Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

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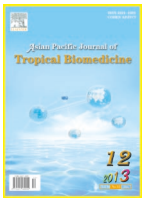

Document heading doi:10.1016/S2221-1691(13)60189-6

Control of human filarial vector, *Culex quinquefasciatus* Say 1823 (Diptera: Culicidae) through bioactive fraction of *Cayratia trifolia* leaf

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PEER REVIEW

Peer reviewer

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Comments

This research work has great commercial application in the field of vector control and human health and wellness. Although the authors used some old analytical techniques, still the outcome is very relevant. This outcome should be scale-up with industry collaboration in to market product.
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ABSTRACT

Objective: To investigate the mosquito larvicidal activity of *Cayratia trifolia* (L.) Domin (Vitaceae: Vitales) (*C. trifolia*) which is distributed in many parts of India with medicinal properties as vector control is facing threat due to the emergence of resistance to synthetic insecticides.
Methods: Young and mature leaves of *C. trifolia* were investigated for larvicidal activity against 3rd instars larvae of *Culex quinquefasciatus* in different seasons throughout the year. The active fractions were extracted using six different solvents in a non-polar to polar fashion *viz* petroleum-ether, benzene, chloroform: methanol (1:1 v/v), acetone, absolute alcohol and distilled water. Dose dependent mortality was recorded against each solvent extract. Determination of LD_{50} and LD_{90} were executed through log-probit analysis using the most bioactive fraction. The fluctuations in mortality were statistically co-related through ANOVA analyses concerning different seasons and types of leaves as random variables. Justification of larvicidal activity was established through student's *t*-test. Costing effects were evaluated on the non-target water fauna under laboratory conditions. Thin layer chromatographic techniques were performed for phytochemical analysis and categorization of chemical personality of the active fractions using the most effective solvent extract following standard methods.
Results: Significant variations in mortality rate were noted with respect to the type of leaves (mature and senescence), concentration of leaf extract and between seasons. The water extract among all the solvent extracts was found to induce cent percent mortality at 50 mg/L in test mosquito species within 24 h with a LD_{50} and LD_{90} value of 10.70 mg/L and 27.64 mg/L respectively. No significant mortality was recorded in non-target water population. Chromatographic analyses of the water extract revealed the presence of steroids, triterpene glycosides, essential oil, phenolics and diterpenes as secondary phytochemicals.
Conclusions: Water extract of *C. trifolia* leaf promised as a cost effective and potent larvicidal agent against *Culex quinquefasciatus*.

KEYWORDS
Cayratia trifolia, *Culex quinquefasciatus*, Larvicidal, Phytochemical analyses

1. Introduction

Synthetic pesticides have been used to reduce pest population since late sixties. Improper dosage and faulty application of synthetic insecticides result resurgence of secondary pest populations and increase resistance to disease vectors[1]. The search for new biodegradable insecticides having no ill effect on non target fauna remains the top priority[2]. Some phytochemicals have been identified to have good mosquito larvicidal properties[3-7]. For isolation of active principle from the plant parts different types of solvents, such as water, petroleum ether, chloroform and methanol are used. Tennyson *et al*[8] screened 150 plant species for their toxicities against

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Foundation Project: Supported by UGC DRS for kind financial support with the grant no: F.3-9/2012(SAP II).

Article history:
Received 9 Sep 2013
Received in revised form 16 Sep, 2nd revised form 20 Sep, 3rd revised form 5 Oct 2013
Accepted 20 Nov 2013
Available online 28 Dec 2013

mosquito and found several to be very effective.

Culex quinquefasciatus Say 1823 (*Cx. quinquefasciatus*) is the principal vector of bancroftian filariasis, and the eradication or control of vector, is regarded as one of the important alternative available in preventing and controlling filariasis[9]. Application of synthetic insecticides is, although, highly efficacious against the target species, vector control is facing a threat due to the development of resistance to chemical insecticides resulting in rebounding vectorial capacity[10]. Furthermore, they are responsible for substantial hazards to a variety of non-target organisms and environment. The researchers therefore have diverted their attention towards plant kingdom, which are ecofriendly and cost effective.

