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Efficacy of *Limonia acidissima* L. (Rutaceae) leaf extract on larval immatures of *Culex quinquefasciatus* Say 1823

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ABSTRACT

Objective: To investigate the role of leaf extract of *Limonia acidissima* L. (Rutaceae) as a biocontrol agent against the larval form of *Culex quinquefasciatus*, and characterization of bioactive component responsible for larvicidal activity. **Methods:** Larval mortality of mosquito species was observed after 24, 48 and 72 hours of exposure to different concentrations of aqueous extract, solvent extract and subsequently bioactive compound. The bioactive compound was subjected to IR and GC-MS analysis. **Results:** Mortality rate at 3% concentration of crude extract were highest (90%) amongst all concentrations tested and subsequently highest (95%) mortality was achieved in chloroform: methanol extract at 100 ppm concentrations. IR and GC-MS analysis of bioactive compound revealed the presence of steroid compound which may act as larvicide. **Conclusions:** The chloroform: methanol extract of mature leaves of Limonia acidissima was found to exhibit considerable mosquito larvicidal activity against Culex quinquefasciatus.

1. Introduction

Repeated use of synthetic insecticides for mosquito control has resulted in the development of resistance[1], undesirable effects on non-target organisms and fostered environmental and human health concern[2] which initiated a search for alternative control measures. Plants are considered as rich sources of bioactive chemicals[3,4] which may be alternative sources of mosquito control agents. Phytochemicals derived from plant sources can act as larvicide, insect growth regulators, repellent and oviposition attractant and have different activities observed by many researchers[5,6]. The leaf extracts of several plant species showed encouraging results against *Aedes aegypti (Ae. aegypti)* [7,8] *Culex quinquefasciatus* (Diptera: Culicidae) (*Cx. quinquefasciatus)* [9] and *Anopheles stephensi* (*An. stephensi)* mosquito larvae[10].

Limonia acidissima L. (Rutaceae) (*L. acidissima*) the Indian wood apple is a multistemed tree, distributed in tropical and temperate regions of the world and is a large tree growing to 9 m tall, with rough, spiny bark. The leaves are pinnate, with 5-7 leaflets, each leaflet 25-35 mm long and 10-20 mm broad, with a citrus-scent when crushed. The fruit is a berry, 5-9 cm in diameter, and may be sweet or sour. The unripe fruit acts as astringent and is used in combination with bail and other medicines, in diarrhoea and dysentery. The fruit is much used in India as a liver and cardiac tonic, and when unripe, as an astringent means of halting diarrhoea and dysentery and effective treatment for hiccough, sore throat and in the diseases of the gums. The ripe fruit is rich in β -carotene, a precursor of Vitamin A; it also contains significant quantities of the B vitamins, thiamine and riboflavin, and small amounts of Vitamin C. The root juice was once popular as a remedy for snakebites. The seed oil is a purgative, and the leaf juice mixed with honey is a folk remedy for fever. The tannin-rich and alkaloid-rich bark decoction is a folk cure for malaria[11-14]. The objective of the present study is to examine the role of leaf extract of *L. acidissima* as a biocontrol agent against the larval forms of *Cx. quinquefasciatus* and the characterization

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of the bioactive components responsible for larval mortality.

2. Materials and methods

2.1. Collection of plant material

Fresh mature leaves of *L. acidissima* were harvested from rural areas of Burdwan (23°16′N, 87°54′E), West Bengal, India, in June , 2009.

2.2. Test mosquitoes

The present study was conducted during June-August of 2009. The larvae of *Cx. quinquefasciatus* were obtained from drains of Burdwan and a laboratory colony was developed and maintained in the Mosquito Research Unit, Department of Zoology, The University of Burdwan. The colony was kept free from exposure to pathogens, and maintained at 25-30 ℃. The larvae were fed on a powdered mixture of dog biscuits and dried yeast powder at a ratio of 3:1. The adult colony was provided with 10% sucrose solution and 10% multivitamin syrup, and was periodically blood-fed on restrained rats.

2.3. Preparation of crude aqueous extracts

Collected leaves were rinsed with distilled water and dried in paper towels. Crude extracts were prepared by grinding the leaves in a mortar and pestle and then passing the ground material through Whatman No 1 filter paper. Proper concentrations of aqueous extracts were prepared by mixing the crude extract with a suitable amount of sterilized distilled water.

