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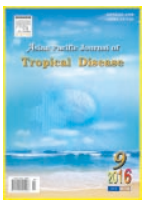

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Effect of seed kernel aqueous extract from *Annona squamosa* against three mosquito vectors and its impact on non-target aquatic organisms

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ABSTRACT

Objective: To evaluate the toxicity of *Annona squamosa* (*A. squamosa*) aqueous (physiological saline) seed soluble extract and its control of mosquito population.
Methods: Ovicidal, larvicidal and pupicidal activity of *A. squamosa* crude soluble seed kernel extract was determined according to World Health Organization. The mortality of each mosquito stage was recorded after 24 h exposured to plant material. Toxicity assay was used to assess the non-target organisms with different concentrations according to Organisation for Economic Co-operation and Development.
Results: The aqueous solubilized extracts of *A. squamosa* elicit the toxicity against all stages of *Aedes aegypti, Anopheles stephensi* and *Culex quinquefasciatus*, and the LC_{50} values against stages of egg, 1st-4th larvae were (1.45 and 1.26–2.5 mg/mL), (1.12 and 1.19–2.81 mg/mL) and (1.80 and 2.12–3.41 mg/mL) respectively. The pupicidal activity also brought forth amended activity against all three mosquitoes species, and the LC_{50} values were consider to be 3.19, 2.42 and 4.47 mg/mL. Ultimately there was no mortality observed from non-target organism of *Chironomus costatus*.
Conclusions: Based on the findings of the study, it suggests that the use of *A. squamosa* plant extract can act as an alternate insecticidal agents for controlling target mosquitoes without affecting the non-target aquatic insect. Further investigation to identify the active compounds and their mechanisms of action is recommended.

1. Introduction

Mosquitoes are more responsible for the spread of several transmitted diseases than any other group of arthropods are[1]. Mosquito-borne diseases still remain a major health problem in both human and veterinary sectors. These diseases are more prevalent in 128 countries, mainly tropical and subtropical countries causing millions of deaths. The biological control of immature stages now appears to be the most powerful tools of control the target populations of Culicidae and other dipteran pests. In worldwide around 3.9 billion people were affected by mosquito disease[2]. *Anopheles stephensi* (*An. stephensi*) is only responsible for the transmissions of malaria in urban regions of India which is endemic in 91 countries with about 40% of the world's inhabitants at risk. Up to 500 million cases occur every year, and 90% of them are in Africa, and there are up to 2.7 million deaths yearly. *Aedes aegypti* (*Ae. aegypti*) is a vector of dengue that carries the arbovirus solely responsible for dengue diseases. Recently, the occurrence of dengue has increased dramatically throughout the world. An estimated 500 000 people with severe dengue require hospitalization each year, a large proportion of whom are children. About 2.5% of those affected die[2]. *Culex quinquefasciatus* (*Cx. quinquefasciatus*) is an important mosquito vector of lymphatic filariasis which is a widely distributed tropical disease. Microfilaria is transmitted to humans by different mosquitoes like *Culex* species, with a special reference to *Cx. quinquefasciatus*. Lymphatic filariasis (elephantiasis) infects about 120 million people in tropical areas of Africa, India and Southeast Asia. In India more than 40 million people were affected by mosquito diseases annually[3]. In earlier days mosquito was controlled using synthetic chemicals known as organophosphate, organochlorine and pyrethroid, *etc.* Although these insecticide applications were highly effective against the target vector species, it was facing a threat due to the progress of resistance

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to chemical insecticides resulting in rebounding vectorial capacity. It has also provoked undesirable effects including toxicity to non-target organisms and fostered environmental and human health concerns. Several investigators have been resorted to explore the plant source as an alternate and to find eco-friendly bio-active compounds that are biodegradable into nontoxic products and potentially suitable for the use of mosquitoes control[4].

Plants have produced numerous organic compounds, many of which have medicinal and insecticidal properties. More than 2 000 plant species have been known to produce chemical factors and metabolites with values in pest control. However, the use of organic solvents extracts is relatively difficult for routine application at community level. Alternatively, aqueous extracts contain desired botanical source with ideal application towards mosquito control[5].

