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Evaluation of mosquito larvicidal activity of *Azolla pinnata* leaf extracts against the filarial vector *Culex quinquefasciatus*

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Vector control is a major challenge now-a-days when they became resistance against commonly available insecticides. As an alternative, preliminary laboratory evaluation of *Azolla pinnata* crude and chloroform: methanol solvent extract was carried out under laboratory trials for control of *Culex quinquefasciatus*. Crude and solvent extract (chloroform: methanol) extracts of *A. pinnata* leaves were examined for the larvicidal activity against all the larval instars (1st to 4th) of *C. quinquefasciatus.* Dose-dependent mortality assays were performed using the extracts. Further, determinations of LC_{50} and LC_{95} values were accomplished through log-probit analyses and regression analyses. The larvicidal activity was statistically justified through ANOVA analyses. Effects of the extracts were examined on non-target water fauna. Exposure to *A. pinnata* crude and chloroform: methanol extract increased the mortality of first to fourth-instar *C. quinquefasciatus*. All the graded concentrations showed significant (P <0.05) larval mortality and the results of the regression equation revealed that the mortality rates were positively correlated with the concentrations of the extracts (R^2 close to 1). LC_{50} values of all instars after 24 h of exposure were between 86.99-294.06 ppm for crude and 48.87-111.44 ppm for chloroform: methanol extract. Chloroform: methanol extract is better than crude because the nature of biological components can be enhanced in presence of solvent and secondly the stronger extraction capacity could have produced a greater number of active constituents. The residual effect is noted even at the end of 72 h. A negligible toxicity to the larvae of *Chironomus circumdatus* was noticed as non-target organisms.

Keywords: *Azolla pinnata*, *Culex quinquefasciatus*, Larvicide, Leaf extract, Non-target organisms.

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Introduction

Mosquitoes are environmentally and economically significant insects because they transmit a variety of diseases that can be fatal. Filariasis is a disease transmitted by the vector *Culex quinquefasciatus* in tropical regions. The vectors of these diseases have long been a focus of disease eradication efforts[1]. Mosquito embryonic and larval stages have been a key target for researchers looking for medications to decrease mosquito populations due to their prevalence in confined spaces (small pools and puddles)[2-4]. For mosquito control, pyrethroids, carbamates, and organophosphates are among the synthetic insecticides available.

Random use of organophosphates such as temephos and fenthion and insect growth regulators such as diflubenzuron and methoprene leads to mosquito resistance against these chemicals[5]. The use of random synthetic pesticides leads to various types of cancer and birth defects in human beings[6]. Some 344 species have been reported to have a variety of activities against mosquitoes[7-14].

However, majority of them are contaminants that impair the ecosystem and non-target creatures[2,15,16]. When *Cx. quinquefasciatus* larvae were treated to the mosquito-control chemicals permethrin and temephos, respectively, they developed resistance[17,18]. As a result, novel medications or drug combinations must be tested in order to manage mosquito populations.

Other biological pest management strategies, such as the use of fungal pathogens, predators, traps, and plant-based medications, are used in addition to synthetic pesticides[2,16]. Plant-based pesticides are popular among biological mosquito control strategies because of their low cost, ease of availability, and environmental friendliness.

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Many species of plants include phytochemicals that can be exploited to build medications to combat disease-causing insects[19]. Chemicals found in plant extracts have been shown to be effective against a variety of medically significant insects[20,21]. Because phytochemicals have a wide range of chemical properties, their use could be a potential way to combat a variety of insect-borne diseases[22,23].

Azolla pinnata is a small aquatic fern, that lives symbiotic with blue-green algae and it provides nourishment material for paddy[24]. There are some scanty reports from different parts of the world having larvicidal properties[25-26]. It also contains several phytochemicals[27]. The purpose of the study is to test the efficacy of crude and solvent (Chloroform: methanol) extract of *A. pinnata* against all instar larvae of *Cx. quinquefasciatus*.

Materials and Methods

Plant collection and identification

Fresh leaves of *A. pinnata* were collected early in the morning during the study period (June-July, 2020) from outskirt ponds of Hatgobindapur, Burdwan, West Bengal, India and maintained in the laboratory in plastic tubs (21 cm diameter) containing a layer of rice field soils (4-5 cm) flooded with tap water. The tubs were kept outdoors in partially shaded places. The herbarium sheet was deposited in the Department of Botany, Dr Bhupendranath Dutta Smriti Mahavidyalaya, Hatgobindapur, West Bengal, India having voucher specimen no. IBB-OTDBNDSMAP1862020 and authenticated by Dr. Raj Narayan Roy, Assistant Professor of Botany, DBNDS Mahavidyalaya, Hatgobindapur, West Bengal, India. Superphosphates were applied once in five days at the rate of 2 g/m^2. Leaves collected were dried at room temperature. They were crushed to fine particle size by the helper of the blender.

