.21, 435.07, 416.20, and 395.50 ppm; *Ae. aegypti* had values of LC_{50}=202.15, 182.58, 160.35, 146.07, and 128.55 ppm, and LC_{90}= 476.57, 460.83, 440.78, 415.38, and 381.67 ppm; *Cx. quinquefasciatus* had values of LC_{50}=198.79, 172.48, 151.06, 140.69, and 127.98 ppm, and LC_{90}=458.73, 430.66, 418.78, 408.83, and 386.26 ppm, respectively.

The current investigation revealed that the crude extract of *O. thymiflorus* possesses remarkable larvicidal activities against three mosquito vectors. This study is the first to report on the mosquito larvicidal activity of *O. thymiflorus* leaf extracts of plant. The result shows that good larvicidal properties of against vector control programs.

Conflict of interest statement

We declare that we have no conflict of interest.

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