During the present piece of study *Cayratia trifolia* (*C. trifolia*) leaves were selected on the basis of preliminary investigation on its mosquito larvicidal activity. Further it was subjected to solvent extraction to find out the most suitable solvent which inflects highest larval mortality. Chemical profile of the leaf extract was determined qualitatively. This is to mention that in traditional Indian medicine, leaves, seeds and roots of *C. trifolia* are used as astringent as well as applied to ulcers and boils. Leaves extract are diaphoretic which recommended in high fever. Roots have an anti-anaemic effect and all the aerial parts can activate CNS and have a hypothermic effect[11].

2. Materials and methods

2.1. Test mosquito larvae

The present study was conducted at Burdwan (23°16′ N, 87°54′ E), West Bengal, India during March 2012 to January 2013. Larvae of *Cx. quinquefasciatus* were collected from concrete drains surrounding the Burdwan University campus and kept in plastic buckets (15 L). The larvae were fed with powdered mixture of dog biscuits and yeast at 3:1 ratio and kept in a germ free condition, away from insecticides or repellants and maintained at (20±2) °C, 75%-85% RH, 14 L: 10D photoperiod cycles in an insectary. During the bioassay experiments, early 3rd instar larvae were taken from the bucket and transferred within the sterile glass dishes or beakers.

2.2. Description of the plant used

C. trifolia (L.) Domin (Vitaceae: Vitales) is a vine that climbs by means of tendrils. Leaves are trifoliate with petioles, 2 to 8 cm long, 1.5 to 5 cm wide, pointed at the tip and coarsely toothed at the margins. Flowers are small greenish white and borne on axillaries solitary cymes. Fruit is fleshy, juicy dark purple or black, subglobose and about 1 cm in diameter. Style short; stigma slightly or inconspicuously expanded. Berry globose shaped 1-4 seeded[12].

2.3. Preparation of crude extract of mature and immature leaves

Fresh, mature and immature leaves of *C. trifolia* were harvested separately from plants growing on outskirts of Burdwan. The plant was identified properly and a voucher specimen (ZGC-S-08) had been deposited at Botany Department, the University of Burdwan. After collection the leaves were initially washed with distilled water and dried on paper towel. Atotal of 50 g leaves were crushed with a Jankel and Kunkel model A10 mill, and the plant juice was filtered by Whatmans No. 1 filter paper and the clear filtrate was used as a stock solution (100% concentration of crude extract) for bioassay experiments. Required concentrations (0.6%, 0.5%, 0.4%, 0.3%, 0.2% and 0.1%) were prepared by mixing up of stock extract with appropriate quantity of sterilized distilled water.

2.4. Preparation of solvent extract of mature leaves

Fresh mature leaves were harvested, flushed with distilled water and dried in the shade at room temperature (20 °C) and crushed into fine powder with a Jankel and Kunkel model A10 mill. The dried leaf-powder (25 g) was put in a Soxhlet apparatus and the plant extracts were prepared according to Adhikari and Chandra[13] by using different 250 mL solvents of analytical grades (Merck) with gradually increasing polarity, *i.e.* petroleum-ether, benzene, chloroform: methanol (1:1 v/v), acetone, absolute alcohol and distilled water, applying one after another with the same leaf powder. The extracted liquid was subjected to rotary evaporator in order to remove the chemicals. The resultant semisolid extract was kept in a deep freeze at -80 °C (REVCO model No, ULT 790-3-V32) for 12 h followed by freeze drying for 24 h at -60 °C. Then the extract was stored in an air tight container at 4 °C for further use. The dried precipitate were weighed and dissolved in suitable volume of distilled water to make different concentrations at mg/L levels.

2.5. Dose-dependent larvicidal bioassay

The larvicidal bioassay followed the WHO standard protocol [14] with suitable change. Each of the prepared concentrations of crude extract and solvent extracts was transferred into the sterile glass beaker (250 mL capacity). Twenty five early 3rd instar larvae of *Cx. quinquefasciatus* were introduced into different beaker containing appropriate concentrations. And 10 mg of larval food (dog biscuits: yeast extract=3:1) was added per beaker. Mortality rate were recorded after 24, 48 and 72 h of post-exposure. The data of mortality in 48 and 72 h were expressed by the addition of the mortality at 24 and 48 h, respectively for both crude and solvent extract experiments. The experiments were replicated four times on four different days.

