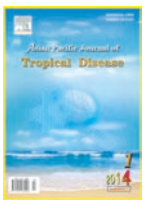
Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Disease

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



Document heading doi: 10.1016/S2222-1808(14)60442-4

Larvicidal, adulticidal, repellency and smoke toxic efficacy of *Ficus krishnae* against *Anopheles stephensi* Liston and *Culex vishnui* group mosquitoes

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PEER REVIEW

Peer reviewer

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Comments

A good initiative to look for alternative natural products for vector control. But needs more studies especially on characterization of active ingredients. Methodology used and interpretations were appropriate and results were conclusive.
Details on Page S219

ABSTRACT

Objective: To establish the plant *Ficus krishnae* as potential antimosquito agent.
Methods: Larvicidal and adulticidal efficacy of ethyl acetate and methanol extracts of leaves of *Ficus krishnae* were evaluated against 3rd instar larvae and adults of *Anopheles stephensi* and *Culex vishnui* group mosquito for 24 h. Smoke toxicity test along with test for repellency on adult forms of the two mosquitoes were performed by the methanol extract.
Results: The mortality rate varies in a dose dependent characteristic. The tests for larvicidal and adulticidal activity with both the solvent extracts showed significant efficiencies against the mosquitoes studied. The outcome of smoke toxicity test and repellency test were impressive.
Conclusion: The study reveals that the solvent extracts of *Ficus krishnae* could be an effective natural alternative to get control over the mosquito population.

KEYWORDS

Ficus krishnae, Solvent extract, Larvicide, Adulticide, Smoke toxicity, Repellency

1. Introduction

Despite the immense development of modern science in the field of human health concern, compared to pre-Dichloro-diphenyl-tricgloroethane era insufficient advancement till date has been achieved for prevention of mosquito borne diseases. The situation has worsened due to biological magnification of Dichloro-diphenyl-tricgloroethane like non-biodegradable chemicals in earth atmosphere with additional complication due to development of resistance in vector mosquitoes. *Anopheles stephensi* Liston (*An. stephensi*) is principal vector of a dreadful mosquito borne disease, malaria, in India and transmit the species of pathogenic protozoa *Plasmodium* during their blood meal on human host. Malaria is transmitted by different species of *Anopheles* mosquitoes in various regions and environments around the world[1]. India alone contributes 77% of total malaria cases among the countries in South-East Asia[2]. Another important acute viral infection, Japanese encephalitis is a parallel health threat and mainly transmitted by *Culex vishnui* (*Cx. vishnui*) group[3]. The fatality rate of Japanese encephalitis range

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Foundation Project: Supported by University Grant Commission with Grant no F.17-8/08 (SA-I).

Article history:
Received 2 Dec 2013
Received in revised form 8 Dec, 2nd revised form 17 Dec, 3rd revised form 21 Dec 2013
Accepted 14 Jan 2014
Available online 28 Jan 2014

from 0.3% to 60%, and 30% of the survivors suffer from lasting damage to the central nervous system, such as memory loss, impaired cognition, behavioral disturbances, convulsion, motor weakness or paralysis and abnormalities of tone and coordination. Beside this, in India, *Culex* mosquitoes, especially *Cx. vishnui* group are tentatively incriminated as vectors of West Nile virus usually responsible for a mild infection called West Nile fever in human and horses[4].

Various commercially available chemically synthesized insecticides and repellants like N,N-diethyl-3-methylbenzamide, d-trans allethrin, transfluthrin, prallethrin are functional against these vectors, but have secondary impacts on environment and human health. In this context, insecticides from botanical origin have gained palpable attention due to their numerous beneficial prospects of being eco-friendly, non-toxic to the non-target organisms, cost effective, as well as natural availability. Through numerous studies, potentiality of various plant extracts against different mosquito species has been evaluated[5-8]. Still the goal is not yet achieved to have biodegradable, target specific, economic as well as accomplished and commercially viable alternatives to get control over the mosquito population.

In this expedition with different botanically derived insecticides, the authors hope was focused in this article on *Ficus krishnae* (*F. krishnae*), an evergreen moderate sized (10-12 ft) tree of family Moracae. It has numerous aerial roots. Branches are much spreading. Leaves are opposite, coriaceous, ovate- cordate, up to 20 cm×10 cm, entire, 3-nerved. Base of the leaves are folded like pockets (Figure 1), petiole with broad smooth greasy gland at the apex. Hypanthodium sessile in axillary pairs, subglobose, downy with numerous male, gall and female flowers, achenes ovoid.

