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Larvicidal efficacy of *Vangueria spinosa* Roxb. (Rubiaceae) leaf extracts against filarial vector *Culex quinquefasciatus*

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Abstract
Culex quinquefasciatus is the transmitter of some dreadful vector-borne diseases. Such diseases can be controlled by managing the vector population. In this study, the mosquito larvicidal activity of the leaf extracts of a deciduous shrub *Vangueria spinosa* was evaluated for the first time. Larval mortalities were assessed using crude and different solvent extracts of young leaves of *V. spinosa* with different concentrations following standard methods. Thin-layer chromatography was done to characterize the major bioactive components liable for larval mortality. Cent percent mortality of the third instar larvae of *Cx. quinquefasciatus* was recorded at 2.5% concentration of crude extract within 48 hrs. Chloroform: Methanol extract was found to be the deadliest and 100% mortality of third instar larvae was reached in 48 hrs at 50 ppm concentration. The active ingredient which seems to be mainly responsible for larval mortality was steroidal in nature. LC_{50} of the fraction was calculated as 13.34 ppm for third instar larvae in 24 hrs. No significant effect of these extracts was noted on non-target organisms. So, the leaf of *V. spinosa* can be used as a potential source for the preparation of mosquito larvicide.

Keywords: Vector biocontrol, *Culex quinquefasciatus*, *Vangueria spinosa*, phytochemicals, chloroform: methanol extract

1. Introduction
Mosquitoes are the transmitting agents of several vector-borne diseases in humans as well as other animals. *Culex quinquefasciatus* (Diptera: Culicidae) is the principal man-biting tropical member of the *Cx. pipiens* complex and known to be transmitting West Nile Virus, Chikungunya, Lymphatic Filariasis, and many more. Disruption of the transmission by either preventing adult mosquitoes to bite humans or by killing the mosquitoes can be approached to manage the diseases transmitted by mosquitoes. The killing of larvae on large scale at the breeding sites of the vectors can be a strategy to manage mosquito populations and mosquito-borne diseases as well [1].
Conventionally chemical synthetic insecticides are used to kill mosquitoes with reported ill effects on the environment and living organisms as well, besides using some biocontrol agents like fishes [2, 3], Insects [4], etc. Environmental management through the replacement of host plants may also reduce some mosquito species [5]. Phytochemicals have the potential to replace synthetic insecticides in the mosquito control program. The use of phytochemicals as mosquitocide [6 - 9], bactericide [10 - 12], anthelmintic [13, 14], pesticide [15, 16], etc. is preferred now for easy availability, biodegradability, and less proneness to develop resistance.
Vangueria spinosa Roxb. (Rubiaceae) (Synonym: *Meyna spinosa*) found in tropical and subtropical regions of the world including India is a small deciduous shrub. It is single to multi-stemmed and can attend a highest up to 7 m, the bark of the tree is smooth and grayish to yellowish-brown in colour. The light green leaves are single, oppositely arranged, and covered with soft-velvety short hairs. Some herbivores graze the leaves. Different parts of this plant are traditionally used as food, to enhance blood purification, as herbal shampoo, and to treat kidney problems, diphtheria, dysentery, helminthiasis, necrosis, etc. Different parts of the tree also possess pharmacological value too, such as antioxidant properties, anti-fungal activity, peroxidase activity, bactericidal properties, etc. [17 - 19].

Etiologies of lymphatic filariasis can affect different groups of the society severely [20, 21]. Millions of people live in the lymphatic filariasis endemic areas and are at risk of getting an infection [22, 23]. *Cx. quinquefasciatus* is the principal transmitter in most regions [24-26] with a role played by other mosquito species as well [27, 28]. To eliminate the disease, along with the application of chemotherapeutic strategies [29], a vector control strategy is vital [8, 23]. The present study was intended to evaluate the larvicidal role of leaf extract of *V. spinosa* on filarial vector *Cx. quinquefasciatus* and primary characterization of the major active element causing the mortality.

2. Materials and Methods

2.1 Collection of Plant Materials

Healthy young (1-3 weeks old) leaves of *V. spinosa* were collected from different localities of the Purba Bardhaman district (West Bengal, India) in March-April, 2021.