2.6. Phytochemical analysis of mature leaves

The phytochemical analysis was carried out using the distilled water extract (as it exhibited highest mortality) following the standard methods of Harborne[15] and Stahl[16]. The extract was dissolved in absolute alcohol and chromatographed using pre-coated and pre-heated (100 °C for 30 min) glass plates (eight glass plates), which were prepared with silica gel G using Unpoplan coating apparatus (Shadon, London). After 5 min of drying, each of the plates was placed in the separate glass chamber for TLC analysis, with different solvent systems as the mobile phase. After the movement of solvent at the top of the plates, each plate was removed from the glass chamber and separately air-dried. After 10 min each of plates was sprayed with a different spraying reagent for the identification of appropriate phytochemical. The phytochemicals included in the study were sapogenins, steroid, terpenoids, flavonoids, alkaloid, essential oils, phenolics and amino acids. The phytochemicals were determined using TLC analysis by the application of suitable solvents and spray reagents and, in each case, R_f value was recorded.

2.7. Effect on non-target organisms

Non target organisms were those animals that share the common habitats of target mosquito larvae and some of them were natural predators of mosquito larvae. The effect of the crude, petroleum-ether, benzene, ethyl acetate, chloroform: methanol (1:1 v/v), acetone, absolute alcohol and distilled water extracts of *C. trifolia* leaves were tested against non-target organisms like *Toxorhynchites* larvae (mosquito predator), *Diplonychus annulatum* (predatory water-bug) and *Chironomus circumdatus* larvae (insect). The predators were exposed to appropriate lethal concentration of crude and solvent extracts at 24 h to observe the mortality and other abnormalities such as sluggishness and reduced swimming activity up to 72 h of exposure. Two replicates were performed for each test concentration along with two replicates of untreated controls.

2.8. Statistical analysis

Two-way replicated ANOVA was carried out on the mortality data of the *Cx. quinquefasciatus* larvae to justify the difference in terms of concentration, leave types and seasons and their interactions. Statistical analyses were done by using computer software SPSS and MS-EXCEL 2003.

3. Results

The efficacy of different concentrations of crude extract of young and matured leaves of *C. trifolia* on early third instar *Cx. quinquefasciatus* larvae is shown in Table 1. The results of two-way ANOVA on the mortality against the mature and young leaves with respect to concentrations and seasons are presented in Table 2. Considering larval death at a particular concentration of aqueous extract of young leaves and comparing with different seasons, statistically it was revealed that a relation between F and F_{crit} value was marginally accepted so pair wise *t* test is necessary (Table 3). For summer, rainy and winter, between young and mature leaves irrespective of doses, the larval mortality rate varied significantly (df=17, t=3.04, P<0.004; df=17, t=6.481, P<0.001; df=17, t=2.034, P<0.03). Absolute mortality was achieved by using mature leaves at 0.4% crude concentration and above (0.5%, 0.6%) after 72 h of exposure in rainy season. Results on toxicity of non-polar to polar solvent extracts against 3rd instars larval form of *Cx. quinquefasciatus* had been presented in Table 4. The results of preliminary phytochemical analysis of the water extract of the leaves of *C. trifolia* were presented in Table 5. No mortality and other abnormalities in swimming behavior were noticed in non target organisms.

Table 1
Effect of crude extract from *C. trifolia* leaf on 3rd instars larvae of *Cx. quinquefasciatus* in different seasons.

