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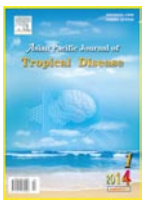

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Mosquitocidal properties of *Morinda citrifolia* L. (Noni) (Family: Rubiaceae) leaf extract and *Metarhizium anisopliae* against malaria vector, *Anopheles stephensi* Liston. (Diptera: Culicidae)

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PEER REVIEW

Peer reviewer

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Comments

This is a good study in which the authors have evaluated *M. citrifolia* leaf extract and *M. anisopliae* individually and in combination against *An. stephensi* under laboratory condition. The results have demonstrated that combined treatment of insecticide was highly effective on medically important vector mosquitoe, *An. stephensi*. This study provided a suitable alternative of synthetic insecticides for the mosquito vector management.

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ABSTRACT

Objective: To evaluate the mosquito larvicidal and pupicidal activity of the ethanolic extracts from *Morinda citrifolia* (*M. citrifolia*) plant and entomopathogenic fungi *Metarhizium anisopliae* (*M. anisopliae*) against malaria vector, *Anopheles stephensi* (*An. stephensi*).

Methods: *M. citrifolia* leaves were collected in and around Alleppy districts, Kerala, India. *M. citrifolia* leaf was washed with tap water and shade dried at room temperature. An electrical blender powdered the dried plant leaves. A total of 500 g leaf powder was macerated with 1.5 L of ethanol sequentially for a period of 72 h and filtered. The crude plant extracts were evaporated to dryness in rotary vacuum evaporator. The larvicidal and pupicidal activity was assayed at various concentrations ranging from 100 to 500 mg/L under the laboratory conditions. The LC_{50} and LC_{90} values of the *M. citrifolia* leaf extract and *M. anisopliae* fungi were determined by Probit analysis.

Results: The plant extract showed larvicidal and pupicidal effects after 24 and 48 h of exposure; all larval instars and pupae have considerably moderate mortality; however, the highest larval and pupal mortality appeared in combined treatment at 24 and 48 h. The LC_{50} and LC_{90} values of *M. citrifolia* and *M. anisopliae* and their combined treatment against the first to fourth instars larvae and pupae of the malaria vector were assessed. *M. citrifolia* had values of LC_{50}=202.47, 95.75, 57.52, 18.30 and 97.78 mg/L; LC_{90}=384.37, 482.91, 631.22, 757.55 and 944.96 mg/L at 48 h. *M. anisopliae* had values of LC_{50}=1.40, 3.99, 5.56, 8.77 and 11.49%; LC_{90}=13.84, 17.62, 22.20, 25.71 and 30.78% at 48 h; Combined treatment had values of LC_{50}=3.71, 16.73, 29.71, 40.60 and 138.10 mg/L; LC_{90}=122.29, 150.15, 156.90, 211.99 and 806.67 mg/L at 48 h, respectively.

Conclusions: The plant and the fungi are promising larvicidal and pupicidal agents against malaria vector, *An. stephensi*. This is a new eco-friendly approach for the control of vector. Therefore, this study provides first report on the combined treatment of this plant extract and fungi from India.

KEYWORDS

Morinda citrifolia, *Anopheles stephensi*, *Metarhizium anisopliae*, larvicidal and pupicidal activity, combined treatment.

1. Introduction

Malaria is a major global health problem. It is estimated that 247 million malaria cases with almost half of the global population are at risk and nearly a million deaths occur each year[1]. Among the 109 malaria endemic countries, India had

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1.5 million confirmed malaria cases in 2009 with over 1,000 deaths[2]. *Anopheles stephensi* (*An. stephensi*) is the primary vector of malaria in India and other West Asian countries, and improved methods of control are urgently needed[3,4]. Malaria caused by *Plasmodium falciparum*, is one of the leading causes of human morbidity and mortality from infectious diseases, predominantly in tropical and subtropical countries[5].

Traditionally, plants and their derivatives were used to kill mosquitoes and other household and agricultural pests. In all probability, these plants used to control insects contained insecticidal phytochemicals that were predominantly secondary compounds produced by plants to protect themselves against herbivorous insects[6].

Morinda citrifolia L. (Noni) (*M. citrifolia*), also known as Indian mulberry, belongs to family Rubiaceae. *M. citrifolia* fruit has a long history of use as a food in tropical regions throughout the world. Written documentation of the consumption of this fruit as a food source precedes the twentieth century. Captain James Cook of the British Navy noted in the late 1700′s that the fruit was eaten in Tahiti[7]. It mainly contains saponins, tannins, triterpenes, alkaloids, flavonoids. It is mainly used for the bowel disorders, including arthritis, atherosclerosis, bladder infections, boils, burns, cancer, chronic fatigue syndrome, circulatory weakness, cold, congestion, constipation, diabetes, eye inflammations, fever, fractures, gastric ulcers, gingivitis, headaches, heart diseases, hypertension, immune weakness, indigestion, intestinal parasites, kidney disease, malaria, menstrual cramps, mouth sores, respiratory disorders, ringworms, sinusitis, sprains, stroke, skin inflammation and wounds[8].