Figure 1. Mature leaves of *F. krishnae*.

Though mosquitocidal and repellency studies on *An. stephensi* Liston is prevalent in a vast volume of literature but similar reports on *Cx. vishnui* group mosquito is substantially rare. Here in, we report for the first time mosquito larvicidal activity and adulticidal efficacy of ethyl acetate and methanol extracts of mature leaves of *F. krishnae* against *An. stephensi* and *Cx. vishnui* group. Smoke toxicity and repellency tests of methanol extract were also performed to explore the effect of the plant extract on adult mosquitoes in laboratory.

2. Materials and methods

2.1. Collection and rearing of test mosquitoes

The larvae of *An. stephensi* were collected from overhead and ground level water tanks, house hold water reservoirs of different locations of Kolkata, India. Larvae of *Cx. vishnui* group were collected through dipping method from the rice fields of surrounding areas. In laboratory they were kept in trays with sufficient water in stress free, pathogen free, hygienic condition. Larvae were provided with finely ground dog biscuit. Colony adults were fed on 10% sucrose and 10% multivitamin syrup, and they were periodically blood-fed on restrained rats.

2.2. Collection of plant material

Mature healthy leaves of *F. krishnae* were collected from the plants growing within the University of Burdwan campus during March-April. The plant was identified properly and voucher specimens were deposited in the Mosquito and Microbiology Research Units, Parasitology Laboratory of The University of Burdwan, Burdwan (23° 16′ N, 87° 54′ E), West Bengal, India.

2.3. Preparation of plant extracts

For preparation of solvent extract, 25 g shed dried leaves of *F. krishnae* were extracted in a Soxhlet apparatus using organic solvent like ethyl acetate and methanol for 72 h in each case using the method of Ghosh and Chandra (2006)[9]. The eluted solvent extracts were filtered through Whatman No. 41 filter paper, then they were lyophilized and the solid residues (stock) were preserved at 4 °C for future use.

2.4. Bioassay for larvicidal activity

For evaluation of larvicidal bioassay we followed the standard protocols of World Health Organization (1981)[10]

with minor modifications. In this study 3rd instar larvae of *An. stephensi* and *Cx. vishnui* group were exposed to appropriate test concentrations (25, 50, 75 and 100 mg/L) of ethyl acetate and methanol extracts of leaves of *F. krishnae* in 100 mL of tap water taken in a series of glass beakers (250 mL). Against each concentration 25 larvae were released into the beakers and each experiment was performed thrice. Control experiments were run in parallel. Mortality rates were recorded after 24 h of exposure.

2.5. Bioassay for adulticidal activity

This bioassay was also performed according to World Health Organization (1981)[10] protocol with minor modifications. Appropriate concentrations (20, 40, 80, 160, 320, 640 mg/L) of the ethyl acetate and methanol extract were prepared by dissolving the solid lyophilized residue in 2.5 mL of acetone and soaked on Whatman No. 1 filter papers (size 12 cm×15 cm) separately then dried following the method of Dua *et al.* (2008)[11]. Control filter papers were treated only with acetone. Batches of 25 *An. stephensi* and *Cx. vishnui* group blood starved and glucose fed adult mosquitoes (2–5 days old) were cautiously transferred into plastic holding tubes separately and kept 1 h for acclimatization inside the tube environment. Then the mosquitoes were exposed to the treated papers for 1 h. They were transferred back to the holding tube and kept there for 24 h for recovery. Mosquitoes were provided with 10% glucose solution soaked in cotton ball as food. Control experiment was performed similarly. The test was repeated four times. Adulticidal efficacy of the plant extract was expressed in terms of mortality rate of the mosquitoes after 24 h of recovery period.

As methanol extract of leaves of *F. krishnae* showed better efficacy in larval and adult mortality than the extract of ethyl acetate, the former was used further for repellency and smoke toxicity tests against adult mosquitoes.