2.2 Rearing of Test Mosquitoes

For the experiments, larvae of *Cx. quinquefasciatus* were obtained from the stock of the mosquito colony maintained at the Mosquito and Microbiology Research Units in the Department of Zoology of The University of Burdwan, West Bengal, India. The colony was protected from insecticides, pathogens, or other harmful agents. The temperature was maintained at 27±2 °C, relative humidity at 80~85%, and a photoperiod of 13:11 (light: dark). The larvae were provided a diet of Brewer yeast, algae, and dog biscuits in a ratio of 3:1:1 [8, 30].

2.3 Preparation of Crude Extract

Collected leaves were initially rinsed in distilled water to remove all dirt and the excess water was absorbed with paper towels. The leaves were crushed in an electric grinder machine. Whatman No. 1 filter paper was used to filter the crushed material and the resulting filtrate liquid was preserved in the freezer at 4°C as the stock solution of 100% concentration. Required crude extract concentrations (0.5%, 1.0%, 1.5%, 2.0%, 2.5%, and 3.0%) for the bioassay were prepared by mixing an appropriate quantity of the stock solution and distilled water [8].

2.4 Preparation of Different Solvent Extracts

Leaves of *V. spinosa* were washed properly and then dried in the shed for a few days. Two hundred grams of dry leaves have been chopped into small pieces and taken in a glass Soxhlet apparatus's porous thimble or extraction chamber. Two liters of each solvent namely petroleum ether, benzene, ethyl acetate, chloroform: methanol (1:1 in v/v), acetone, and absolute alcohol in a non-polar to polar fashion were loaded separately one after another on the solvent-boiling glass flask with the same leaves. The extraction time set for each solvent was 72 hrs. After 72 hrs the extracts from the flask were collected separately in separate beakers, filtered through Whatman No. 1 filter paper, and condensed in a rotary evaporator. These extracts were weighed and preserved in the refrigerator at 4 °C. Graded concentrations (10, 30, and 50 ppm) of the solvent extracts for the bioassay were prepared by dissolving them in desirable volumes of distilled water.

2.5 Dose-Response Larvicidal Bioassay

100 ml of each concentration of the plant extracts (first the crude and then extracts prepared with different solvents) were transferred into separate 100 ml size sterile glass beakers. Twenty-five larval *Cx. quinquefasciatus* were brought into each of those beakers containing graded concentrations of extracts. After 24, 48, and 72 hrs exposure the number of dead larvae was recorded. The larvae were considered as dead when they remained unmoved on probing [8]. During the experiments, the ambient temperature was 28±2 °C, relative humidity at 85±2%, and photoperiod of 13:11 (light: dark). The control experiment without any extract was also run in parallel.

2.6 Characterization of the Plant Extract (TLC analysis)

Extracts exhibiting the highest mortality in the larvicidal bioassay (which happens to be the chloroform: methanol extract in this experiment) was subjected to thin-layer chromatography (TLC). The mobile phase used was Benzene: ethyl acetate (1:1 in v/v) against the silica gel as a solid or stationary phase. After chromatography, for visualization of the bands, the TLC plates were kept in an iodine chamber. Distance traveled by the solute (spots) and the solvent (solvent front) from the baseline was noted for the calculation of Retention (Rf) values of the main positive spots. The TLC plates were also sprayed with different identifying reagents (Liebermann - Burchard reagent, Anthrone reagent, Antimony chloride, Ceric sulphate - sulphuric acid, Dragendorff's reagent, Folin - Ciocalteu reagent, Formaldehyde - phosphoric acid, Ninhydrin, Silver nitrate, Vanillin - phosphoric acid, etc.) to identify the chemical nature of the positive spots developed from the extract, which were supposed to contain the active ingredient. About 50 TLC plates were prepared and analyzed.

2.7 Isolation and Bioassay with the Active Ingredients

From about 40 chromatogrammed plates the major positive spots (with similar Rf value, which happened to be 0.58 in this experiment) were scrapped and dissolved in absolute alcohol and the silica gel fraction deposited at the bottom of the container was discarded. After evaporation of the absolute alcohol, the solid mass was collected and weighed and considered the most effective fraction. That mentioned fraction has been dissolved in suitable volumes of distilled water to obtain desirable concentrations (5, 10, 15, 20, and 25 ppm) for the bioassay. To test the larval toxicity, 100 ml of each concentration was taken separately in 100 ml size sterilized glass beakers and twenty-five larval *Cx. quinquefasciatus* were introduced in each. Larval deaths were noted in 24, 48, and 72 hrs.