Purification of a *n*-BuOH-soluble partition of the MeOH extract of *M. citrifolia* (Noni) fruits led to the isolation of two new iridoid glucosides, 6 alpha-hydroxyadoxoside and 6 beta, 7 beta-epoxy-8-epi-splendoside, as well as 17 known compounds, americanin A, narcissoside, asperuloside, asperulosidic acid, borreriagenin, citrifolinin B epimer a, citrifolinin B epimer b, cytidine, deacetylasperuloside, dehydromethoxygaertneroside, epi-dihydrocornin, d-glucose, d-mannitol, methyl alpha-d-fructofuranoside, methyl beta-d-fructofuranoside, nicotifloroside, and beta-sitosterol 3-O-beta-d-glucopyranoside. The structures of the new compounds were determined by spectroscopic data interpretation. Compound 4, borreriagenin, cytidine, deacetylasperuloside, dehydromethoxygaertneroside, epi-dihydrocornin, methyl alpha-d-fructofuranoside, and methyl beta-d-fructofuranoside were isolated for the first time from *M. citrifolia*[9].

Metarhizium anisopliae (*M. anisopliae*) and *Beauveria bassiana* (*B. bassiana*) are two of the most widely used hyphomycete species for insect pest control. They are ubiquitous worldwide and comprise a large number of different strains and isolates of different geographical origins and host specificities[10]. Under natural conditions, *Metarhizium* and *Beauveria* are found in the soil where moist conditions allow filamentous growth and the production of infectious spores, called conidia, which infect soil-dwelling insects upon contact. Fungal sporulation was observed in more than 95% of mosquito cadavers in the treatment groups. The results indicate that *M. anisopliae* IP 46 has the potential to be a bio-control agent for African malaria vector species, and is a suitable candidate for further research and development[11].

Under suitable moist conditions they can germinate and produce germ tubes that penetrate the insect cuticle using mechanical pressure and cuticle-degrading enzymes[12]. The effect of relative humidity (43%, 75%, 86% and >98%) on *Aedes aegypti* (*Ae. aegypti*) eggs treated with *M. anisopliae* or water only was tested for up to a six months with exposure at 25 °C. Survival of larvae inside eggs was clearly affected by the lowest humidity (43%) tested, and eclosion diminished at all humidities after increasing periods of exposure[13]. The impact of persistence of entomopathogenic fungi on insects and on filage has not been extensively studied. Conidia of hyphomycetous fungi strongly adhere to insect cuticle, and the attachment of conidia to cuticles is through involving nonspecific adhesion mechanisms mediated by the hydrophobicity of the cell wall[14]. Entomopathogenic fungi, *M. anisopliae* and *B. bassiana*, are promising bio-pesticides for application against adult malaria mosquito vectors[15].

The fungus multiplies within the insect; death is due to toxin production by the fungus or multiplication to inhabit the entire insect. Under favourable environmental condition, the fungus grows out of the cadaver, and forms conidiophores or analogous structure and sporulates. Alternatively, many species form some types of resting stages capable of forming or releasing a type of spore. Spores need new hosts, so the fungus needs a strategy for dissemination. Therefore, the important point is that the environment and host are crucial to the survival and reproduction of the fungus. Insect pathogens have a long history of recognition despite the relatively recent understanding of microbial infections.

The present study would be useful in promoting research aiming at the development of new agent for mosquito control based on plant source of natural products. In view of the recent increased interest in developing plant-based insecticides as an alternative to chemical insecticides, this study was undertaken to assess the mosquitocidal properties of *M. citrifolia* leaf extracts and fungi, *M. anisopliae* against the medically important malaria vector, *An. stephensi*.

2. Materials and methods

2.1. Collection of plants and preparation of extracts

The *M. citrifolia* plants were collected from in and around Alleppy (sea sources) districts in Kerala, India. The plants were identified by Taxonomist, Department of Botany, University of Madras, Chennai, Tamil Nadu, India. *M. citrifolia* leaves were washed with tap water and shade dried at room temperature (28 ±2 °C) for 10 to 15 d. The air-dried plant leaves were powdered by an electrical blender. A total of 500 g leaf powder was macerated with 1.5 L of ethanol sequentially for a period of 72 h and filtered. The yield of extracts was 14.68 g. The extracts were concentrated at reduced temperature in a rotary vacuum evaporator and stored at a temperature of 4 °C. One gram of the plant residue was dissolved in 100 mL of acetone (stock solution), which was considered as 1% stock solution. From this stock solution different concentrations were prepared ranging from 100, 200, 300, 400 and 500 mg/L, respectively.

2.2. Fungal bioassay

Entomopathogenic fungi, *M. anisopliae* (Metsch.) were supplied by T-Stanes & Company Ltd., Research and Development Centre, Coimbatore, Tamil Nadu, India. The

required quantity of entmopathogenic fungi, *M. anisopliae* liquid formulation was thoroughly mixed with distilled water to prepare various conidia concentrations ranging from 1×10^2 to 5×10^{10} viable conidia/mL, respectively.