2.6. Repellency test

This test was performed following the percentage of protection in relation to dose method. For this test in net cages (45 cm×30 cm ×45 cm dimension) one hundred 3–4 days old blood starved female *An. stephensi* and *Cx. vishnui* group mosquitoes were kept separately. Four different concentrations (30 mg/L, 60 mg/L, 90 mg/L, 120 mg/L) of the methanol extract were prepared by dissolving the stock solid residue in ethanol. After cleaning the forehand of the volunteer with ethanol different test concentrations of plant extracts (one at a time) were applied from the elbow to the tip of the fingers of one hand. The other hand was treated with ethanol only and that served as control. Simultaneously the control and treated hands were introduced into the cage containing adult mosquitoes. The numbers of bites were counted for each 5 min exposure after 10 min, 30 min, 60 min and 120 min interval. All the experiments were conducted three times along with control. The percentage protection was calculated by using the following formula of Venketachalam and Jebasan (2001)[12].

$$\% \text{ Protection} = \frac{\text{Number of bites received by control hand} - \text{Number of bites received by treated hand}}{\text{Number of bites received by control hand}} \times 100$$

2.7. Smoke toxicity test

The smoke toxicity test was performed by following the method of Saini *et al.* (1986)[13] with minor modifications. A total of50 mg of solid, stock methanol extract residue of the leaf, 0.5 g sawdust (as binding material) and 0.5 g charcoal powder (as burning material) along with 1–2 drops of adhesive (Fevicol) were thoroughly mixed with distilled water to make a semisolid paste from which 0.4 cm thick and 1.0 cm long mosquito coils were prepared manually and dried in shade. Control coil was prepared similarly except the plant materials and was designated as control 1. Commercial mosquito coil (containing d-Trans Allethrin) of same dimension was used as control 2. Hundred blood fed adult *An. stephensi* and *Cx. vishnui* group female mosquitoes (age-3 or 4 days) were released into separate experimental glass chambers (measuring 100 cm × 60 cm × 60 cm, with a window measuring 30 cm × 15 cm) and exposed to the smoke produced from burning of the prepared coil from methanol extract of *F. krishnae* for 1 h. Similar experiments with control 1 and control 2 coils were also performed for same time interval. The number of the dropped down and dead mosquitoes were counted after 10, 20, 40 and 60 min of exposure. Each experiment was repeated three times on three successive days with mosquitoes of same age. All data were pooled and means were used for calculation.

2.8. Statistical analysis

The percentage of corrected mortality was calculated following Rawani *et al*[14]. Experimental data were statistically analyzed by using the computer software "STAT PLUS 2007 (Trial version)" and MS excel 2002 to find the LC_{50}, LC_{90} values, regression equations (Y=mortality; X=concentrations) and regression coefficient values. 95% confidence interval values were calculated following the method adopted by Zar (2008)[15].

3. Results

Results of 24 h larvicidal bioassay are presented in Table 1. It shows that in case of both the species of mosquitoes methanol extract was more effective than the ethyl acetate extract. The rate of mortality increased with increased dose and 100 mg/L concentration caused maximum mortality among the tested concentrations. Result of log-probit analysis showed that LC_{50} values (*i.e.* lethal concentration for 50% mortality) for the two mosquitoes ranged in between 34.03 mg/L to 56.64 mg/L of the extracts and LC_{90} values (*i.e.* lethal concentration for 90% mortality) ranged in between 97.69 mg/L to 213.48 mg/L after 24 h of exposure. It is manifested from regression equation that Y (mortality rate, dependent variable) was positively related to its corresponding X (dose, independent variable) and the R value in all cases were nearer to 1 which indicates that the rate of mortality linearly increases with the increasing dose.

Table 2 expresses the result of adulticidal activities of the extracts of *F. krishnae* leaves on *An. stephensi* and *Cx. vishnui* group after 24 h of exposure. Similar to larval stage, mortality rates of adult mosquitoes are also increased in a dose dependent pattern (in every cases the regression equations maintain the formula of straight line) which is

Table 1

Mean larval mortality (%) at 24 h with 95% confidence interval, Log-probit analysis and regression analysis of larvicidal activity of solvent extracts from mature leaf of *F. krishnae* against 3rd instar larval form of *An. stephensi* and *Cx. vishnui* group.

