Efficacy of larvicidal and pupicidal properties of *Acalypha alnifolia* Klein ex Willd. (Euphorbiaceae) leaf extract and *Metarhizium anisopliae* (Metsch.) against *Culex quinquefasciatus* Say. (Diptera: Culicidae)

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ABSTRACT

The present study was carried out to establish the properties of *Acalypha alnifolia* leaf extract and microbial insecticide, *Metarizhium anisopliae* on larvicidal and pupicidal activity against the lymphatic filarial vector, *Culex quinquefasciatus*. The methanol extract of *A. alnifolia* leaf showed larvicidal and pupicidal effects after 24 h of exposure; with, the highest larval and pupal mortality was recorded against the first- to fourth-instar larvae and pupae of values LC_{50}= 5.67% 1st instar, 6.62% 2nd instar, 7.53% 3rd instar and 9.05% 4th instar, and 10.20% pupae respectively, and microbial insecticide, *M. anisopliae* against the first to fourth instar larvae and pupae with LC_{50} values 1st instar was 10.53%, 2nd instar was 15.57%, 3rd instar was 23.06%, and 4th instar was 31.36%, and pupae was 42.54%, respectively. Moreover, combined treatment of values of LC_{50} values of 1st instar was 3.73%, 2nd instar was 4.72%, 3rd instar was 5.55%, and 4th instar was 7.66%, and pupae was 9.16%, respectively. No mortality was observed in the control. The results shows the leaves extract of *A. alnifolia* and the entomopathogenic fungi, *M. anisopliae* are candidates for controlling lymphatic filarial vector, *C. quinquefasciatus*. Hence, *A. alnifolia* and *M. anisopliae* can be considered for eco-friendly vector control programs.

Key words: *Acalypha alnifolia, Metarizhium anisopliae, Culex quinquefasciatus,* larvicidal, pupicidal, lymphatic filarial vector.

INTRODUCTION

Culex quinquefasciatus is one of the most annoying vectors which transmit lymphatic filariasis and Japanese encephalitis in India (Mourya *et al.,* 1989; Das *et al.,* 2002). Pandian *et al.* (1989) observed the repellent activity of herbal smoke on the biting activity of *C. quinquefasciatus*. Thangam and Kathiresan (1992a) stated that smoke from burning various dry materials has been used since early times to deter insects especially mosquitoes. *C. quinquefasciatus* and many other Culex species bite their hosts at night. *Cx. quinquefasciatus* commonly rest indoors both before and after feeding, but also shelter in outdoor resting places (Service, 2000).

Nirmal Sharma *et al.* (1998) reported larvicidal activity of *Gliricidia sepium* crude ethanol extracts of dried leaves, fresh leaves, dried petioles and stem bark were tested for their activities against third instar larvae of *Anopheles stephensi, Aedes aegypti* and *Culex quinquefasciatus*. El Hag *et al.* (1999) observed the effect of methanolic extracts of neem seeds on egg hatchability and larval development of *Cx. pipiens*. The leaf extract of *Acalypha indica* with different solvents: benzene, chloroform, ethyl acetate, and methanol has ben tested for larvicidal, ovicidal activity, and oviposition attractancy against *An. stephensi* (Govindarajan *et al.,* 2008a). The leaf extract of *Acalypha alnifolia* with different solvents of tested for larvicidal activity against three important mosquitoes such as malarial vector, *An. stephensi*, dengue vector, *A. aegypti* and *Bancroftian filariasis* vector, *Cx. quinquefasciatus* (Kovendan *et al.,* 2012b).

Acalypha alnifolia Klein ex Willd. (Family: Euphorbiaceae) known as Sirukurunjan in Tamil. *Acalypha alnifolia* is a shrub known as Cat-tail and Copperleaf found in the wild in South India (Garg, 2009).