2.3. Mosquito culture

The eggs of *An. stephensi* were collected from National Centre for Disease Control field station, Mettupalayam, 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 feed with pedigree dog biscuits and yeast at 3:1 ratio. The feeding was continued until the larvae transformed into pupal stage. The pupae were collected from the culture trays and transferred to plastic containers (12 cm×12 cm) containing 500 mL 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) °C, 75%-85% relative humidity, under a photoperiod of 14:10 (light/dark). A 10% sugar solution was provided for a period of 3 d before blood feeding. The adult female mosquitoes were allowed to feed on the blood of a rabbit (one 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. Larval and pupal toxicity test

Twenty five numbers of first to fourth instars larvae and pupae were introduced into 500 mL glass beaker containing 249 mL of dechlorinated water and 1 mL of desired concentrations of leaf extract and fungi was added. Larval food was given to the test larvae. At each tested concentration two to five trials were made and each trial consisted of five replicates. The control was setup by mixing 1 mL of acetone with 249 mL of dechlorinated water. The larvae and pupae were exposed to dechlorinated water without acetone which was served as blank control. The control mortalities were corrected by using Abbott's formula[16].

The LC_{50} and LC_{90} were calculated from toxicity data by using probit analysis[17].

2.5. Statistical analysis

All data were subjected to analysis of variance. The means were separated using Duncan's multiple range tests by Alder and Rossler[18]. The average larval and pupal mortality data were subjected to probit analysis for calculating LC_{50}, LC_{90}, and other statistics at 95% fiducial limits of upper fiducial limit and lower fiducial limit. *Chi*-square values were calculated using the SPSS statistical software package 13.0 version. Results with $P<0.05$ were considered to be statistically significant.

3. Results

The present study investigated the mortality effects of ethanol leaf extract of *M. citrifolia*, *M. anisopliae* and their combinations against *An. stephensi* at 24 and 48 h, respectively (Tables 1-3). The *M. citrifolia* were studied and used as ecofriendly

Table 1

Mortality effects of ethanol leaf extract of *M. citrifolia* against different larval instars and pupae of *An. stephensi* treated for 24 and 48 h.

Concentrations (mg/L)	Hours	First instars	Second instars	Third instars	Fourth instars	Pupae
100	24	42.70±2.21^{e}	35.50±2.88^{e}	28.50±2.98^{e}	25.70±2.21^{e}	22.50±2.08^{e}
	48	74.20±2.75^{d}	64.50±2.64^{d}	59.70±2.62^{d}	54.70±2.21^{d}	50.70±3.77^{d}
200	24	54.50±2.08^{de}	48.50±2.64^{de}	42.20±2.16^{de}	34.00±1.82^{de}	30.50±2.08^{de}
	48	80.20±2.50^{cd}	74.50±2.08^{c}	67.20±2.90^{c}	59.00±3.16^{cd}	51.70±1.70^{cd}
300	24	75.70±1.70^{c}	71.50±1.29^{cd}	64.70±2.50^{cd}	57.50±2.64^{cd}	55.50±2.38^{c}
	48	89.20±2.50^{b}	85.20±1.70^{b}	78.70±2.98^{bc}	74.00±2.94^{bc}	66.70±3.7^{7bc}
400	24	85.00±2.16^{bc}	81.70±1.70^{bc}	73.70±2.75^{c}	72.50±2.08^{c}	65.20±2.50^{bc}
	48	88.70±2.21^{b}	86.00±2.58^{b}	82.70±2.21^{b}	77.50±2.38^{b}	69.20±1.70^{b}
500	24	92.50±2.08^{ab}	89.20±2.50^{ab}	83.20±2.75^{ab}	78.70±2.21^{ab}	73.20±2.75^{ab}
	48	94.00±1.82^{a}	89.00±2.16^{a}	81.70±1.70^{a}	76.00±1.82^{a}	70.50±2.64^{a}

Control: Nil mortality. Data followed by the same letter(s) within rows indicates no significant difference by Duncan's multiple range test.

Table 2

Mortality effects of entomopathogenic fungi, *M. anisopliae* against different larval instars and pupae of *An. stephensi* treated for 24 and 48 h.