Preparation of crude extract

Initially, the leaves were rinsed with distilled water and then dried on a paper towel. Extracts were prepared by grinding the leaves in a mortar and pestle, and passing through a cheese cloth. Then the requisite concentration of crude extract which was required to test larval mortality was prepared by mixing a suitable amount of sterilized water with the crude extract.

Preparation of solvent (Chloroform: methanol) extract

After collecting and processing, leaves were ground to a fine powder with the help of a mixer grinder. Then, 200 g of finely ground leaves were poured into grease-free Soxhlet apparatus and passed successively through a solvent extract from non-polar to polar of five organic solvents. The extracts were collected separately and filtered through a Whatman filter paper (No. 41). Each extract was subjected to a rotary evaporator below 40 °C and then concentrated to 100 mL. The resulting concentrated extract was kept in a deep freezer at -80 °C (REVCO, model no. ULT 790-3-V32) overnight, and then subjected to freeze to dry for 24 h at -60 °C. The resulting semi-solid extract was stored in a freezer until further used for bioassay.

Bioassay

The entire study was conducted according to standard test protocols[15]. Bioassays were performed on first to fourth-instar laboratory-bred *Cx. quinquefasciatus* species using all the above-mentioned concentrations. Then, following standard protocols of WHO[28], twenty-five larvae of different instars (1^{st}, 2^{nd}, 3^{rd}, and 4^{th}) were transferred into a beaker of 100 mL capacity. Distilled water was used as a control. The experiment was repeated thrice.

Graded concentrations of crude extracts (200, 100, 50, 30, and 10 ppm) were applied against all larval instar of *Cx. quinquefasciatus*. Before experimentation, the crude solvent extract (after lyophilization) was dissolved in distilled water to make 500 mL of volume. The stock solution was serially diluted to prepare test solutions of 200, 100, 50, 30, and 10 ppm solvent extract. One drop of emulsifier (Tween 20, Sigma Chemical Company) was added to ensure the complete solubility of the material in water. Petri dishes were kept at room temperature (28±2 °C) and 88±2% relative humidity. The mortality rates were recorded after every 24 hours. The larvae were supposed to be dead when they failed to move after probing with a needle in the siphon or cervical region[29].

Effect on non-target organism

The effect of the crude and solvent extract of the plant was tested against *Chironomus circumdatus* Kieffer (*Diptera:Chironomidae*). Ten larvae of *C. circumdatus* were exposed to LC_{50} value of 3^{rd} instar and mortality rate or other anomalies like listlessness or abridged swimming activity were observed after 72 h of post-exposure.

Phytochemical analyses

The phytochemical analyses of the plant extractive were carried out using the standard protocol of Harborne[30] and Stahl[31].

Ethical clearance

Ethical clearance for the study was obtained from IAEC, Approval No. 23/IAEC (06)/RNLKWC/2020, dt. 08.02.2020.

Statistical analysis

Lethal concentrations (LC_{50} and LC_{95}) were calculated using the process of Probit Analysis[32]. Percentage mortality in the treatments was corrected if necessary for mortality in the control using Abbot's formula[33]. The slope, lethal concentrations in ppm, probit regression, 95% confidence intervals, and chi-square were calculated. Univariate analysis was done to calculate the relationship of mortality with dose. The toxic effect of crude and solvent extracts was analyzed by multivariate analysis followed by Tukey's HSD test. Tests of between-subject effects were done to analyze how dependent variables differ from independent variables.