2.8 Effect on Non-target Organisms

Larvae of Chironomids and nymphs of *Diplonychys annulatum* share similar aquatic habitats as mosquito larvae and were carefully chosen as the non-target organisms to assess the effect of phytochemicals under trial. They were exposed for 72 hrs to LC_{50} value of crude and solvent extract of 3rd instar *Cx. quinquefasciatus* larvae separately.

Each set of bioassays/experiments with extracts and the control set was performed in triplicate for each larval instars (1st, 2nd, 3rd, and 4th).

All the chemicals used in the experiments were of analytical grade (Merck). Extract preparation with Soxhlet apparatus, phytochemical analysis, TLC, and bioassay were conducted following standard protocols with little modifications [31 - 35]. The results obtained were analyzed statistically [36 - 38].

3. Results

Leaf extract of *V. spinosa* exhibited notable mortality against all instars larvae of *Cx. quinquefasciatus* in laboratory experiments. The 2.5% crude extract brought 100% mortality to the 1st instar larvae and 3.0% crude extract showed 100% mortality to the 2nd instar larvae within 24 hrs, while 2.5% crude extract resulted in 100% mortality to the 3rd instar larvae and 3.0% crude extract showed 100% mortality to the 4th instar larvae within 48 hrs (Table 1).

Bioassay with different polar and non-polar solvents against 3rd instar larvae showed the highest mortality in chloroform: methanol extract, followed by the absolute alcohol extract and acetone extract (Table 2). The percent mortality with chloroform: methanol extract was significantly higher than those with other solvent extracts (p<0.05).

Application of identifying reagents on TLC plates revealed that the major component of the chloroform: methanol extract was steroid in nature. The Rf value of the positive spots was noted to be 0.58, which also pointed out the existence of steroid compounds as active biochemical largely responsible for the highest larval mortality.

The isolated positive fraction of chloroform: methanol extract of *V. spinosa* leaves from the TLC plates, which seems to be steroids, showed high mortality against *Cx. quinquefasciatus* larvae (Table 3). Probit analysis of the percent mortality in chloroform: methanol extract fraction indicated LC_{50} values 2.18, 3.48, 4.03, and 4.14 ppm against 1st, 2nd, 3rd, and 4th instars larvae respectively on 72 hrs of exposure. The regression analysis confirmed that percent mortality (Y) was positively correlated with the concentration (X) of the extract (Table 4).

Multivariate ANOVA (Table 5) considering concentrations, hours of exposure, and larval instars as three parameters indicated no significant difference (P > 0.05) of the larvicidal effect of chloroform: methanol extract fraction of *V. spinosa* leaves against *Cx. quinquefasciatus*.

Treatment of non-target organisms with crude and solvent extracts depicted no significant abnormality or mortality in 72 hrs of exposure.

4. Discussion

Larvicides are used for decades to kill mosquitoes in their breeding sites and thus to control mosquitoes. But most of the conventional vector larvicides are synthetic, non-ecofriendly, non-specific toxic chemicals and moreover known to develop resistance in vectors. In recent times, vector biologists are trying to find answers to these problems by developing mosquito larvicides of botanical origin. Many plants develop secondary metabolites to protect themselves from predators which can be used as mosquito larvicides. Phytochemicals from thousands of plants are reported to possess larvicidal properties [39]. These botanical insecticides or phytochemicals are easily biodegradable, eco-friendly, relatively safe, less expensive, easily available, and above all, less prone to the development of resistance, and hence offer an advantage over synthetic insecticides [40, 41]. Probably the only disadvantage of the phytochemicals is their lesser stability to heat and light in comparison to synthetic insecticides which may be one of the causes that very few botanical products practically moved to the field for use from the laboratory.

Crude extract of young leaves of *V. spinosa* reveals strong larvicidal action against the larvae of *Cx. quinquefasciatus* in the present study. Maximum death was noted among the instars larvae and survivability was found to be positively correlated with the maturity of the larvae and negatively correlated with the time of exposure. Among the extracts with different polar and non-polar solvents, the maximum efficiency as a larvicidal agent was brought about by the 1:1 (v/v) chloroform: methanol extract, which was higher than that of the crude extract. This also indicates that any secondary metabolite present in the leaves of *V. spinosa* may be responsible for the mortality, which is found in some other studies also [42, 43].