Concentrations (conidia/mL)	Hours	First instars	Second instars	Third instars	Fourth instars	Pupae
1×10^2	24	40.00±2.58^{e}	31.70±2.50^{e}	27.00±1.82^{e}	23.50±2.08^{e}	20.20±2.21^{e}
	48	67.50±2.64^{d}	58.20±2.75^{d}	52.70±2.21^{d}	43.00±2.58^{d}	36.70±2.75^{d}
2×10^4	24	58.20±2.21^{de}	51.00±1.82^{cd}	44.70±2.50^{de}	37.20±2.75^{de}	27.70±1.70^{de}
	48	89.50±3.10^{bc}	82.50±2.08^{bc}	73.00±2.58^{c}	66.20±2.50^{c}	56.70±2.75^{c}
3×10^6	24	80.50±2.08^{c}	72.70±2.75^{c}	62.20±2.21^{cd}	55.50±3.10^{cd}	48.00±2.94^{cd}
	48	92.00±1.82^{b}	81.00±3.36^{b}	76.00±2.58^{bc}	66.50±3.69^{c}	59.20±1.70^{c}
4×10^8	24	92.50±2.50^{ab}	89.20±1.70^{ab}	81.00±2.58^{ab}	73.20±2.21^{bc}	71.20±2.75^{bc}
	48	100.00±0.00^{a}	100.00±0.00^{a}	90.70±2.21^{b}	83.00±1.82^{b}	75.50±2.98^{b}
5×10^{10}	24	100.00±0.00^{a}	100.00±0.00^{a}	100.00±0.00^{a}	86.50±2.08^{ab}	83.70±3.09^{ab}
	48	100.00±0.00^{a}	100.00±0.00^{a}	100.00±0.00^{a}	93.20±0.00^{a}	91.00±2.58^{a}

Control: Nil mortality. Data followed by the same letter(s) within rows indicates no significant difference by Duncan's multiple range test.

Table 3
Mortality effects of ethanol leaf extract of *M. citrifolia* and *M. anisopliae* against different larval instars and pupae of *An. stephensi* treated for 24 and 48 h.

Concentrations	Hours	First instars	Second instars	Third instars	Fourth instars	Pupae
50 mg/L+1×10^2 conidia/mL	24	45.00±3.00^e	42.60±3.05^e	35.30±2.51^e	32.30±1.52^e	26.00±3.60^e
	48	74.60±2.51^c	70.00±2.00^c	65.00±2.00^c	61.60±1.52^c	56.00±3.00^{cd}
100 mg/L+1×10^4 conidia/mL	24	60.00±3.00^d	53.00±2.00^d	50.60±3.05^d	42.60±2.08^d	35.30±2.51^d
	48	87.00±2.00^b	77.30±1.52^b	76.60±2.51^b	67.00±2.64^b	58.30±3.05^c
150 mg/L+1×10^6 conidia/mL	24	76.30±2.51^{bc}	72.00±2.64^{bc}	66.00±3.00^{bc}	60.60±3.05^{bc}	53.20±2.08^{bc}
	48	94.00±0.00^{ab}	89.00±0.00^{ab}	87.00±0.00^{ab}	75.00±1.52^{ab}	67.00±3.60^b
200 mg/L+1×10^{8}conidia/mL	24	100.00±0.00^a	100.00±0.00^a	82.00±0.00^b	90.30±2.51^{bc}	68.00±2.00
	48	100.00±0.00^a	100.00±0.00^a	100.00±0.00^a	91.60±2.08^b	78.30±3.05^b
250 mg/L+1×10^{10}conidia/mL	24	100.00±0.00^a	100.00±0.00^a	100.00±0.00^a	100.00±0.00^a	86.00±3.60^{ab}
	48	100.00±0.00^a	100.00±0.00^a	100.00±0.00^a	100.00±0.00^a	93.00±2.64^a

Control: Nil mortality. Data followed by the same letter(s) within rows indicates no significant difference by Duncan′s multiple range test.

Table 4
Lethal concentration values of ethanol leaf extract of *M. citrifolia* against different larval instars and pupae of *An. stephensi* treated for 24 and 48 h.

Mosquito life stages	Exposure hours	Regression equation	LC_{50} (mg/L) (LFL–UFL)	LC_{90} (mg/L) (UFL–UFL)	x^2 (*df*=4)
First instars	24	Y= −0.637 62+0.004 19x	152.05 (105.98–186.32)	457.66 (413.26–522.31)	0.88^a
	48	Y= −0.442 17+0.002 18x	202.47 (45.71–613.16)	384.37 (17.18–316.70)	1.15^a
Second Instars	24	Y= −0.802 49+0.004 22x	190.22 (151.42–220.98)	494.00 (447.14–562.01)	1.21^a
	48	Y= −0.212 06+0.002 21x	95.75 (22.96–368.84)	482.91 (403.39–650.92)	1.73^a
Third Instars	24	Y= −0.932 38+0.003 93x	237.43 (202.21–267.56)	563.77 (507.05–648.55)	1.45^a
	48	Y= −0.107 04+0.001 86x	57.52 (62.62–361.01)	631.22 (508.57–941.97)	2.29^a
Fourth instars	24	Y= −0.003 94+1.077 17x	273.12 (241.45–302.65)	598.06 (537.75–688.19)	2.24^a
	48	Y= −0.031 73+0.001 73x	18.30 (121.79–242.45)	757.55 (596.55–1192.10)	3.15^a
Pupae	24	Y= −1.118 27+0.003 66x	305.25 (273.11–337.78)	655.09 (583.16–766.44)	3.07^a
	48	Y= −0.147 92+0.001 51x	97.78 (146.56–190.14)	944.96 (711.63–1684.17)	2.14^a

LC_{50}: Lethal concentration that kills 50% of the exposed larvae and pupae, LC_{90}: Lethal concentration that kills 90% of the exposed larvae and pupae, LFL: Lower fiducial limit, UFL: Upper fiducial limit, x^2: *Chi*–square value, *df*: Degrees of freedom, ^{a}: Significant at $P<0.05$ level.