Mosquito	Solvent extract	Concentration (mg/L)	Percent mortality rate (95% *CI*)	LC_{50}	LC_{90}	Regression equation	*R* value
An. stephensi	Ethyl acetate	25	26.67±2.25	49.33	160.63	$Y=0.74X_{+}8.17$	0.99
		50	43.33±2.99				
		75	68.00±1.96				
		100	80.33±3.75				
	Methanol	25	31.67±2.99	38.99	130.51	$Y=0.68X_{+}20.67$	0.96
		50	61.00±1.96				
		75	76.33±2.99				
		100	83.00±1.96				
Cx. vishnui group	Ethyl acetate	25	25.00±1.96	56.64	213.48	$Y=0.68X_{+}6.50$	0.99
		50	38.67±1.13				
		75	58.67±4.08				
		100	75.67±2.99				
	Methanol	25	36.67±2.99	34.03	97.69	$Y=0.72X_{+}24$	0.97
		50	66.67±2.99				
		75	81.67±2.99				
		100	92.00±3.39				

CI- confidence interval, LC- lethal concentration

Table 2

Mean percent mortality (confidence interval 95%) of adult mosquitoes of *An. stephensi* and *Cx. vishnui* group with Log-probit analysis and regression analysis upon exposure of solvent extracts of leaf of *F. krishnae* for 24 h.

Mosquito	Solvent extract	Concentration (mg/L)	Percent mortality rate (95% *CI*)	LC_{50}	LC_{90}	Regression equation	*R* value
An. stephensi	Ethyl acetate	20	0.00±0.00	241.43	727.50	$Y=1.64X_{+}0.14$	0.97
		40	0.00±0.00				
		80	16.00±6.40				
		160	27.00±7.50				
		320	63.10±7.50				
		640	87.00±9.86				
	Methanol	20	0.00±0.00	134.56	431.31	$Y=13.8X_{+}0.15$	0.93
		40	14.00±10.12				
		80	32.00±14.31				
		160	49.00±13.38				
		320	79.00±13.38				
		640	100.00±0.00				
Cx. vishnui group	Ethyl acetate	20	0.00±0.00	286.26	886.55	$Y=-0.52X_{+}0.13$	0.97
		40	0.00±0.00				
		80	13.00±7.50				
		160	19.00±7.50				
		320	57.00±3.92				
		640	82.00±10.12				
	Methanol	20	0.00±0.00	185.59	705.00	$Y=8.52X_{+}0.14$	0.96
		40	11.00±7.50				
		80	25.00±7.50				
		160	35.00±7.50				
		320	68.00±14.31				
		640	92.00±6.40				

Table 3

Effect of smoke toxicity of methanol extract of *F. krishnae* leaf against *An. stephensi* and *Cx. vishnui* group.

Mosquito	Observation time (min)	% dropped down mosquitoes in treated	% dead mosquitoes in treated	% dead mosquitoes in control 1	% dead mosquitoes in control 2	% dead mosquito over control 1
An. stephensi	10	29.67±0.88	14.00±0.58	1.33±0.33	49.67±0.88	12.67
	20	50.33±0.88	28.67±0.88	2.00±0.57	62.00±0.57	26.67
	40	66.67±0.88	42.67±1.20	4.00±0.57	83.33±1.20	38.67
	60	89.00±0.58	70.67±1.20	5.33±0.33	88.33±0.88	65.34
Cx. vishnui group	10	32.67±0.33	22.00±0.58	1.67±0.33	53.00±0.58	20.33
	20	56.00±1.15	31.33±1.20	2.33±0.33	67.33±1.20	29.00
	40	71.00±0.58	53.33±0.88	5.00±0.58	85.00±1.15	48.33
	60	92.67±1.45	77.00±1.54	6.00±0.58	90.33±0.88	71.00

Test coil: coil with plant material, Control 1: Negative control-blank without plant material, Control 2: Positive control-coil containing d-Trans Allethrin.

signified by the values of R (nearer to 1) in each case. The LC_{50} values for methanol extract were 134.56 mg/L and 185.59 mg/L for *An. stephensi* and *Cx. vishnui* group mosquitoes respectively whereas for ethyl acetate extract the parameter values were 241.43 mg/L and 286.26 mg/L respectively.

Figure 2(a) and (b) embodies the three dimensional graphical representations to display the simultaneous variation of the repellent efficiency at different concentrations of methanol extract and at different time intervals for both the mosquitoes.