TLC of chloroform: methanol extract indicated the existence of steroidal compounds and bioassay reveals a significant death rate in that fraction (p<0.05). Chloroform: methanol extract from some other plants is also reported to exhibit mosquito larvicidal potential [35, 44, 45] and comparisons show that the LC_{50} value of the chloroform: methanol extract fraction of *V. spinosa* leaves against *Cx. quinquefasciatus* larvae are quite acceptable. The minimum adverse effect of the test plant on the non-target organisms proves its specificity in action as a mosquito larvicide.

Table 1: Mortality of *Culex quinquefasciatus* larvae in the crude extract of *Vangueria spinosa* leaves

Larval Instars	Concentration (%)	Percentage Mortality (± SE)		
		24 hrs	48 hrs	72 hrs
1st	0.5	73.33±0.88	88.00±0.58	100.00±0.0
	1.0	80.00±1.16	98.67±0.33	100.00±0.0
	1.5	84.00±0.58	100.00±0.0	100.00±0.0
	2.0	96.00±1.00	100.00±0.0	100.00±0.0
	2.5	100.00±0.0	100.00±0.0	100.00±0.0
	3.0	100.00±0.0	100.00±0.0	100.00±0.0
	Control	0.00	1.33±0.33	1.33±0.33
2nd	0.5	65.33±0.33	88.00±0.58	100.00±0.0
	1.0	68.00±1.16	89.33±0.33	100.00±0.0
	1.5	73.33±0.88	92.00±0.58	100.00±0.0
	2.0	76.00±1.00	98.67±0.33	100.00±0.0
	2.5	96.00±1.00	100.00±0.0	100.00±0.0
	3.0	100.00±0.0	100.00±0.0	100.00±0.0
	Control	0.00	0.00	1.33±0.33
3rd	0.5	58.67±0.88	86.67±0.88	100.00±0.0
	1.0	64.00±1.00	88.00±1.16	100.00±0.0
	1.5	66.67±0.33	93.33±1.20	100.00±0.0
	2.0	65.33±0.67	97.33±0.67	100.00±0.0
	2.5	78.67±0.88	100.00±0.0	100.00±0.0
	3.0	88.00±1.00	100.00±0.0	100.00±0.0
	Control	0.00	0.00	0.00
4th	0.5	53.33±0.88	85.33±0.33	98.67±0.33
	1.0	60.00±0.0	86.67±0.33	100.00±0.0
	1.5	65.33±0.67	92.00±0.58	100.00±0.0
	2.0	74.67±0.67	96.00±0.58	100.00±0.0
	2.5	77.33±1.20	97.33±0.67	100.00±0.0
	3.0	86.67±0.33	100.00±0.0	100.00±0.0
	Control	0.00	0.00	0.00

Table 2: Mortality of 3rd instar *Cx. quinquefasciatus* larvae in different solvent extracts of *V. spinosa* leaves

Solvents	Concentration (ppm)	Percentage Mortality (± SE)		
		24 hrs	48 hrs	72 hrs
Petroleum ether	10	4.00±0.58	6.67±0.33	13.33±0.67
	30	0.00	9.33±0.33	16.00±0.58
	50	6.67±0.33	20.00±0.58	26.67±0.88
	Control	0.00	0.00	0.00
Benzene	10	0.00	6.67±0.33	9.33±0.67
	30	6.67±0.33	13.33±0.33	22.67±0.67
	50	13.33±0.88	16.00±1.00	29.33±1.20
	Control	0.00	1.33±0.33	2.67±0.33
Ethyl acetate	10	0.00	6.67±0.88	9.33±1.20
	30	6.67±0.33	16.00±1.00	25.33±0.33
	50	20.00±0.0	36.00±0.58	38.67±0.88
	Control	0.00	0.00	0.00
Chloroform: Methanol	10	73.33±0.88	82.67±0.67	92.00±0.58
	30	80.00±0.58	96.00±1.00	100.00±0.0
	50	93.33±0.33	100.00±0.0	100.00±0.0
	Control	0.00	0.00	0.00
Acetone	10	6.67±0.88	13.33±0.88	26.67±0.67
	30	13.33±0.88	26.67±0.88	40.00±1.16
	50	20.00±0.0	30.67±0.88	53.33±0.88
	Control	0.00	0.00	0.00
Absolute alcohol	10	13.33±0.88	26.67±0.88	49.33±0.88
	30	16.00±1.00	40.00±1.16	66.67±0.88
	50	22.67±0.67	49.33±0.88	82.67±0.67
	Control	0.00	0.00	0.00