Table 5
Lethal concentration values of entomopathogenic fungi, *M. anisopliae* against different larval instars and pupae of *An. stephensi* treated for 24 and 48 h.

Mosquito life stages	Exposure hours	Regression equation	LC_{50} (%) (LFL–UFL)	LC_{90} (%) (UFL–UFL)	x^2 (*df*=4)
First instars	24	Y= −1.959 01+0.103 51x	9.26 (7.46–10.71)	21.64 (19.90–23.96)	2.74^a
	48	Y=−0. 145 17+0.103 08x	1.40 (3.05–4.06)	13.84 (12.14–16.04)	5.25^a
Second Instars	24	Y= −1.205 18+0.105 88x	11.38 (9.87–12.67)	23.48 (21.72–25.80)	4.01^a
	48	Y= −0.375 68+0.094 04x	3.99 (0.37–110.39)	17.62 (11.50–86.17)	16.16^a
Third Instars	24	Y= −1.328 54+0.099 40x	13.36 (8.25–16.99)	26.25 (21.67–37.27)	8.99^a
	48	Y= −0.076 99+0.428 28x	5.56 (10.61–10.68)	22.20 (17.30–36.79)	7.80^a
Fourth instars	24	Y= −1.362 25+0.088 24x	15.43 (13.96 −16.81)	29.96 (27.61–33.15)	4.44^a
	48	Y= −0.075 63+0.663 39x	8.77 (14.66–16.71)	25.71 (19.15–63.06)	14.11^a
Pupae	24	Y= −1.438 13+0.080 31x	17.90 (16.39–19.41)	33.86 (31.01–37.84)	2.00^a
	48	Y= −0.764 07+0.764 07x	11.49 (2.04–16.06)	30.78 (24.36–51.15)	7.33^a

LC_{50}: Lethal concentration that kills 50% of the exposed larvae and pupae, LC_{90}: Lethal concentration that kills 90% of the exposed larvae and pupae, LFL: Lower fiducial limit, UFL: Upper fiducial limit, x^2: *Chi*–square value, *df*: Degrees of freedom, ^{a}: Significant at $P<0.05$ level.

Table 6
Lethal concentration values of methanol leaf extract of *M. citrifolia* and *M. anisopliae* against different larval instars and pupae of *An. stephensi* treated for 24 and 48 h.

Mosquito life stages	Exposure hours	Regression equation	LC_{50} (mg/L) (LFL–UFL)	LC_{90} (mg/L) (UFL–UFL)	x^2 (*df*=4)
First instars	24	Y= −0.966 02+0.011 92x	81.03 (37.27–122.80)	188.54 (144.18–354.48)	14.22^a
	48	Y= −0.037 83+0.010 17x	3.71 (26.68–58.80)	122.29 (104.88–3.70))	2.49^a
Second Instars	24	Y= −1.981 63+0.011 56x	84.89 (30.73–114.99)	195.72 (161.67–270.12)	6.97^a
	48	Y= −0.160 71+0.009 61x	16.73 (70.01–245.21)	150.15 (107.17– 269.47)	7.68^a
Third Instars	24	Y= −1.036 21+0.009 54x	108.59 (39.82–146.73)	242.90 (195.72–373.26)	9.50^a
	48	Y= −0.299 40+0.010 08x	29.71 (77.01–157.98)	156.90 (117.05–265.66)	7.70^a
Fourth instars	24	Y= −1.324 69+0011 23x	117.99 (50.11–159.63)	232.15 (184.46–380.68)	13.87^a
	48	Y= −0.303 57+0.007 48x	40.60 (101.90–334.95)	211.99 (155.15–516.15)	10.81^a
Pupae	24	Y= −1.116 62+0.007 19x	155.19 (138.02–171.44)	333.31 (300.17–382.55)	0.73^a
	48	Y= −0.264 72+0.001 92x	138.10 (21.43–206.39)	806.67 (645.27–1193.78)	2.73^a

LC_{50}: Lethal concentration that kills 50% of the exposed larvae and pupae, LC_{90}: Lethal concentration that kills 90% of the exposed larvae and pupae, LFL: Lower fiducial limit, UFL: Upper fiducial limit, x^2: *Chi*–square value, *df*: Degrees of freedom, ^{a}: Significant at $P<0.05$ level.