2.4. Preparation of plant extracts in different solvent systems

Shade dried 25 g leaves were put in a Soxhlet apparatus and extracts were prepared according to the method of Ghosh & Chandra[10] using six solvents, namely petroleum ether, benzene, ethyl acetate, chloroform: methanol (1:1, v/v), acetone and absolute alcohol, applying successively (extraction period 72 hour in each case) with the same leaves. Serially, the extracts were collected separately and the Soxhlet apparatus was washed with 200 mL of water and 100 mL of a similar solvent as an eluent after each type of solvent extraction procedure. The eluted materials and each type of extract were concentrated in combination at 40 ℃ to 100 mL of extract by evaporation in a rotary evaporator. After that, each of the extracts was filtered through Whatman No. 41 filter paper, solvents were lyophilized and the solid residues were weighed and then dissolved in a suitable amount of sterilized distilled water to make the different graded concentrations.

2.5. Bioassay experiments

To examine the larvicidal bioassay we followed the standard protocols of World Health Organization[15] with slight modifications. Each of the concentrations of aqueous leaf extract (0.5%-3%) was transferred into sterile glass Petri dishes (9 cm diameter/150 mL capacity).Ten third instar larvae of *Cx. quinquefasciatus* were separately released into different Petri dishes containing graded concentrations and the likewise mortality were recorded after 24, 48 and 72 hours of the exposure period. The data of mortality in 48 and 72 hours were expressed by compiling the mortality at 24 and 48 hours, respectively. The larvae were considered dead when they failed to move after probing with a needle in the siphon or cervical region and unable to reach the water surface. The experiments were conducted under laboratory conditions at an ambient temperature ranging from 25-30 ℃ and 80%-90% relative humidity with three replicates. Control experiments were run without extract in parallel.

2.6. Preparation of samples for isolation of bioactive part responsible for larval mortality

The phytochemical analysis was carried out using chloroform: methanol (1:1 v/v) extract (as it exhibited highest mortality against *Culex* larvae) of the mature leaves of *L. acidissima* using the standard methods of Harbone[16] and Stahl[17]. The extract was chromatographed using silica gel 'G' TLC plates. The plates (thickness 0.5 mm) were prepared with silica gel G (Sigma, USA) and a thin- layer coating apparatus (Unoplan-Shandon, London). The mobile phase was chloroform: methanol (1:1, v/v). The thin layer chromatography (TLC) plates (50 in number) were sprayed with different spraying reagents for identification of class or nature of phytochemicals and the Rf values were also measured.

Then purified fractions were made in different concentrations and treated against third instar larvae of *Cx. quinquefasciatus* and larval death was recorded after 24, 48, and 72 h.

2.7. Bioassay with active ingredient

Preparative thin layer chromatography was done to separate the compounds of identified region of definite Rf values. Twelve number of plates were used for this purpose. The fractions obtained from preparative TLC were dissolved in distilled water to prepare different concentrations. Then 1st, 2nd, 3rd, and 4th instars larvae were introduced separately to different graded concentrations and the larval death rates were determined after 24, 48 and 72 h of exposure

2.8. Preparation of active ingredient for IR and GCMS analysis

As the spots exhibited positive response in Lieberman Burchard reagent recorded highest larval mortality during further bioassay experiments, were scrapped from preparative silica gel 'G' plates and dissolved in absolute alcohol. The fraction was collected discarding the silica gel G and filtered through Whatman No.1 filter paper. The purified fraction was dried and subjected to infrared (IR) spectroscopy. The IR spectroscopy analysis of the active spot was performed with the aid of an Infrared spectroscope (JASCO FT-IR Model-420) using KBr plates.

The sample was analysed by Gas Chromatography Mass Spectrometry on a Shimadzu - GC MS - QP-5050A fitted with a ZB- 5 (Phenomesex Company, Japan) capillary column (300 m long, 0.25 mm in diameter, film thickness 0.25 mm). MS condition; ionization voltage 70 eV; Ion source temperature 270 ℃ and mass range 30-700 mass units. The individual peaks were identified by comparison of their retention indices by comparing their mass spectra with the

NIST/ Wiley Library mass spectral data base.

2.9. Statistical analysis

The percentage mortality observed (%M) was corrected using Abbott's formula[18]. The statistical analysis was performed using computer software Stat plus 2007, SPSS ver. 11 and MS EXCEL 2002 to find the LC_{50}, regression equations (Y = mortality; X = concentrations) and regression coefficient values.

3. Results

The results of the present study indicate that the mortality rates at 3% concentration of crude extract were highest (90%) amongst all concentrations tested and it was significantly higher (P<0.05) than the mortality rates at 0.5%, 1%, 1.5%, 2% and 2.5 % after 24 hours of exposure (Table 1).