Table 3: Mortality of *Cx. quinquefasciatus* larvae using the major fraction of Chloroform: Methanol extract of *V. spinosa* leaves

Larval Instars	Concentration (ppm)	Percentage Mortality (± SE)		
		24 hrs	48 hrs	72 hrs
1st	5	33.33±0.88	76.00±1.00	90.67±0.88
	10	42.67±0.33	84.00±0.58	92.00±0.58
	15	56.00±0.58	86.67±0.88	96.00±0.58
	20	82.67±0.67	94.67±0.33	100.00±0.0
	25	94.67±0.33	100.00±0.0	100.00±0.0
	Control	0.00	0.00	1.33±0.33
2nd	5	16.00±1.00	52.00±1.00	78.67±0.88
	10	25.33±0.67	72.00±0.58	90.67±0.33
	15	45.33±0.67	85.33±0.67	94.67±0.88
	20	80.00±0.00	93.33±0.67	100.00±0.0
	25	90.67±0.67	98.67±0.33	100.00±0.0
	Control	0.00	0.00	0.00
3rd	05	13.33±0.88	46.67±1.20	74.67±0.67
	10	24.00±0.58	70.67±0.88	86.67±0.88
	15	42.67±0.67	84.00±0.58	93.33±0.33
	20	76.00±1.00	90.67±0.67	100.00±0.0
	25	88.00±0.0	96.00±0.58	100.00±0.0
	Control	0.00	0.00	0.00
4th	05	9.33±0.33	36.00±1.00	72.00±1.00
	10	25.33±0.67	65.33±0.67	85.33±0.67
	15	42.67±0.67	81.33±0.33	92.00±0.58
	20	65.33±0.67	86.67±0.33	98.67±0.33
	25	84.00±0.58	93.33±0.67	100.00±0.0
	Control	0.00	0.00	0.00

Table 4: LC_{50} and LC_{90} values of the major fraction of Chloroform: Methanol extract of *V. spinosa* leaves calculated by log-probit and regression analysis

Larval Instars	Period of Exposure	LC_{50} (ppm)	LC_{90} (ppm)	Regression Equation	R^2 value
1st	24 hrs	9.19	26.31	y = 2.803x + 2.299	0.813
	48 hrs	3.66	10.94	y = 2.693x + 3.480	0.615
	72 hrs	2.18	6.36	y = 2.751x + 4.068	0.737

2nd	24 hrs	12.34	29.86	y = 3.335x + 1.359	0.865
	48 hrs	5.6	15.01	y = 2.992x + 2.760	0.899
	72 hrs	3.48	8.12	y = 3.479x + 3.115	0.846
3rd	24 hrs	13.34	32.41	y = 3.319x + 1.266	0.892
	48 hrs	5.77	18.27	y = 2.557x + 3.052	0.982
	72 hrs	4.03	8.62	y = 3.717x + 2.747	0.823
4th	24 hrs	14.63	36.73	y = 3.203x + 1.267	0.953
	48 hrs	6.96	21.75	y = 2.587x + 2.819	0.997
	72 hrs	4.14	9.86	y = 3.395x + 2.904	0.856

Table 5: Three-way ANOVA analysis using Concentration, Hours, and Instars as parameters

Source of Variation	df	Sum of Squares	Mean Squares	F value	P level
Concentration (C)	4	49398.756	12349.689	623.022	0.000
Hours (H)	2	51275.200	25637.600	1293.377	0.000
Instars (I)	3	3980.356	1326.785	66.934	0.000
C * H	8	13075.911	1634.489	82.457	0.000
C * I	12	1796.978	149.748	7.555	0.000
H * I	6	563.378	93.896	4.737	0.000
C * H * I	24	553.956	23.081	1.164	0.289
Residual	120	2378.667	19.822		
Total	179	123023.200			

5. Conclusion

So, it can be concluded that the leaf of *V. spinosa* can be used as a potential biocontrol agent (either crude, extracted, or isolated) against the larval form of medically important mosquito *Cx. quinquefasciatus*. However, further investigation on the identification and chemical structure of the active molecules, their mode of action, and proper field assessment are desired before applying them in the mosquito control program.

6. Conflict of interests

The author declares that they have no conflict of interest.

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