Entomopathogenic imperfect fungus like *Metarhizium anisopliae* show considerable promise for use in integrated pest management (IPM) programmes (Butt *et al.*, 2001). Hyphomycetes fungal isolates of *M. anisopliae* and *Beauveria bassiana* is known to infect and kill adults of the African malaria vector *Anopheles gambiae* sensu stricto through tarsal contact in laboratory containers (Scholte *et al.,* 2003; Blanford *et al.,* 2005). *M. anisopliae* uses a combination of enzymes and mechanical force to penetrate the host cuticle and access the nutrient-rich haemolymph (Wang *et al.,* 2002). Conidia of hyphomycetous fungi strongly adhere to insect cuticle, and the attachment of conidia to cuticles is through to involve non-specific adhesion mechanisms mediated by the hydrophobicity of the cell wall (Bouclas *et al.*, 1988; 1991). Survival of entomopathogenic fungi requires a delicate balance of interaction between the fungus, host and the environment. In general, the life cycle of the entomopathogenic fungi involves an infective spore stage, which germinates on the cuticle of the host, forming a germ tube that penetrate the cuticle and invades the hemocoel of the insect host (Hajek and Leger, 1994).

Hence, in the present investigation an attempt has been made evaluate the *A. alnifolia* leaves and fungal pathogen, *M. anisopliae* on the larvicidal, pupicidal effect of on lymphatic filarial vector, *Cx. quinquefasciatus*.

MATERIALS AND METHODS

Collection and maintenance of insect

The eggs of *Cx. quinquefasciatus* 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 18×13×4-cm enamel trays containing 500 ml of water for hatching. The mosquito larvae were fed with pedigree dog biscuits and yeast at 3:1 ratio. The feeding was continued until the larvae transformed into the pupal stage.

The pupae were collected from the culture trays and transferred to plastic containers (12×12 cm) containing 500 ml of water with the help of a dipper. The plastic jars were kept in a 90×90×90-cm mosquito cage for adult emergence. Mosquito larvae were maintained at 27°C±2°C, 75–85% relative humidity, under a photoperiod of 14:10 hrs light:dark. A 10% sugar solution was provided for a period of 3 days before blood feeding.

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 days to ensure adequate blood feeding for 5 days. After blood feeding, enamel trays with water from the culture trays were placed in the cage as oviposition substrates.

Collection and preparation of plant extract

A. alnifolia were collected from the Kallar Hills (Western Ghats), Mettupalyam, Coimbatore, India. The plants were identified at BSI (Botanical Survey of India), and the plants were deposited at Zoology Department, Bharathiar University, Coimbatore, Tamil Nadu, India. *A. alnifolia* plant was washed with tap water and shade-dried at room temperature. The dried plant materials (leaves). The powder (500 g) of the leaf was extracted with 1.5 litre of organic solvents of methanol using a Soxhlet apparatus at 60–80°C for 8 hrs (Vogel, 1978). The extract was concentrated under reduced pressure 22–26 mm Hg at 45°C and the residue obtained was stored at 4°C. The extracts were filtered through a Buchner funnel with Whatman No. 1 filter paper. The crude plant extracts were evaporated to dryness in rotary vacuum evaporator. One gram of the plant residue was dissolved in 100 mL of acetone (stock solution) and considered as 1% stock solution. From this stock solution, different concentrations were prepared ranging from 4 to 12%, respectively.

Fungal preparation

The commercial fungal formulations of *Metarhizium anisopliae* (Metsch.) obtained from T- Stanes & Company Limited, Research Development Centre, Coimbatore, Tamil Nadu, India was used for the study. The required quantity of entmopathogenic fungi, *M. aniospliae* liquid formulation was thoroughly mixed with distilled water to prepare at various conidia concentrations were adjusted $1x10^2$ to $5x10^{10}$ viable conidia/mL, respectively.