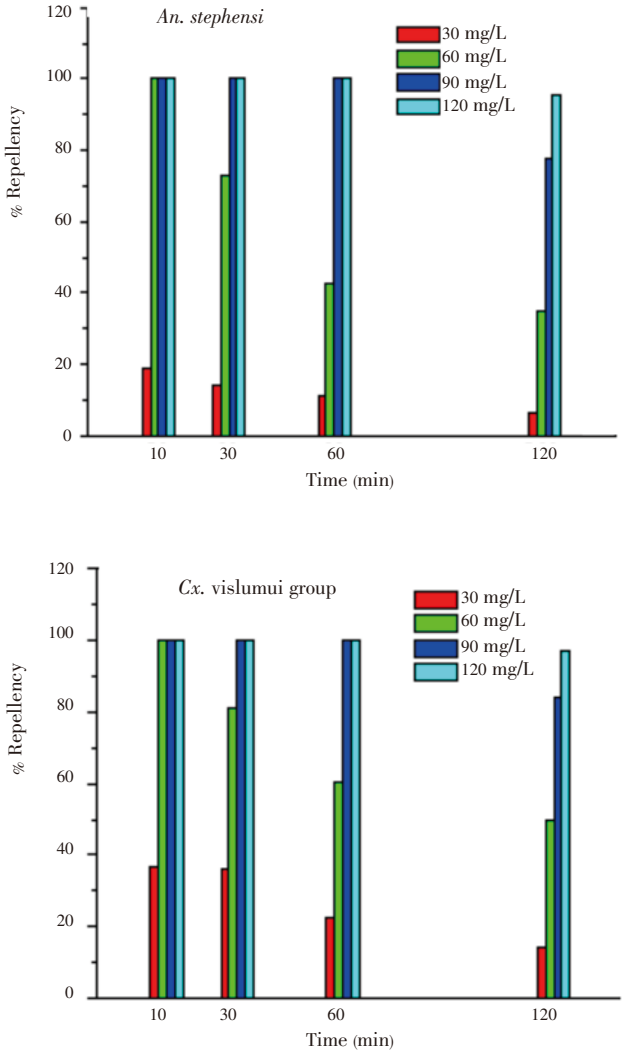

Figure 2. Repellent activity of methanol extracts of *F. krishnae* leaf at different concentrations against adult (a) *An. stephensi* and (b) *Cx. vishnui* group at different time interval.

Table 3 represents the effect of smoke toxicity of methanol extract of *F. krishnae* leaf. For control 1 *i.e.* coils prepared without extract produced only 5.33% and 6.00% mortality for 1 h exposure in case of *An. stephensi* and *Cx. vishnui* group respectively. Mosquito coils prepared from the extract exhibited 70.67% and 77.00% mortality for same time intervals which were 65.34% and 71.00% additional mortality over the control coil 1. In comparison to control 2, a commercial coil containing d-Trans Allethrin, the results were almost comparable considering that the test coils were prepared with crude methanol extract. Regarding the fact that the test coils were prepared with crude methanol extract, the results were almost comparable with the commercial coil containing d-trans allethrin (control 2).

4. Discussion

Crude plant materials and botanical derivatives had primary role for pest control in agriculture, veterinary and public health in ancient era. Chemically synthesized insecticides, due to their magical success replaced the popularity at intermediate time. Over and injudicious application of synthetic insecticides for long term causing various impairment of eco-system has renewed the importance of insecticides of green origin recently. Different plants contain different complex chemicals with unique biological activities. Literature provides a good volume of documents on efficacy of plant derived materials against different mosquitoes[16-20]. The peel methanol extract of *Citrus sinensis* and the leaf and flower ethyl acetate extracts of *O. canum* were tested against the larvae of *An. stephensi* (LC_{50}=95.74, 101.53 and 28.96 mg/L; LC_{90}=303.20, 492.43 and 168.05 mg/L respectively), by Kamaraj *et al.* 2008[21]. Mathew *et al.* (2009)[22] observed LC_{50} value 116.8 mg/L against *An. stephensi* while working with methanol extract of *Clitoria ternatea* L. seed. Prabhu *et al.* (2011)[23] in a recent work with methanol extract of *Moringa oleifera* seed on 3rd instar larvae of the same mosquito species found LC_{50} and LC_{90} values 72.45 mg/L and 139.82 mg/L respectively. In our study the methanol extract of *F. krishnae* leaf against *An. stephensi* produces LC_{50} and LC_{90} values 38.99 mg/L and 130.51 mg/L respectively which fairly establishes its efficacy. In case of

Cx. vishnui group LC_{50} and LC_{90} value of methanol and ethyl acetate extract were estimated to be 34.03, 97.69 mg/L and 56.64, 213.48 mg/L respectively in the present study.

In case of adulticidal test a clear dose-dependent mortality was observed, as the rate of mortality (*Y*) was positively correlated with the concentration (*X*) of the extract as evident from established regression equations.