Seasons	Concentrations(%)	Young leaves						Mature leaves					
		24h		48h		72h		24h		48h		72h	
		M	S	M	S	M	S	M	S	M	S	M	S
Summer	0.6	75.0	25.0	10.0	15	7.5	7.5	85.0	15.0	3.5	11.5	9.5	2.0
	0.5	77.5	22.5	0	22.5	12.5	10.0	82.5	17.5	2.5	15.0	7.5	7.5
	0.4	75.0	25.0	5.0	20	5.0	15.0	75.0	25.0	10	15.0	7.5	7.5
	0.3	67.5	32.5	7.5	25	10.0	15.0	72.5	27.5	7.5	20.0	7.5	12.5
	0.2	67.5	32.5	10.0	22.5	12.5	10.0	65.0	35.0	15	20.0	5.0	15.0
	0.1	67.5	32.5	10.0	22.5	7.5	15.0	70.0	30.0	15	22.5	2.5	20.0
	Control	2.5	97.5	0	97.5	0	97.5	0	100	2.5	97.5	2.5	95.0
Rainy	0.6	90.0	10.0	5	5	2.5	2.5	90.0	10.0	7.5	2.5	2.5	0
	0.5	87.5	12.5	7.5	5	2.5	2.5	95.0	5.0	5	0	0	0
	0.4	82.5	17.5	12.5	5	0	5.0	90.0	10.0	7.5	2.5	2.5	0
	0.3	75.0	25.0	12.5	12.5	5.0	7.5	82.5	17.5	7.5	10.0	7.5	2.5
	0.2	75.0	25.0	7.5	17.5	10.0	7.5	82.5	17.5	10	7.5	5.0	2.5
	0.1	72.5	27.5	10.0	17.5	7.5	10.0	75.0	25.0	10	15.0	5.0	10.0
	Control	0	100	0	100	5.0	95.0	2.5	97.5	0	97.5	0	97.5
Winter	0.6	0	100	0	100	30.0	70.0	0	100	0	100.0	30.0	70.0
	0.5	0	100	0	100	20.0	80.0	0	100	5	95.0	22.5	72.5
	0.4	0	100	0	100	12.5	87.5	0	100	5	95.0	10.0	85.0
	0.3	0	100	0	100	0	0	0	100	0	100.0	5.0	95.0
	0.2	0	100	0	100	0	0	0	100	0	100.0	7.5	92.5
	0.1	0	100	0	100	0	0	0	100	0	100.0	0	100.0
	Control	2.5	97.5	0	97.5	0	97.5	0	100	0	100.0	5.0	95.0

M: % Mortality, S: % Survivality

Table 2
Result of two-way ANOVA between mature and young leaves in respect to seasonal mortality and concentration.

Source of variation	*df*	Sum of squares	Mean sum of squares	*F* value
Types of leaves and concentrations as two random variables				
Concentrations	5	7326.16	1465.23	29.35**
Types of leaves	17	656803.94	38635.53	773.91**
Concentrations×Types of leaves	85	5828.01	68.56	1.37*
Residual	324	16175.00	49.92	
Total	431	686133.10		
Concentrations and seasons as two random variables				
Concentrations	5	1836.99	167470.63	5.76**
Seasons	5	160821.71	32164.34	504.43**
Concentrations×Seasons	25	220.93	8.84	0.14 (N.S.)
Residual	72	4591.00	63.76	
Total	107	167470.63		

*P<0.05, **P<0.001

Table 3
Result of *t*-tests between mature and young leaves related to seasonal mortalit.

Season	*df*	*t*-value	*P*-value
Summer	17	3.03	0.003
Rainy	17	6.48	0.001
Winter	17	2.03	0.03

Table 4
Competence of seven solvent extracts of *C. trifolia* leaf against 3rd instars *Cx. quinquefasciatus* larvae.

Solvent extracts	Concentration (mg/L)	Exposures					
		24 h		48 h		72 h	
		M	S	M	S	M	S
Petroleum ether	100	0	100	0	100	0	100
	50	0	100	0	100	0	100
	30	0	100	0	100	0	100
	10	0	100	0	100	0	100
	control	0	100	0	100	0	100
Benzene	100	10	90	20	70	10	60
	50	10	90	20	70	0	70
	30	0	100	0	100	20	80
	10	0	100	0	100	10	90
	control	0	100	0	100	0	100
Ethyl acetate	100	0	100	40	60	10	50
	50	10	100	30	70	20	50
	30	0	100	30	70	0	70
	10	0	100	20	80	10	70
	control	0	100	0	100	0	100
Chloroform: methanol (1:1 v/v)	100	0	100	10	90	10	80
	50	0	100	0	100	15	85
	30	0	100	0	100	0	100
	10	0	100	0	100	0	100
	control	0	100	0	100	0	100
Acetone	100	0	100	0	100	40	60
	50	0	100	0	100	30	70
	30	0	100	0	100	0	100
	10	0	100	0	100	0	100
	control	0	100	0	100	0	100
Absolute alcohol	100	10	90	10	80	10	70
	50	10	90	0	90	10	80
	30	0	100	10	90	0	90
	10	0	100	0	100	0	100
	control	0	100	0	100	0	100
Distilled water	100	100	0	0	0	0	0
	50	100	0	0	0	0	0
	30	90	0	0	0	0	0
	10	50	50	10	40	10	30
	control	0	100	0	100	0	100

4. Discussion

Mosquito control is chiefly directed against larvae and only against adults when necessary. Larval control can be an effective control tool due to the low mobility of larval mosquitoes, especially where the principal breeding habitats are manmade and can be easily identified[17].