Results

Exposure to *A. pinnata* crude and chloroform: methanol extract increased the mortality of first to fourth-instar larvae of *Cx. Quinquefasciatus* in a concentration-dependent manner (Fig. 1). The 200

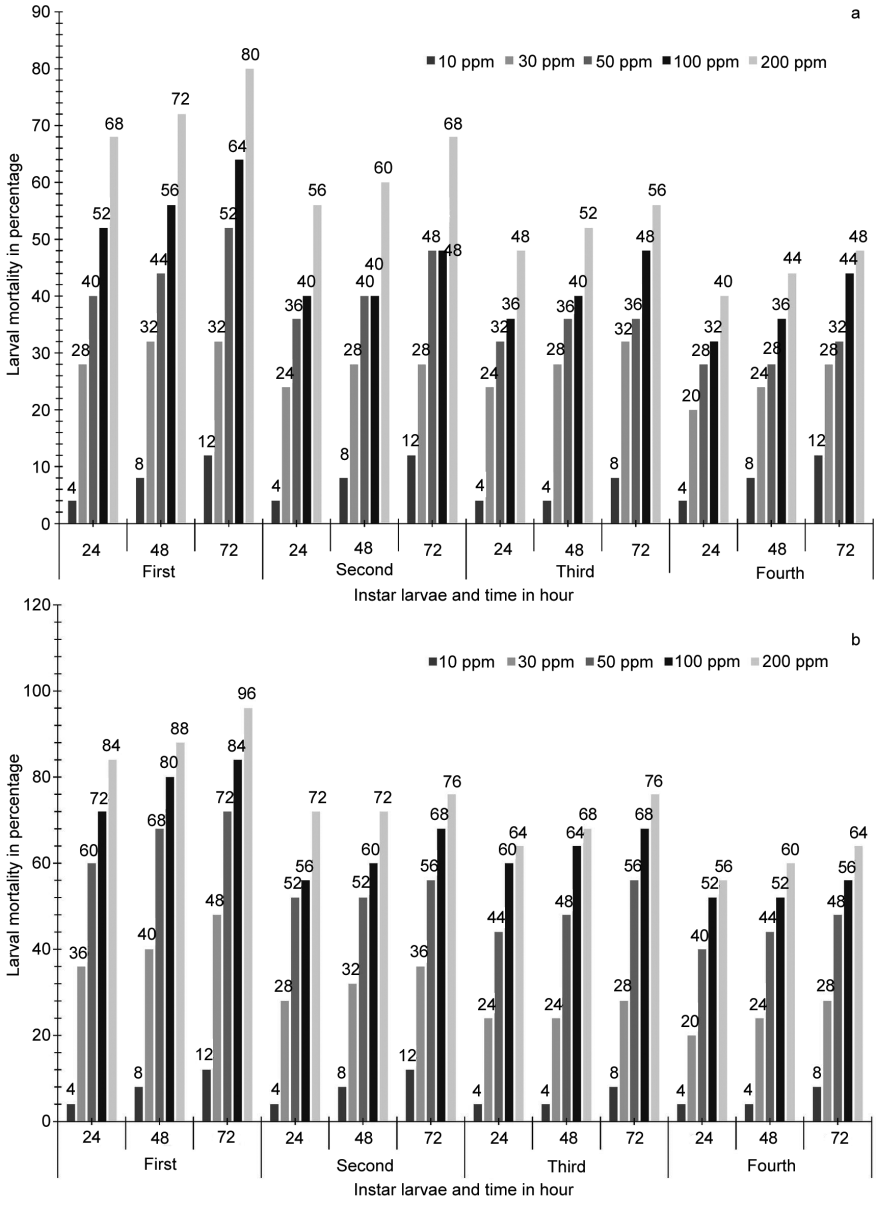

Fig. 1 — Mortality *of Culex quinquefasciatus* (first, second, third and fourth) immatures after treatment with *Azolla pinnata* at five different concentrations in three different hours (24, 48, 72), a) Crude extract and, b) Chloroform:methanol extract.

ppm concentration of leaf extract of crude and chloroform: methanol extract was most effective and killed 68 and 84% of the first instar in crude and chloroform:methanol extract within 24 h; 72 and 88% in 48 h and 80 and 96% within 72 h; 56 and 72% of the second instar in crude and chloroform:methanol extract within 24 h; 60 and 72% in 48h and 68 and 76% within 72 h; 48 and 64% of the third instar in crude and chloroform:methanol extract within 24 h; 52 and 68% in 48h and 56 and 76% within 72 h; 40 and 56% of the fourth instar in crude and chloroform:methanol extract within 24 h; 44 and 60% in 48 h and 48 and 64% within 72 h. This shows that chloroform:methanol extract is preferable to crude because the nature of biological components can be improved in the presence of solvent, and the stronger extraction capability could have resulted in a bigger number of active compounds.