Where application of any other method to protect oneself from mosquito bite is impossible, repellents could be used for personal protection. From the result it is evident that methanol extract of *F. krishnae* has a comparable repellent activity at laboratory condition. In the three dimensional graphical representation the X axis indicates different concentrations (30, 60, 90, 120 mg/L) of methanol extract tested against the adult mosquitoes. Z axis designates the time (10, 30, 60, 120 min) of exposure of mosquitoes in the smoke and the percent of repellency is depicted along the *Y* axis. For *An. stephensi* Liston, 30 mg/L concentration of methanol extract gave only 6.35% protection against mosquito bite after 2 h of exposure while 60 mg/L concentration of the same initially gave 100% protection but with time it gradually decreased to 34.92% after 2 h. 90 mg/L and 120 mg/L concentrations provided 100% protection up to 1 h, whereas at the end of 2 h exposure 77.78% and 95.24% protection have been achieved respectively. In case of *Cx. vishnui* group 60 mg/L concentration gave 100% protection for 10 minutes while 90 mg/L and 120 mg/L conferred the same for 1 h. And 96.83% protection against mosquito bite was detected at 2 h exposure in case of 120 mg/L concentration of methanol extract. The methanol-extracted *F. krishnae* did not cause any burning sensation or dermal irritation when applied to human skin. No adverse effects were observed on the skin or other parts of the human volunteers' body. Therefore, *F. krishnae* can be a potential candidate for use in the development of commercial repellents that may be an alternative to conventional synthetic chemicals, particularly in community vector control applications.

As plant materials contain several chemical components, smoke burning from it also contains some of those active ingredients. Murugan *et al.* (2007)[24] reported that smoke from Albizza amara had more toxic effect on *Ae. aegypti* than *Ocimum bascilicum*. The methanol extract of *F. krishnae* have smoke toxicity on both *An. stephensi* and *Cx. vishnui* mosquitoes. Though the efficiency of this extract in respect to its dropdown activity was excellent, adulticidal property was lower than the chemical based commercial mosquito coil. Further study with active ingredients after purification of the methanol extract may fulfill this discrepancy and show increased efficiency.

It can be concluded safely that methanol and ethyl acetate solvent extracts of leaf of *F. krishnae* can be used as larvicidal and adulticidal agents against *An. stephensi* and *Cx. vishnui* group mosquitoes. Methanol extract could also be used as skin repellent and smoke toxic material against those two mosquitoes. So from these results the authors are encouraged to investigate for the active ingredient(s) that is (are) responsible for the larvicidal, adulticidal, smoke toxic and repellent activity of *F. krishnae* extracts against the mosquito species and the studies are under progress.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

The authors are grateful to University Grant Commission (UGC) for providing financial support with Grant no F.17-8/08 (SA-I).

Comments

Background

The presented study gains importance in view of the present day situation wherein the possibilities of control of vector borne diseases are getting fewer mainly due to lessened efficacy of the chemical insecticides due to development of resistance. Resistance proof alternatives are needed and the preferred tools will be of biological / natural origin such as the present study plant *F. krishnae*. A vast literature exist on the potentiality of various plant extracts against different mosquito species but the need for availability of biodegradable, target specific, economic as well as commercially viable alternatives still exist to get control over the mosquito population.

Research frontiers

The authors have worked on a new plant extract to find out larvicidal and adulticidal properties of the crude extract. The results presented in the present manuscript are encouraging and may lead to commercially available antilarval and repellent products as an alternatives to chemical insecticides. But the study is of routine experimental studies.

Related reports

There is a vast literature on botanical extracts having larvicidal and adulticidal properties against disease vectors. Authors have tried to include relevant literature in the manuscript. The study included determination of efficacy against larvae and adult, repellency against adults and smoke emanation from coils. Studies are conducted using crude extracts the lethal values are high and are not feasible for use in field. In applications on humans for repellency authors may have considered ethical issues and clearances obtained and needs clarification. The studies on smoke were little encouraging as knock down was possible within 120

min. These studies may be further continued for more clear evidence for application in field.

Innovations & breakthroughs

The reported study is claimed to be for first such study and with evidenced smoke toxicity. However more evidence is needed for feasibility for use in field.

Applications

This is a preliminary study and the indications of the results may lead to development of products for control of disease vectors.

Peer review

A good initiative to look for alternative natural products for vector control. But needs more studies especially on characterization of active ingredients. Methodology used and interpretations were appropriate and results were conclusive.

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