The aqueous (physiological saline) extract of *Sapindus emarginatus* (*S. emarginatus*) has elicited maximum mortality against three vectors of *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus*[6]. Similarly the toxicity of water extract of *Moringa oleifera* against third instar larvae of *Ae. aegypti* was studied[7]. Also, the aqueous extract of *Lantana camara* var. *aculeata* obtained maximum mortality among other solvent extract against fourth instar larvae of three mosquitoes species[8].

Annona squamosa L. (Annonaceae) (*A. squamosa*), commonly known as the custard apple tree is a native of West Indies but cultivated throughout India. *A. squamosa* showed a variety of biological activities including insecticidal, anti-tumor, anti-diabetic, anti-oxidant, anti-inflammatory and antibacterial activities[9]. The present study was to evaluate the ovicidal, larvicidal and pupicidal activity of physiological saline aqueous soluble extract of *A. squamosa* seed against *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus* and non-target aquatic organisms.

2. Materials and methods

2.1. Collection and processing of plant materials

The seeds of *A. squamosa* were purchased from local herbal shop and market and the seeds were identified by plant authentic taxonomist. The seed coat was manually removed, and seed kernel was grinded into fine powder and stored in refrigerator (4 ℃) for further use.

2.2. Preparation of extract

Ten grams of *A. squamosa* seed flour was mixed with 100 mL of 0.9% physiological saline solution and kept overnight at 4 ℃ and then the sample was stirred and centrifuged at 15 000 r/min for 30 min at 4 ℃. Supernatant was lyophilized for further bioassay experiment[6].

2.3. Determination of dry weight

The dry weight of the extract was determined gravimetrically using a sample (1 mL) with known weight of *A. squamosa* extract, which was completely dried in a desiccators containing fused calcium chloride. The dry weight was determined to the *A. squamosa* extract for successive bioassays. Although 10 g of kernels from the plant source was used to prepare the aqueous extract, the dry weight per milliliter of supernatant obtained from *A. squamosa* seed kernel extract considerably varied, evidently due to variation in the amount of water-soluble biochemical components present in kernel from different plant species.

2.4. Insect rearing

An. stephensi, *Ae. aegypti* and *Cx. quinquefasciatus* (Figure 1) mosquitoes eggs were collected from mosquito breeding site, Chennai, and brought to laboratory, then placed in water-filled tray (30 cm × 15 cm × 6.5 cm) and maintained under laboratory condition [(26 ± 2) ℃] with photoperiod of 12:12 h (Light: Dark) Feed of dog biscuits: yeast (3:1; w/w) was regularly provided. The larvae of *Chironomus costatus* (*C. costatus*) (Chironomidae: Diptera) were collected from Adyar River.

2.5. Ovicidal bioassay

The ovicidal bioassay was carried out according to World Health Organization recommended procedure. Three mosquito species eggs of *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus* were introduced into 50 mL of tap water containing different concentrations of (1–10 mg/mL) aqueous soluble extracts. The bioassay was left for 96 h with humidity temperature, and between these intervals the test medium was freshly replaced with the same concentration. After 96 h post-exposure, the eggs were transferred into normal tap water and the larval hatching was analyzed. The percentage of mortality of ovicidal bioassay was calculated by the number of unhatched eggs from the number of eggs exposued to extracts.

2.6. Larvicidal and pupicidal bioassay

The larvicidal and pupicidal activity was assessed and ten organisms in each assay were introduced into soluble extract with various concentrations (1–10 mg/mL) for 24 h exposure according to the procedure of World Health Organization[10] and method by Koodalingam *et al.*[6] with slight modifications.

2.7. Non-toxicity assessment

The collected larvae of *C. costatus* were exposed to different

concentrations of extracts according to Organisation for Economic Co-operation and Development[11]. Each test included a set of control groups (0.9% saline) with two duplicates for each individual concentration.

2.8. Determination of lethal concentration

Lethal concentrations (LC_{50} and LC_{90}) represented the concentrations of the test material that caused 50% and 90% mortality of the test organisms (target and non-target) within the specified period of test medium exposed, and it was also determined by exposing various developmental stages of mosquitoes with different concentrations of the crude test medium. Based on these mortality of the test organisms recorded in this bioassays, LC_{50} was calculated along the fiducial limits at 95% confidence level by probit analysis and *Chi*-square values were calculated by using the software developed by Reddy *et al.*[12].