LC_{50} values of *A. pinnata* extracts against *Cx. quinquefasciatus* are shown in Table 1. LC_{50} values of all instar after 24 h of exposures were between 86.99-294.06 ppm for crude and 48.87-111.44 ppm for chloroform:methanol extract, after 48 h the values were between 73.00-255.68 ppm for crude and 39.28 - 97.53 ppm for chloroform:methanol extract and after 72 h the values were between 55.48-180.99 ppm for crude and 31.57-80.99 ppm for chloroform:methanol extract. Differences in mortality rate among instar were statistically significant in 24, 48, and 72 h of exposures. First instar larvae were most susceptible in bioassay experiments with the lowest LC_{50} value after 24 h of exposures.

The regression equations, R^2 values of the mosquito species against crude and chloroform: methanol extract is presented in Table 1 using the mortality rates as the dependent variable, 'Y' and the

Table 1 — LC_{50} , LC_{95}, Regression equation and chi-square value on the larval mortality on the exposure of crude and solvent extract (chlorofom: methanol) of *Azolla pinnata* leaves

Solvent Used	Instars	Hours	LC_{50}			LC_{95}			Regression Equation	R^2	χ^2	DF	P
				LCL	UCL		LCL	UCL					
Crude	First	24	86.99	58.60	129.12	1050.69	307.53	3587.36	1.77+1.65X	0.960	0.769	3	0.856
		48	73.00	49.18	108.34	1023.45	295.01	3550.52	2.21+1.49X	0.981	0.343	3	0.951
		72	55.48	38.89	79.15	643.87	235.57	1759.85	2.28+1.55X	0.991	0.162	3	0.983
$ChCl_3$: MeOH		24	48.87	36.10	66.17	340.23	168.09	686.64	1.39+2.09X	0.952	1.165	3	0.761
		48	39.28	29.21	52.82	258.35	141.04	473.21	1.73+2.02X	0.960	0.721	3	0.868
		72	31.57	23.62	42.19	175.66	104.56	295.10	1.66+2.21X	0.992	0.146	3	0.985
Crude	Second	24	135.14	75.36	242.33	2815.97	400.81	19783.80	2.04+1.39X	0.931	1.070	3	0.784
		48	119.54	65.42	218.45	3458.17	404.91	29534.85	2.53+1.19X	0.935	0.874	3	0.831
		72	82.98	51.22	134.43	1939.73	344.88	10909.76	2.64+1.22X	0.952	0.677	3	0.878
$ChCl_3$: MeOH		24	71.60	49.85	102.84	773.69	261.75	2286.88	1.70+1.76X	0.933	1.070	3	0.784
		48	65.05	44.33	95.45	881.68	269.27	2886.95	2.21+1.52X	0.956	0.443	3	0.801
		72	51.68	35.58	75.05	691.07	234.57	2035.96	2.44+1.47X	0.968	0.140	3	0.932
Crude	Third	24	186.93	83.48	418.56	6274.00	416.82	94436.36	2.22+1.24X	0.903	1.200	3	0.753
		48	146.64	74.53	288.53	4280.82	408.89	44817.43	2.20+1.30X	0.882	1.639	3	0.650
		72	116.90	62.91	217.23	3870.64	393.22	38100.22	2.59+1.16X	0.934	0.806	3	0.848
$ChCl_3$: MeOH		24	84.34	57.13	124.52	995.01	297.02	3333.16	1.75+1.68X	0.938	1.194	3	0.754
		48	74.15	52.00	105.73	744.74	263.87	2101.89	1.64+1.78X	0.937	1.333	3	0.721
		72	57.69	41.12	80.94	571.46	227.02	1438.48	2.00+1.69X	0.954	0.924	3	0.819
Crude	Fourth	24	294.06	92.30	936.82	15210.05	394.06	587077.83	2.33+1.11X	0.903	0.967	3	0.809
		48	255.68	81.51	801.97	18859.46	402.07	884613.83	2.77+0.93X	0.955	0.359	3	0.948
		72	180.99	67.67	484.08	15659.17	381.83	642190.47	3.03+0.87X	0.963	0.261	3	0.967
$ChCl_3$: MeOH		24	111.44	68.95	180.13	1741.56	366.46	8276.55	1.89+1.52X	0.930	1.334	3	0.721
		48	97.53	62.14	153.07	1501.60	336.92	6692.30	1.90+1.55X	0.923	1.455	3	0.692
		72	80.99	51.91	126.35	1460.44	325.34	6555.80	2.35+1.37X	0.943	0.912	3	0.822

dose as the independent component 'X'. Mortality rate increases with the increasing rate of dose (R^2 closer to 1).