The total yield of each solvent extract from 25 g of leaves were as follows: petroleum ether extract 1.12g; benzene extract, 1.98 g; chloroform: methanol (1:1, v/v) extract, 3.94 g; acetone extract, 2.34 g and absolute alcohol extract 2.21g. The present study revealed the highest mortality in chloroform: methanol (1:1 in v/v) extract at 100 ppm concentration (Table 2). We found significant difference in larval mortality between extract with chloroform: methanol (1:1 in v/v) and extracts with petroleum ether (t=147.47),

Table 1

Effect of crude extract of mature leaves of *L. acidissima* on third instar larvae of *Cx. quinquefasciatus.*

Concentration (mL)	Mortality (M%±SE)		
	24 hrs	48 hrs	72 hrs
0.5	12.00±0.00	18.00±0.57	21.66±0.66
1.0	22.66±0.88	24.66±0.88	36.33±0.66
1.5	40.66±0.66	55.00±0.00	60.00±0.00
2.0	63.33±0.66	66.00±0.57	75.00±0.57
2.5	74.33±0.33	78.00±0.57	90.00±0.00
3.0	90.33±0.66*	95.33±0.66*	99.66±0.33*
Control	0.00±0.00	0.00±0.00	0.00±0.00

*P<0.05, comparing with mortality in the same row.

Table 2

Efficacy of different concentrations of polar and non-polar solvent extracts of mature leaves of *L. acidissima* on third instar larvae of *Cx. quinquefasciatus.*

Solvents	Concentration(ppm)	Mortality(M±SE)		
		24 hrs	48 hrs	72 hrs
Petroleum ether	100	9.33±0.33	10.00±0.57	11.00±0.57
	75	7.33±0.33	8.00±0.57	7.66±0.33
	50	4.00±0.57	3.66±0.88	3.66±0.88
Distilled water	Control	0.00±0.00	0.00±0.00	0.00±0.00
Benzene	100	10.00±1.15	15.00±0.57	15.33±0.88
	75	5.00±0.57	8.33±0.33	14.00±0.57
	50	4.00±0.57	7.66±0.33	11.66±0.66
Distilled water	Control	0.00±0.00	0.00±0.00	3.00±0.57
Ethyl acetate	100	14.66±0.66	17.33±0.88	22.66±0.88
	75	12.00±1.00	18.00±0.57	20.66±0.88
	50	3.00±0.00	8.00±0.57	8.66±0.33
Distilled water	Control	3.00±0.57	3.00±0.57	3.33±0.88
Chloroform & methanol(1:1 v/v)	100	90.00±0.57*	94.33±0.33*	95.33±0.33*
	75	81.33±0.33	83.66±0.33	84.66±0.66
	50	69.33±0.88	71.00±0.57	72.33±0.33
Distilled water	Control	0.00±0.00	0.00±0.00	0.66±0.66
Acetone	100	37.33±0.66	42.00±0.57	42.33±0.66
	75	24.33±0.88	29.66±0.88	30.66±0.66
	50	20.66±0.57	22.33±0.33	25.66±0.88
Distilled water	Control	0.00±0.00	0.00±0.00	1.00±1.00
Absolute alcohol	100	18.66±0.57	19.66±0.88	22.66±0.33
	75	13.33±0.33	14.66±0.33	15.00±0.57
	50	3.00±0.00	9.33±0.88	9.00±0.57
Distilled water	Control	0.00±0.00	0.00±0.00	1.66±0.88

*P<0.05, comparision of mortality between extracts with chloroform : methanol (1:1 v/v) and extracts with other solvents at 100 ppm contration.

benzene (t=61.02), ethyl acetate (t=117.71), acetone (t= 82.29) and absolute alcohol (t=115.81) at 100 ppm concentration. (Table 2). The mortality rate at 100 ppm concentration of chloroform: methanol (1:1 in v/v) extract was higher than mortalities at 75 ppm (t=16.03) and 50 ppm (t=17.22) concentrations in 24h study period against the tabulated value of 3.82 at 5 degrees of freedom.

The fraction showing mortality gave an Rf value of 0.63. Results of the bioassay tests with that fraction of bioactive compounds against 1st, 2nd, 3rd, 4th instars larvae are presented in the Table 3. Mortality rate at 25 ppm was significantly higher (P<0.05) than those at 20 ppm and 15 ppm for 1st (t= 2.60; t =17.10), 2nd (t = 5.80; t = 20.02) 3rd (t = 13.53; t = 22.17) and 4th (t = 15.62; t = 15.82) instars larvae. The results of regression analysis indicate that the mortality rate (Y) was positively correlated with the test concentration (X) having a regression coefficient close to one in each case (Table 4). The results of log probit analysis (95% confidence level) revealed that LC_{50} values gradually decreased with the exposure periods having the lowest value at 72 hours of experiment.