3. Results

In the present study, we aimed to evaluate the mosquito control using aqueous seed extract of *A. squamosa* against all stages of *Ae. aegypti*. *An. stephensi* and *Cx. quinquefasciatus* (Figure 1). The ovicidal, larvicidal and pupicidal mortality was observed with maximum level within around 5 to 7 mg/mL using aqueous seed extract of *A. squamosa*. The ovicidal effect of *A. squamosa* aqueous seed extract appeared to be effective against *Ae. aegypti* (LC_{50} = 1.45 mg/mL; LC_{90} = 4.35 mg/mL), *An. stephensi* (LC_{50} = 1.12 mg/mL; LC_{90} = 3.36 mg/mL) and *Cx. quinquefasciatus* (LC_{50} = 1.80 mg/mL; LC_{90} = 5.40 mg/mL) (Table 1).

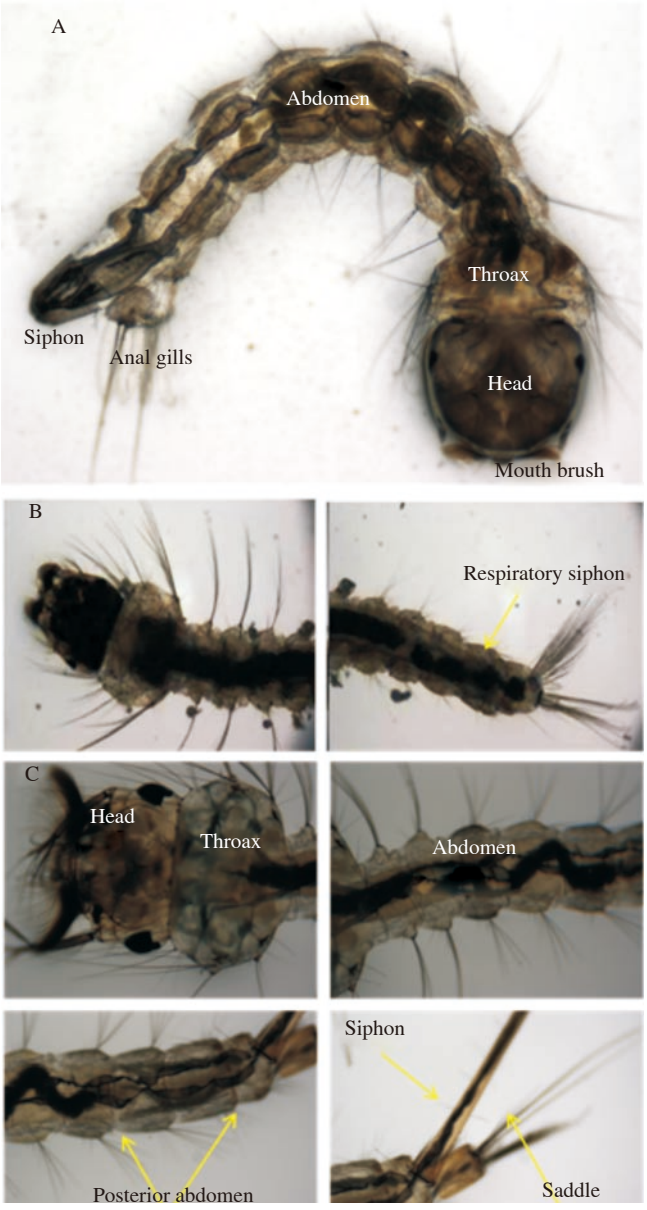

Figure 1. Three mosquitos species tested in this study.
A: *Ae. aegypti*; B: *An. stephensi*; C: *Cx. quinquefasciatus*.

Table 1
Ovicidal, larvicidal and pupicidal activity of aqueous extract of *A. squamosa* against *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus*.