The result of chi-square is presented in Table 1. Chi-square shows a relationship between two categorical variables. All the calculated values are far less than the tabulated chi-square value 7.82 at a 0.05 level of significance. A low value of chi-square means there be a high correlation between sets of data. Therefore, chi-square is significant in all cases.

The result of the univariate analysis (Table 2) revealed that both instar and test concentrations have significant relations with mortality against both the extracts tested. But there was no significant difference when the interactions of the factors are considered.

The data of multivariate analysis (Table 3) revealed there was a statistically significant difference in mortality against test concentrations F (8,228)=44.68, P <0.0005, Wilk's Λ=0.152, partial η^2=0.61. To determine how the dependent variables (mortality and instar) differ for the independent variable (Test concentration), we need to look at the tests of the between-subjects effects table which is presented in Table 4. From this table we found that test concentration has a statistically significant effect with instar (F (4,115) = 107.965; P < 0.0005; partial η^2 = 0.790).

Table 2 — Univariate analysis

Source	Type III Sum of Squares	df	Mean Square	F	Sig	Partial Eta Square
Corrected Model	56348.80	19	2965.726	41.039	0.001	0.886
Intercept	194568.53	1	194568.53	2692.36	0.001	0.964
Instar (I)	4621.867	3	1540.622	21.319	0.001	0.390
Test conc (T)	50206.13	4	12551.53	173.68	0.001	0.874
I × T	1520.80	12	126.73	1.754	0.067 (N.S.)	0.174
Error	7226.66	100	72.26			
Total	258144.00	120				
Corrected Total	63575.46	119				

Table 3 — Multivariate analysis

Effect		Value	F	Hypothesis df	Error df	Sig	Partial Eta Square
Intercept	Pillai's Trace	0.978	2491.220	2.00	114.00	0.001	0.978
	Wilk's Lambda	0.022	2491.220	2.00	114.00	0.001	0.978
	Hotelling's Trace	43.706	2491.220	2.00	114.00	0.001	0.978
	Roy's Largest Root	43.076	2491.220	2.00	114.00	0.001	0.978
Test Conc	Pillai's Trace	0.848	21.179	8.00	230.00	0.001	0.424
	Wilk's Lambda	0.152	44.689	8.00	228.00	0.001	0.611
	Hotelling's Trace	5.595	79.027	8.00	226.00	0.001	0.737
	Roy's Largest Root	5.595	160.851	4.00	115.00	0.001	0.848

Table 4 — Test of between subject effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig	Partial Eta Square
Corrected Model	Mortality	50206.133	4	12551.33	107.965	0.001	0.790
	Instar	0.0001	4	0.00	0.000	1.000	0.000
Intercept	Mortality	194568.53	1	194568.33	1673.63	0.001	0.936
	Instar	750.00	1	750.00	575.00	0.001	0.833
Test Conc	Mortality	50206.13	4	12551.53	107.965	0.001	0.790
	Instar	0.000	4	0.000	0.000	1.000	0.000
Error	Mortality	13369.33	115	116.25			
	Instar	150.00	115	1.30			
Total	Mortality	258144.00	120				
	Instar	900.00	120				
Corrected Total	Mortality	63575.46	119				
	Instar	150.00	119				

Post-Hoc Tukey's test is presented in Table 5. Considering the mean difference or studentized range between instar two with three and instar three with four and vice versa are not significant at 0.05 level, but when test concentrations are considered all the values of mean difference are significant at 0.05 level.

No toxicity was recorded to the non-target *C. circumdatus* larvae in the bioassay test containing the crude and solvent extract that causes 50% mortality in the mosquito larvae after 24 h of exposure, but after 48 h, 1% mortality, and after 72 h of exposure, 1% mortality was recorded for crude and solvent extract after 48 h, 1% mortality and after 72 h of exposure 1.33% mortality was recorded (Table 6).

The result of the preliminary screening of phytochemicals from *A. pinnata* leaves is given in Table 7. Phytochemicals like tannin, saponin, steroid, flavonoids and, alkaloid-freeglycoside-bound anthraquinones were detected from the plant leaves. No abnormalities related to the sluggishness of swimming activity were observed but a little number of mortalities in the non-target organism after 72 h of exposure.