Among the different phytochemical analyses, the Libermann-Burchard, Vanillin-phosphoric acid and Ceric sulphate-sulphuric acid tests were positive, which suggests that the purified fraction was steroid in nature.

From IR spectroscopy (Figure 1) we observed the O-H stretching, a C=C stretching and C=O stretching vibrations of ester group. The GCMS analysis revealed the presence of six major bioactive compounds with their distinctive amount and retention times (Figure 2). The identified compounds were 1,1 - diethoxy, 3-methyl butane (peak no 6; M.W=160), Benzoic acid (peak no 9; M.W=122), Thymol (peak no 10; M.W=150), Dibutyl phthalate (peak no 15; M.W=278), Stigmasta-5,22-dien-3-ol (peak no 38; M.W=454), Stigmasta-3,5-dien-7-one (peak no 45; M.W=410).

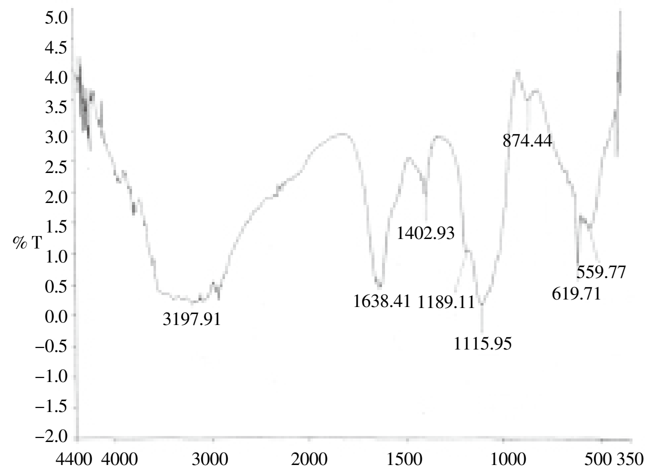

Figure 1. Interpretation of IR spectra of the bioactive compound. Frequency range and probable functional groups of the compound: Strong bond at 3197 cm^{-1} (broad and multiple) indicates a O-H stretching vibration which may be a part of -COOH group, Strong vibration bond at 1638 cm^{-1} may be due to double bond, two bonds at 1189 cm^{-1}, 1 115 cm^{-1} are due to C=O stretching of ester.

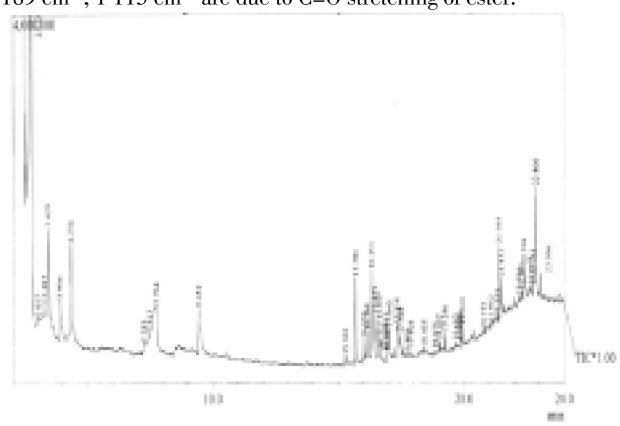

Figure 2. GCMS-EI analysis (GCMS-SHIMADZU-QP5050A) of the bioactive compound with distinctive retention time.

Table 3

Mortality of different larval instars of *Cx. quinquefasciatus* in different concentrations of bioactive compound.

Instars	Concentration (ppm)	Mortality(M%±SE)		
		24 hrs	48 hrs	72 hrs
First	25	56.00±0.57*	74.66±0.33*	82.33±0.33*
	20	54.33±0.66	70.33±0.33	80.00±0.57
	15	44.66±0.66	62.00±0.57	77.33±0.33
	Control	0.00±0.00	2.00±0.57	2.66±0.33
Second	25	50.66±0.66*	59.66±0.33*	63.00±0.57*
	20	47.33±0.33	54.00±0.57	59.66±0.66
	15	40.33±0.33	52.66±0.88	59.00±0.57
	Control	0.00±0.00	0.00±0.00	2.00±0.57
Third	25	40.66±0.66*	51.33±0.33*	55.66±0.33*
	20	32.00±0.57	42.00±0.57	53.33±0.33
	15	27.00±0.57	37.66±0.88	47.00±0.57
	Control	0.00±0.00	0.00±0.00	0.00±0.00
Fourth	25	34.66±0.33*	39.00±0.57*	43.33±0.33*
	20	27.33±0.33	33.00±0.57	36.66±0.33
	15	17.33±0.88	20.00±0.57	25.66±0.33
	Control	0.00±0.00	0.00±0.00	0.00±0.00

*P<0.05 comparing with other contrations.