insecticides instead. The LC_{50} and LC_{90} values against the first to fourth instars larvae and pupae for the control of malaria vector were calculated. *M. citrifolia* had values of LC_{50}=152.05, 190.22, 237.43, 273.12 and 305.25 mg/L at 24 h; 202.47, 95.75, 57.52, 18.30 and 97.78 mg/L at 48 h; and LC_{90} = 457.66, 494.00, 563.77, 598.06 and 655.09 mg/L at 24 h; 384.37, 482.91, 631.22, 757.55 and 944.96 mg/L at 48 h (Table 4). *M. anisopliae* had values LC_{50}=9.26, 11.38, 13.36, 15.43 and 17.90% at 24 h; 1.40, 3.99, 5.56, 8.77 and 11.49% at 48 h; LC_{90}=21.64, 23.48, 26.25, 29.96 and 33.86% at 24 h; 13.84, 17.62, 22.20, 25.71 and 30.78% at 48 h (Table 5). Combined treatment of *M. citrifolia* leaf extract and *M. anisopliae* had values of LC_{50} = 81.03, 84.89, 108.59, 117.99 and 155.19 mg/L at 24 h; 3.71, 16.73, 29.71, 40.60 and 138.10 mg/L at 48 h; and LC_{90} = 188.54, 195.72, 242.90, 232.15 and 333.31 mg/L at 24 h; 122.29, 150.15, 156.90, 211.99 and 806.67 mg/L at 48 h, respectively (Table 6).

4. Discussion

Malaria now is responsible for the estimated more than 300 million cases and one million deaths per year[19]. Dengue fever is a mosquito-borne disease of major global public health concern. It is endemic to tropical and subtropical countries, especially in the urban and suburban areas[20]. Mosquito control is being strengthened in many areas, but there are still many challenges, including an increasing mosquito resistance to insecticides and a lack of alternative, cost-effective, and safe insecticides. The effect of three citrus species and enantiomers of α- and β-pipenes were also studied against third instar larvae of *Culex pipenes*[21].

The direct and indirect contributions of such effects to treatment efficacy through reduced larval feeding and fitness need to be properly understood in order to improve the use of botanical insecticides against *An. stephensi*. Some naturally occurring insecticides may play a more prominent role in mosquito control programs in the future[22]. The methanolic extracts of *Solanum suratence*, *Azadirachta indica*, and *Hydrocotyl javanica* exhibited larvicidal activity against *Culex quinquefasciatus* (*Cx. quinquefasciatus*)[23]. Murugan and Jeyabalan have reported that the effect of some indigenous plants on the larvicide and ovipositional properties on *An. stephensi*[24].

Previous reports on extracts of *Psammaplysilla purpurea* and *Haliclona cribricutis* showed LC_{50} values of less than 50 mg/L against *Ae. aegypti*[25], whereas fucoidan derived from *Undaria pinnatifida* seaweed showed LC_{50} values of 9.17 μg/mL against *Plasmodium falciparum*[26]. The leaf extract of *Amelanchier alnifolia* (*A. alnifolia*) with different solvents-hexane, chloroform, ethyl acetate, acetone and methanol were tested for larvicidal activity against malaria vector. The early fourth instar larvae of *An. stephensi* had values of LC_{50}=197.37, 178.75, 164.34, 149.90 and 125.73 mg/L and LC_{90}=477.60, 459.21, 435.07, 416.20, and 395.50 mg/L, respectively. The results of the leaf extract of *A. alnifloia* are promising as good larvicidal activity against the mosquito vector, *An. stephensi*[27]. Earlier authors reported that the third larvae of *An. stephensi* had values of LC_{50}=345.10, 324.26, 299.97, 261.96, and 284.59 mg/L and LC_{90}=653.00, 626.58, 571.89, 505.06, and 549.51 mg/L; *Ae. aegypti* had values of LC_{50}=361.75, 343.22, 315.40, 277.92, and 306.98 mg/L and LC_{90}=687.39, 659.02, 611.35, 568.18, and 613.25 mg/L and *Cx. quinquefasciatus* had values of LC_{50}=382.96, 369.85, 344.34, 330.42, and 324.64 mg/L and LC_{90}=726.18, 706.57, 669.28, 619.63, and 644.47 mg/L, respectively. The results of the leaf extract of *M. citrifolia* are promising as good larvicidal agent against the mosquito vectors *An. stephensi*, *Ae. aegypti*, and *Cx. quinquefasciatus*[28].

In a study of *Calotropis procera* against *An. stephensi* we observed more than 95% mortality after 24 h at 256 mg/L. Tests with latex showed 99% mortality at 64 mg/L for *An. stephensi*, only 44% mortality against *Cx. quinquefasciatus* and a maximum mortality of 67% at 256 mg/L were observed, respectively[29]. Sharma *et al.*[30] reported that the acetone extract of *Nerium indicum* and *Thuja orientelis* has been studied with LC_{50} values of 200.87, 127.53, 209.00, and 155.97 mg/L against third instars larvae of *An. stephensi* and *Cx. quinquefasciatus*, respectively. Mathew N *et al.*[31] have reported that leaf chloroform extracts of *Nyctanthes arbortristis* showed lethal values LC_{50}=526.3 and 780.6 ppm (24 h) and LC_{50}=303.2 and 518.2 ppm (48 h) against *Ae. aegypti* and *An. stephensi*, respectively. Flower methanol extracts of the above plants showed lethal values LC_{50}=679.4 and 244.4 ppm and LC_{90}=1 071.3 and 433.7 mg/L against *An. stephensi* after 24 and 48 h, respectively. Larvicidal activity of flower methanol extract showed LC_{50} values 233.0 and 302.5 mg/L against *An. stephensi* and *Ae. aegypti*, respectively, after 48 h treatment. Methanol extract showed the lowest LD values against several instars of larvae and 50 adults (121.59, 142.73, 146.84, 202.98, 290.65, 358.42, and 300.03 μg/cm^2, respectively) which indicates the highest toxicity or insecticidal activity[32].