Larval/pupal toxicity test

Laboratory colonies of mosquito larvae/pupae were used for the larvicidal/pupicidal activity. Twenty-five numbers of 1^{st} to 4^{th} instars larvae and pupae were introduced into 500 ml glass beaker containing 249 mL of dechlorinated water and 1 mL of desired concentrations of plant extract, and fungi (liquid formulation) were added. Larval food was given for the test larvae. Each tested concentration, was thrice replicated. The control was set up by mixing 1 mL of acetone with 249 mL of dechlorinated water. The larvae and pupae which were exposed to dechlorinated water without acetone served as control. The control mortalities were corrected by using Abbott's formula (Abbott's 1925). The LC_{50} and LC_{90} were calculated from toxicity data by using probit analysis (Finney, 1971).

Statistical analysis

All data were subjected to analysis of variance; the means were separated using Duncan's Multiple Range Tests by Alder and Rossler (1977). SPSS (Statistical software package) 9.0 version was used. Results with $P<0.05$ were considered to be statistically significant.

RESULTS

Larval and pupal mortality of *Cx. quinquefasciatus* after the treatment of methanolic extract of *A. alnifolia* leaf was observed. Forty one percent mortality was noted at 1^{st} instar larvae by the treatment of *A. alnifolia* at 4%, whereas it has been increased to 12% of *A. alnifolia* leaf extract treatment. Similar trend has been noted for all the instars of *Cx. quinquefasciatus* at different concentration of *A. alnifolia* treatment (Table 1).

Forty percent mortality was noted in 1st instar larvae treated with *M. anisopliae* at $1x10^2$ conidia/mL, whereas it has been increased to 86% at $5x10^{10}$ conidia/mL of *M. anisopliae* treatment, similarly 38% pupal mortality was noted in *M. anisopliae* treatment at $1x10^2$ conidia/mL and it has been increased to 51% at $5x10^{10}$ conidia/mL. Similar trend was also noted in all the instars of *C. quinquefasciatus* at different concentrations of *M. anisopliae* treatment (Table 2). The LC_{50} and LC_{90} values were dose and time dependent one..

The concentration at 1.8% *A. alnifolia* + $1x10^{10}$ *M. anisopliae* conidia/ml combination for 1st instar larvae mortality was recorded 96% (Table 3). The LC_{50} value of 1st instar was 3.73%, 2nd instar was 4.72%, 3rd instar was 5.55%, and 4th instar was 7.66%. The LC_{90} values were also dose and time dependent one.

DISCUSSION

Similarly, the methanolic extracts of *Solanum suratence*, *Azadirachta indica* and *Hydrocotyl javanica* exhibited larvicidal activity against *Cx. quinquefasciatus* (Venkatachalam and Jebanesan, 2001). The larvicidal activity of various plant extracts such as *Pedalium murax*, *Cleome icosondra* and *Dictyosa dietotoma* have been found to be promising against *Cx. quinquefasciatus* and *An. stephensi* (Kalyanasundaram and Das, 1985) naturally occurring insecticides may play a more prominent role in mosquito control programs in the future (Wandscheer *et al.*, 2004). Vahitha *et al.* (2002) and Rajkumar and Jebanesan (2004) studied the larvicidal efficacy of plants against *Cx. quinquefasciatus*.

A 23% mortality was noted at I instar larvae by the treatment of *A. ilicifolius* at 20 ppm, whereas it was increased to 89% at 100 ppm of *A. ilicifolius* leaf extract treatment (Kovendan and Murugan, 2011). Kovendan *et al.* (2011a, b) recently have reported that the leaf extract of methanol *Jatropha curcas* against *Cx. quinquefasciatus* and *Leucas aspera* leaf extract against *An. stephensi*, respectively. The results of the leaf extract of *A. alnifloia* are promising as good larvicidal activity against the mosquito vector, *An. stephensi*, *Ae. aegypti*, *Cx. quinquefasciatus* (Kovendan *et al.* 2012 b). A very recent study by Murugan *et al.* (2012) reported that the combination of *A. alnifolia* and *M. anisopliae* against the malarial vector, *An. stephensi* as target species.