Botanicals have preference over synthetic chemicals as these are target specific, biodegradable, cheap and easily available. Secondary metabolites such as alkaloids[18], essential oils[19], steroids, phenolics[20], triterpenes[21], *etc.* have been isolated from plant extracts having good mosquito larvicidal properties.

The results of the present study showed that larval mortality rate was higher in rainy than those of summer and winter seasons both in mature and young leaves of *C. trifolia*. No significant larval mortality was found in winter. A significant variation in mortality rate was noted with respect to the type of leaves, concentration and between seasons. The interaction between the concentration and the type of leaves affected the mortality rate considerably, signifying that the concentration of the active principle might differ between leaf age, young or mature. Cent percent mortality was recorded at 50 mg/L concentration of distilled water extract after 24 h of exposure. The plant extracts were safe to those non-target organisms that share the same habitat of *Cx. quinquefasciatus* mosquito larvae.

In conclusion, *C. trifolia* offers potential larvicidal activity against *Cx. quinquefasciatus*. *C. trifolia* is liberally available and as mosquito larvae can be controlled with the distilled water extract of leaves of this plant at a low dose rate without causing harm to the non-targets, this extract may be used efficiently cost effectively for controlling mosquitoes in the field. Further studies are in progress to evaluate the effect of purified extract on larval mortality.

Conflict of interest statement

We proclaim that we do not have any disagreement of interest.

Acknowledgements

Authors are thankful to plant taxonomist Dr. A. Mukherjee,

Table 5
Detection of biochemical present in TLC glass plate following treatment of different spraying reagents.

Solvents Used	Name of spraying reagents	R_f values and the color of positive spots	Conclusion
Acetone: Hexane (4:1)	Antimony chloride in concentrated hydrochloric acid	–	Absence of sapogenins
Methanol concentrated: Ammonium hydroxide (200:3)	Dragendroff's reagent (Muiner and Macheboeuf)	–	Absence of alkaloids
Chloroform	Acetic anhydride : Sulphuric acid (Lieberman-Burchard reagent)	0.957 (deep pink)	Presence of steroids/ triterpene glycosides
Chloroform: Benzene (1:1)	Vanillin-sulphuric acid	0.987 (pink red)	Presence of essential oil
Chloroform: Acetic acid: Water (90:45:6)	Saturated alcoholic sodium acetate	–	Absence of flavonoids
Ethyl acetate : Benzene (1:1)	Folin	0.983 (deep violet)	Presence of phenolics
Chloroform on silica gel plate treated with silver nitrate	Antimony chloride in chloroform	0.976 (orange yellow)	Presence of diterpenes

Department of Botany, Burdwan University, Burdwan, W.B., for identifying the plant. We are indebted to UGC DRS for kind financial support. The grant no is No. F.3-9/2012(SAP II) under UGC DRS.

Comments

Background

Vector control is one of the most challenging and important task to control communicable diseases due to its increasing resistance of vectors to synthetic insecticides. So the world needs natural way of doing this job without affecting other species.

Research frontiers

The water extract (most acceptable media by regulators) of leaves of *C. trifolia* among all the solvent extracts was found to be most effective at very low concentration within 24 h. No significant mortality was recorded in non-target water population.

Related reports

The plant *C. trifolia* has been used traditionally for insect control, but this study demonstrated first time the active extraction phase (water) at low dose is effective as larvicidal against *Cx. quinquefasciatus*.

Innovations and breakthroughs

The authors used 6 different extraction methods for isolation of active components and have determined both LD_{50} and LD_{90} values. They also identified wide range of actives in water extract.

Peer review

This research work has great commercial application in the field of vector control for communicable diseases and human health and wellness. Although the authors used some old analytical techniques, still the outcome is very relevant. This outcome should be scale-up with industry collaboration in to market product.

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