Larvicidal studies were carried out against *Cx. quinquefasciatus* and the results were compared with bulk permethrin. The LC_{50} of nanopermethrin and bulk permethrin to *Cx. quinquefasciatus* was 0.117 and 0.715 mg/L respectively[33]. Sakulku U, *et al.*[34] have reported the low release rate of nanoemulsion with large droplet size that resulted in prolonged mosquito repellant activity compared to the nanoemulsion with small droplet size. The plant extract showed larvicidal and pupicidal effects after 24 and 48 h of exposure; all larval instars and pupae have considerably moderate mortality; however, the highest larval and pupal mortality was methanol extract of *M. citrifolia* observed in three mosquito vectors at 48 h. In a study of *M. citrifolia* against the first to fourth instar larvae and pupae against mosquito vectors, *An. stephensi*, the plant had values of LC_{50}=146.08, 159.07, 172.16, 185.08 and 202.68 mg/L at 24 h; 117.83, 133.07, 139.44, 146.04 and 149.58 mg/L at 48 h; and LC_{90}=322.12, 363.48, 388.56, 436.51 and 513.56 mg/L at 24 h; 281.22, 310.16, 329.70, 361.74 and 419.19 mg/L at 48 h, respectively[35]. In the present results, *M. citrifolia* ethanol leaf extract had values of LC_{50}=152.05, 190.22, 237.43, 273.12 and 305.25 mg/L at 24 h; 202.47, 95.75, 57.52, 18.30 and 97.78 mg/L at 48 h; and LC_{90}= 457.66, 494.00, 563.77, 598.06 and 655.09 mg/L at 24 h; 384.37, 482.91, 631.22, 757.55 and 944.96 mg/L at 48 h against *An. stephensi*, respectively.

Scholte *et al.*[36] have reported that reduced the longevity of adult female *Anopheles gambiae* mosquitoes to 3.49 d from 9.30 d by applying the spores of *M. anisopliae*, which was similar to the present study. Blanford *et al.*[37] studied for the

first time using the impregnated spores of *M. anisopliae* for interrupting the malaria transmission in Tanzania and reduced the transmission by a factor of 80. Biological control at the larval stages of development of mosquitoes is one of the techniques which is cheap, easy to use and environmental friendly. Natural insecticides are phytotoxic and do not accumulate chemical residue in the flora, fauna and soil. Furthermore, *M. anisopliae* and *B. bassiana* kill mosquitoes in a slower manner than insecticides kill insecticide-susceptible mosquito populations[15,38,39].

The resistant VKPER strain was significantly more susceptible to fungal infection than the insecticide-susceptible SKK strain. Furthermore, *B. bassiana* was significantly more virulent than *M. anisopliae* for both mosquito strains, although this may be linked to the different viabilities of these fungal species. The viability of both fungal species decreased significantly one day after application onto polyester netting when compared to the viability of conidia remaining in suspension[40]. 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 1×10^8 conidia/mL. In order to achieve the highest possible impact of the fungus on the mosquito population, it was desirable that the other pathways besides the primary mode of contamination are utilized. The results of this study show that laboratory condition is more significant to the field[41].

Bacillus thuringiensis, the LC_{50} values of first to fourth larval instars and pupae of *An. stephensi* were 37.24, 45.41, 57.82, 80.09, and 98.34 mg/L; *Ae. aegypti* values were 42.38, 51.90, 71.02, 96.17, and 121.59 mg/L; and *Cx. quinquefasciatus* values were 55.85, 68.07, 94.11, 113.35, and 133.87 mg/L. *Bacillus spaericus* was tested against the first to fourth instars larvae and pupae, which had the LC_{50} and LC_{90} values represented as follows: LC_{50}=0.051, 0.057, 0.062, 0.066 and 0.073% and the LC_{90}=0.114, 0.117, 0.120, 0.121 and 0.142%, respectively[42,43]. Spinosad tested against the *An. stephensi* had values of LC_{50}=384.19, 433.39, 479.17, 519.79, and 572.63 mg/L, and *Ae. aegypti* had values of LC_{50}=210.68, 241.20, 264.93, 283.27, and 305.85 mg/L, respectively[44]. Microbial insecticide, *M. anisopliae* was tested against the first to fourth instars larvae and pupae with values of LC_{50}=7.917, 10.734, 17.624, 26.590 and 37.908%, respectively[45]. In the present results, *M. anisopliae* had values LC_{50}=9.26, 11.38, 13.36, 15.43 and 17.90% at 24 h; 1.40, 3.99, 5.56, 8.77 and 11.49% at 48 h; LC_{90}=21.64, 23.48, 2625, 29.96 and 33.86% at 24 h; 13.84, 17.62, 2220, 25.71 and 30.78% at 48 h against *An. stephensi*, respectively.