Table 4

Log probit analysis of the larvicidal activity of chloroform:methanol extract of the mature leaves of *L. acidissima* on different instar larvae of *Cx. quinquefasciatus*.

Instars	Period of bioassay (hrs)	LC50 Values(ppm)	Regression equations	R2
First	24	18.22	Y=2.37x−3.10	0.95
	48	9.65	Y=3.05x−6.46	0.94
	72	1.73	Y=3.38x−8.11	0.96
Second	24	21.09	Y=2.12x−2.72	0.95
	48	14.74	Y=2.47x−4.44	0.91
	72	5.01	Y=2.56x−7.41	0.89
Third	24	36.18	Y=1.61x−0.63	0.99
	48	24.73	Y=2.06x−1.74	0.97
	72	17.37	Y=2.35x−3.71	0.93
Fourth	24	36.07	Y=1.38x−0.88	0.98
	48	31.35	Y=1.58x−0.78	0.98
	72	29.19	Y=1.76x−0.01	0.99

LC, lethal concentration; R, coefficient of regression equations.

4. Discussions

Natural botanicals are playing an important role as a suitable alternative to synthetic pesticides, whose application is safe due to vast availability and their easy degradable property. Although several plants from different families have been reported for mosquitocidal property[19,20] only a few botanicals have moved from laboratory to field use like *Chrysanthemum cinerarifolium* (Family: Compositae)[21] which has also been used in indoor sprays[22].

Different types of biological activities are played by the wide variety of secondary metabolites of plants. Most studies reported active compounds responsible for mosquito larvicidal property as steroidal saponins. Wiesman & Chapagain[23] revealed that saponin extracted from the fruit of *Balanites aegyptica* showed 100% mortality against larvae of *Stegomyia aegypti* (*S. aegypti*). The larvicidal property of a saponin mixture isolated from *Cestrum diurnum* was also evaluated against *An. stephensi* mosquito by Ghosh & Chandra[10]. Alkaloids derived from *Piper longum* fruit and *Triphyophyllum pellatum* reported by Lee[24] and Francois *et al*.[25], exhibited larvicidal activity against *Culex pipiens* (*Cx. pipiens*) and *An. stephensi*, respectively. Joseph *et al*.[26], showed isoflavonoids from tubers of Neorautanenia mitis had a larvicidal effect against the mosquitoes, *Anopheles gambiae* and *Cx. quinquefaciatus*, respectively. The impact of phenolic compounds on the mosquito larvae has also been evaluated[27, 28]. Cavalcanti *et al*.[29] reported that the essential oils extracted from Brazilian plants having larvicidal activity against *S. aegypti*. Khanna & Kannabiran[30] reported the role of tannin compounds extracted from *Hemidesmus indicus*, *Gymnema sylvestre* and *Eclipta prostrate* that causes mortality of *Cx. quinquefasciatus* larvae.

In the present study, the crude extract of mature leaves of *L. acidissima* was found to exhibit considerable mosquito larvicidal activity against *Cx. quinquefasciatus*. The highest mosquito larvicidal activity was noted in chloroform: methanol extract. The qualitative and chromatographic study exhibited the presence of several bioactive compounds and the probit analysis of bioassay experiment revealed the LC_{50} values of the bioactive compounds against different instars of *Cx. quinquefasciatus* larvae which were significant. However, the IR spectra and GCMS analysis of the bioactive compounds during the present study also indicated that presence of steroid compound(s) which may be responsible for larval toxicity.

In the present study *L. acidissima* leaf extract produced high mortality against the target mosquito species which might be due to the actions of a particular bioactive compound or synergistic effects of others. Phytochemical analysis of the leaf extract revealed the presence of some other compounds in addition to steroidal compounds. From GCMS analysis six major compounds have been identified and of them; thymol[31], benzoic acid[32] and dibutyl phthalate[33] have been previously reported for their larvicidal activity. Thus, the identified steroid compounds or the synergistic activity are responsible for larval mortality in the bioassay experiment. Further studies are required to identify the particular compound (s) and the specific mechanism of action of the bioactive principle present in the leaves of *L. acidissima*.

Conflict of interest statement

We declare that we have no conflict of interest.

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