Scholte *et al.* (2005) reduced the longevity of adult female *An. gambiae* mosquitoes to 3.49 days from 9.30 days by applying the spores of *M. anisopliae*, which is similar to the present study. Blanford *et al.* (2005) for the first time used the impregnated spores of *M. anisopliae* for interrupting the

Table 1. Larval and pupal toxicity effect of *A. alnifolia* methanol leaf extract against lymphatic filarial vector, *C. quinquefasciatus*

Mosquito larval instars and pupae	% of larval and pupal mortality					LC_{50} (LC_{90})	95% confidence limit		x^2 (df = 4)
	Concentration of *A. alnifolia* (%)						LFL	UFL	
	4	6	8	10	12		LC_{50} (LC_{90})	LC_{50} (LC_{90})	
1st Instar	41a	53a	62a	73a	91a	5.67 (13.03)	4.68 (11.78)	6.41 (15.01)	3.77*
2nd Instar	38ab	46b	55b	67b	78b	6.62 (16.18)	5.51 (14.10)	7.47 (19.98)	0.51*
3rd Instar	34b	41c	51b	64b	71b	7.53 (17.69)	6.53 (15.20)	8.43 (22.41)	0.35*
4th Instar	29c	36d	43c	55c	64c	9.05 (20.13)	8.07 (16.90)	10.29 (26.68)	0.27*
Pupa	18d	31e	38d	53c	56d	10.20 (19.81)	9.29 (16.97)	11.54 (25.11)	1.77*

Control-Nil mortality, *LFL* = Lower Fiducidal Limit, *UFL* = Upper Fiducidal Limit, x^2–*Chi*-square value, *df* - degrees of freedom, Within a column means followed by the same letter(s) are not significantly different at 5% level by DMRT. *Significant at $P < 0.05$ level.

Table 2. Larval and pupal toxicity effect of microbial insecticide, *M. anisopliae* against lymphatic filarial vector, *C. quinquefasciatus*

Mosquito larval instars and pupae	% of larval and pupal mortality: Concentration of *M. anisopliae* (conidia/ml/liter)					LC_{50} (LC_{90})	95% confidence limit: LFL	95% confidence limit: UFL	x^2 (df = 4)
	1×10^{2}	2×10^{4}	3×10^{6}	4×10^{8}	5×10^{10}		LC_{50} (LC_{90})	LC_{50} (LC_{90})	
1st Instar	38[a]	51[a]	58[a]	71[a]	86[a]	10.53 (57.70)	4.72 (48.89)	14.97 (72.18)	0.94*
2nd Instar	35[ab]	47[ab]	53[ab]	65[b]	74[b]	15.57 (78.54)	8.69 (63.55)	21.10 (107.40)	1.48*
3rd Instar	31[bc]	43[b]	47[c]	58[c]	67[c]	23.06 (94.17)	16.56 (74.16)	29.92 (136.22)	1.59*
4th Instar	27[cd]	35[c]	46[bc]	53[c]	59[d]	31.36 (108.18)	24.59 (83.43)	41.34 (163.74)	2.49*
Pupa	22[d]	31[c]	39[d]	47[d]	51[e]	42.54 (125.94)	33.83 (94.56)	59.93 (202.94)	2.77*

Control-Nil mortality, *LFL* = Lower Fiducidal Limit, *UFL* = Upper Fiducidal Limit, x^2–*Chi*-square value, *df* - degrees of freedom, Within a column means followed by the same letter(s) are not significantly different at 5% level by DMRT. *Significant at $P < 0.05$ level.

Table 3. Combined treatment of larval and pupal toxicity effect of *A. alnifolia* of methanol leaf extract and microbial insecticide, *M. anisopliae* against lymphatic filarial vector, *C. quinquefasciatus*