Table 5 — Post Hoc Tukeys test for instar and test concentrations

Instar (I)	Instar (J)	Mean Difference (I-J)	Sig	Test Conc (I)	Test Conc (J)	Mean Difference (I-J)	Sig
1	2	9.06*	0.001	1	2	-22.00*	0.001
	3	12.40*	0.001		3	-38.50*	0.001
	4	16.93*	0.001		4	-47.50*	0.001
2	1	-9.06*	0.001		5	-58.33*	0.001
	3	3.33	0.430 **(N.S.)**	2	1	22.00	0.001
	4	7.86*	0.001		3	-16.50*	0.001
3	1	-12.40*	0.001		4	-25.50*	0.001
	2	-3.33	0.430 **(N.S.)**		5	-36.33*	0.001
	4	4.53	0.172 **(N.S.)**	3	1	38.50	0.001
4	1	-16.93*	0.001		2	16.50	0.001
	2	-7.86*	0.003		4	-9.00*	0.036
	3	-4.53	0.172 **(N.S.)**		5	-19.83*	0.001
				4	1	47.50*	0.001
					2	25.50*	0.001
					3	9.00*	0.036
					5	-10.83*	0.006
				5	1	58.33*	0.001
					2	36.33*	0.001
					3	19.83*	0.001
					4	10.83*	0.006

*. The mean difference is significant at the 0.05 level.

*. The mean difference is significant at the 0.05 level.

Table 6 — Toxicity of crude and solvent extract of *A. pinnata* to fourth instar *Chironomus* sp. larvae at the lowest concentration that produced more than 50% larval mortality in larvicidal test.

Concentrations	Solvents Used	24 h		48 h		72 h	
		Mortality of the third instar mosquito larvae (%)	Mortality of the non-target organism (%)	Mortality of the third instar mosquito larvae (%)	Mortality of the non-target organism (%)	Mortality of the third instar mosquito larvae (%)	Mortality of the non-target organism (%)
200 ppm (lowest concentration causing more than 50% mortality)	Crude	51	0	52	2	56	3
Control		0	0	0	1	1	1
73 ppm (lowest concentration causing more than 50% mortality)	Choloroform: methanol	53	0	57	3	63	4
Control		0	0	1	1	1	1.33

Table 7 — Qualitative analyses of phytochemicals of crude extract of tested plant leaves

Tanin	Saponin	Steroid	Flavonoid	Terpenoid	Cardic glycosides	Alkaloid free glycoside bound anthraquinones
++	++	++	++	--	--	++

Discussions

The development of insecticide resistance is very common. To overcome this alternate approach it is very much necessary which leads scientists all over the world to search for insecticides having a biological origin, which are effective but with fewer side effects, easily biodegradable in nature[34].

Secondary metabolites of plants are associated with a wide range of biological activities. Several review articles are published by scientists all over the world mention that different secondary biochemicals such as steroids, alkaloids, terpenoids, saponins, phenolics, and essential oils play a vital role in controlling mosquito immatures[35-39]. Ghosh *et al.*[40] reviewed the current state of knowledge on phytochemical sources and mosquitocidal activity, their mechanism of action on the target population, variation of their larvicidal activity.

Azolla plant is easily available in large quantities in Burdwan, West Bengal, India so if we explore it for mosquito control programs then it may reduce the dependence on expensive synthetic insecticides. However, further studies on the mode of action, active ingredients present in them, their effects on other non-target organisms, and formulations for improving their insecticidal potency are to be carried out for their standardization. Further research work is needed to carry out the on-field evaluation, and search of the active ingredient of this leaf against *Cx. quinquefasciatus*, species for environmentally safer botanical insecticide inventions.

Conclusion

Environmental protection is now considered to be of paramount importance. Eco-friendly insecticides with adequate mortality on target species should be promoted to keep pest populations below the threshold level. *A. pinnata* leaf extract had larvicidal efficacy against *Cx. quinquefasciatus* and could be used as a bio-larvicide for *Culex* mosquito vector control. Commercial exploitation of *A. pinnata*, which is extensively spread in West Bengal, could be an essential step toward producing a novel plant-based pesticide. Screening locally accessible plants for bio-insecticides could create jobs, reduce reliance on imported goods, and stimulate local efforts to improve public health.

Conflict of interest

The authors declare that they have no conflict of interest.

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