The results from the current study showed that the daily survival rates of *M. anisopliae* infected adult as well as larval mosquitoes at any given moment in the mosquito life span, was lower than non-infected mosquitoes, and that their life span was reduced, provided that the conidia dose was high enough. Prospects for developing this adult and larvae mosquito control strategy are promising and may in due course be developed into a mosquito control tool. Kamalakannan *et al*.[39] proved that the entomopathogenic fungus, *M. anisopliae* was being considered as a biocontrol agent for the adult mosquito of *An. stephensi* (malarial vector). The present experiment was carried out in the laboratory with 30-50 male and female adult mosquitoes exposed to *M. anisopliae* (exposed to 1×10^6 conidia/mL of oil or water suspension). In our results, 96% and 94% adult mortality was observed in oil and water formulated conidia of *M. anisopliae*. Similarly, adult emergency rate was also decreased with increasing concentration (1×10^8 conidia/mL). Finally, we conclude that the fungal spores or cells developed within insect cuticle which suppress the cellular defence system and also fungal grow on the legs and wings to arrest the mosquito movement. Earlier, Kamalakannan and Murugan[46] investigations were undertaken on ten microbial products to develop a strategy to control mosquito larval and pupal population in the lab and field. The highest larval mortality was evident in the lab with LC_{50} and LC_{90} at 0.25 and 0.50 mg/L at 24 h for *Ae. aegypti*, respectively. The LC_{50} values of *Aspergillus flavus*, *Aspergillus parasiticus*, *Penicillium falicum*, *Fusarium vasinfectum* and *Trichoderma viride* were 38.34, 40.39, 44.97, 50.03 and 54.16 mg/L, respectively. Among the five different fungi, the culture filtrate of *A. flavus* was found to be more toxic than the other four species of fungi against *Cx. quinquefasciatus*[47].

A. alnifolia was tested against the first to fourth instars larvae and pupae and the values LC_{50}=5.388, 6.233, 6.884, 8.594 and 10.073%. Microbial insecticide, *M. anisopliae* was tested against the first to fourth instars larvae and pupae with values LC_{50}=7.917, 10.734, 17.624, 26.590 and 37.908%. Combined treatment of *A. alnifolia* and *M. anisopliae* gave values of LC_{50}=3.557, 4.373, 5.559, 7.223 and 8.542%, respectively. *A. alnifolia* and microbial insecticide, *M. anisopliae* are promising and good larvicidal and pupicidal agents against malaria fever mosquito, *An. stephensi*[45]. In the present results, combined treatment of *M. citrifolia* leaf extract and fungi, *M. anisopliae* gave values of LC_{50}=81.03, 84.89, 108.59, 117.99 and 155.19 mg/L at 24 h; 3.71, 16.73, 29.71, 40.60 and 138.10 mg/L at 48 h; and LC_{90} = 188.54, 195.72, 242.90, 232.15 and 333.31 mg/L at 24 h; 122.29, 150.15, 156.90, 211.99 and 806.67 mg/L at 48 h, against *An. stephensi*.

In conclusion, the larvicidal and pupicidal properties of *M. anisopliae* was showed to be a good bio-control agent against *An. stephensi*. Finally, we discussed about fungal pathogen *M. anisopliae* and *M. citrifolia* leaf extract interacting with mosquito as an attempt to control the mosquito in the laboratory level. This is a new eco-friendly approach for the vector control programs. Therefore, this study provides the first report on the mosquitocidal activity of combined treatment against malaria vector from India.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

M. citrifolia leaf extract and entomopthogenic fungi, *M. anisopliae* were against all larval instars and pupae of malaria vector mosquitoes under laboratory experiments. In the present study, additional scientific information in the combined treatment plant extract and fungi against malaria vector, *Anopheles stephensi* was assessed.

Research frontiers

The main cutting edge in this paper is the laboratory evaluation of *M. citrifolia* leaf extract and *M. anisopliae* individually and in combination against malaria vector mosquito of *An. stephensi*.

Related reports

Entomopathogenic fungi, *M. anisopliae* against vectors have been performed by Murugan *et al.*, 2012, and Kamalakannan *et al.*, 2011. Authors have taken note of earlier studies to carry out the experiments of laboratory in the vector species of mosquitoes.

Innovations & breakthroughs

The article is the report of combined treatment against vector mosquitoe of *An. stephensi* under the laboratory condition.

Applications

It is important to study this plant extract and fungi in detail against *An. stephensi*. In the present scenario of microbial insecticides developing resistance against vector mosquitoes, it has been important field of research to find out new sources. This study may lead to new control method of vector mosquito *An. stephensi*.

Peer review

This is a good study in which the authors have evaluated *M. citrifolia* leaf extract and *M. anisopliae* individually and in combination against *An. stephensi* under laboratory condition. The results have demonstrated that combined treatment of insecticide was highly effective on medically important vector mosquitoe, *An. stephensi*. This study provided a suitable alternative of synthetic insecticides for the mosquito vector management.

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