Mosquito larval instars and pupae	% of larval and pupal mortality: Concentration of *A. alnifolia* (%) + *M. anisopliae* (conidia/ml/liter)					LC_{50} (LC_{90})	95% confidence limit: LFL	95% confidence limit: UFL	x^2 (df = 4)
	1.0 + 1×10^{2}	1.2 + 1×10^{4}	1.4 + 1×10^{6}	1.6 + 1×10^{8}	1.8 + 1×10^{10}		LC_{50} (LC_{90})	LC_{50} (LC_{90})	
1st Instar	47[a]	61[a]	71[a]	82[a]	96[a]	3.73 (10.90)	2.54 (9.86)	4.57 (12.47)	3.41*
2nd Instar	42[b]	50[b]	65[b]	76[b]	84[b]	4.72 (13.83)	3.44 (12.19)	5.63 (16.56)	0.41*
3rd Instar	39[b]	48[b]	56[c]	70[c]	79[c]	5.55 (15.87)	4.29 (13.71)	6.49 (19.76)	0.55*
4th Instar	28[c]	39[c]	48[d]	61[d]	68[d]	7.66 (18.26)	6.71 (15.60)	8.67 (23.15)	0.25*
Pupa	20[d]	30[d]	45[d]	54[e]	60[e]	9.16 (19.26)	8.25 (16.49)	10.38 (24.26)	1.35*

Control-Nil mortality, *LFL* = Lower Fiducidal Limit, *UFL* = Upper Fiducidal Limit, x^2–*Chi*-square value, *df* - degrees of freedom, Within a column means followed by the same letter(s) are not significantly different at 5% level by DMRT. *Significant at $P < 0.05$ level.

malaria transmission in Tanzania and reduced the transmission by a factor of 80. The fungal cells developing within the insects may possess an outer coat, which is neutral to circulating heamocytes or they are effectively masked by host proteins or by producing immuno-modulating substances which suppress the cellular defence mechanism, the fungal cells may be tolerant to the humoral and cellular defence system of the insects. The *M. anisopliae* showed to be pathogenicity of larvae of *Cx. quinquefasciatus*, of the mosquito larvae when exposed to 1 x 10^6 dry conidia. For the successful conidial attachment and in the end, killing of a mosquito, a threshold number of conidia per unit surface area are required. In our lethal dose response experiment the lowest dose resulting in a significant effect on mosquito survival was 1 x 10^8conidia/ml. The results of this study show that laboratory condition is more significant to the field (Scholte *et al.* 2003).

Kamalakannan *et al.* (2008) proved that the entomopathogenic fungus, *M. anisopliae* is being considered as a biocontrol agent for the adult mosquito of *A. stephensi*. In our results, 96% and 94% adult mortality was observed in oil and water formulated conidia of *M. anisopliae*. Similarly, adult emergency rate also decreased with increasing concentration (1x10^8conidia/ml). Finally, we conclude that the fungal spores or cells developed within insect cuticle which suppresses the cellular defence system and also fungal growth on the legs and wings to arrest the mosquito movement. Recently, Kamalakannan and Murugan (2011) investigations were undertaken on 10 microbial product to develop a strategy to control mosquito larval and pupal population in the laboratory and field. Highest larval mortality was evident in the lab with LC_{50} and LC_{90} at 0.25 and 0.5 at 24 h for *Ae. aegypti* as observed for the the larvae of *C. quinquefasciatus* were more susceptible than the larvae of *A. stephensi* and *A. aegypti* (Mohanty *et al.*, 2008).

In conclusion, the evaluation of larvicidal, pupicidal activity of *M. anisopliae* and *A. alnifolia* against the vector *Culex quinquefasciatus* depicted as a good biocontrol agent Entomopathogenic fungi are considered excellent candidates for bio-pesticides due to their safety, relatively limited host range, ease of production and suitability of large scale production.

ACKNOWLEDGMENTS

The authors are thankful to the Department of Science and Technology (DST), Govt. of India, New Delhi, India and Tamil Nadu State Council for Science and Technology (TNSCST), Chennai, Tamil Nadu for providing financial support for the present work. The authors are grateful to Mr. N. Muthukrishnan, Technician and Mr. A. Anbarasan, Lab Assistant, National Centre for Diseases Control (NCDC), Mettupalayam, Tamil Nadu for helping in mosquito sample collection and in the identification of mosquito species of samples provided for the experiment work.

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Received: October 17, 2011 Revised: January 31, 2012 Accepted: February 3, 2012