Species	Mosquito stages	LC_{50} (LCL–UCL) (mg/mL)	LC_{90} (LCL–UCL) (mg/mL)	Slope	r^2
Ae. aegypti	Egg	1.45 (1.06–1.98)	4.35 (3.09–5.07)	57	0.993
	1st	1.26 (0.90–1.77)	3.78 (2.92–4.02)	60	0.988
	2nd	1.86 (1.49–2.33)	5.67 (4.53–6.04)	65	0.983
	3rd	2.09 (1.81–2.40)	6.27 (5.49–6.99)	45	0.986
	4th	2.51 (2.27–2.79)	7.59 (6.49–8.32)	50	0.999
	Pupae	3.19 (2.73–3.74)	9.57 (7.43–11.70)	55	0.987
An. stephensi	Egg	1.12 (0.81–1.56)	3.36 (3.13–4.94)	77	0.990
	1st	1.19 (0.91–1.56)	3.57 (2.99–4.01)	65	0.899
	2nd	1.80 (1.54–2.10)	5.40 (4.02–6.41)	30	0.786
	3rd	2.07 (1.80–2.37)	6.21 (5.03–6.98)	95	0.989
	4th	2.81 (2.50–3.17)	9.51 (7.93–11.37)	65	0.789
	Pupae	2.42 (2.01–2.92)	7.26 (5.94–9.07)	30	0.999
Cx. quinquefasciatus	Egg	1.80 (1.41–2.29)	5.40 (4.32–6.37)	82	0.987
	1st	2.12 (1.69–2.67)	6.36 (5.43–7.78)	45	0.979
	2nd	2.43 (2.03–2.92)	7.29 (6.44–8.37)	70	0.986
	3rd	2.97 (2.65–3.33)	8.91 (7.33–9.03)	75	0.999
	4th	3.41 (3.06–3.81)	10.23 (8.11–11.37)	45	0.878
	Pupae	4.47 (3.82–5.23)	12.41 (10.33–15.98)	45	0.983

LC_{50}: Lethal concentration that kills 50% of the exposed eggs, larvae and pupae; LC_{90}: Lethal concentration that kills 90% of the exposed eggs, larvae and pupae; LCL: Lower confidence limit; UCL: Upper confidence limit; r^2: Regression coefficient; 1st–4th: The first to fourth larvae instar.

The larvicidal activity of *A. squamosa* aqueous seed extract against first to fourth instars of *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus* was tested. The results showed that the percent mortalities were as followed: 100, 100, 75, 60 and 45; 100, 85, 65, 50 and 35; 90, 80, 65, 45 and 25; 90, 70, 50, 40 and 15 respectively against 1st, 2nd, 3rd and 4th instar larvae of *Ae. aegypti* (Figure 2); 100, 100, 85, 65 and 45; 100, 100, 80, 50 and 30; 100, 95, 65, 45 and 25; 85, 65, 50, 30 and 20 respectively against 1st, 2nd, 3rd and 4th instar larvae of *An. stephensi* using plant extract exposured (Figure 3) and 90, 80, 60, 45 and 35; 85, 70, 55, 40 and 30; 75, 65, 50, 30 and 15; 70, 55, 45, 25 and 10 respectively against 1st, 2nd, 3rd and 4th instar larvae of *Cx. quinquefasciatus*, respectively (Figure 4). The lethal effect against first to fourth instars larvae of *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus* is shown in Table 1.

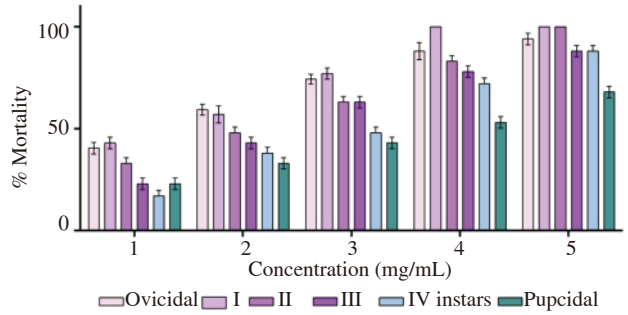

Figure 2. Percent mortality of all stages of *Ae. aegypti* mosquito for 24 h.

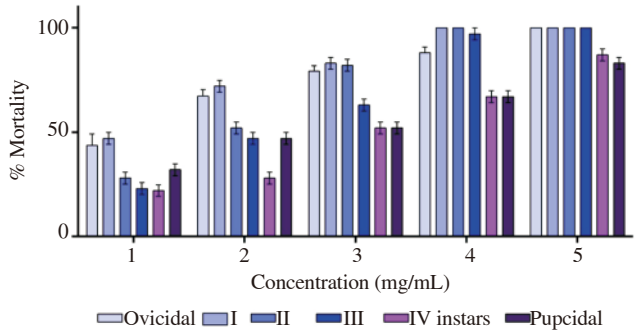

Figure 3. Percent mortality of all stages of *An. stephensi* mosquito for 24 h.

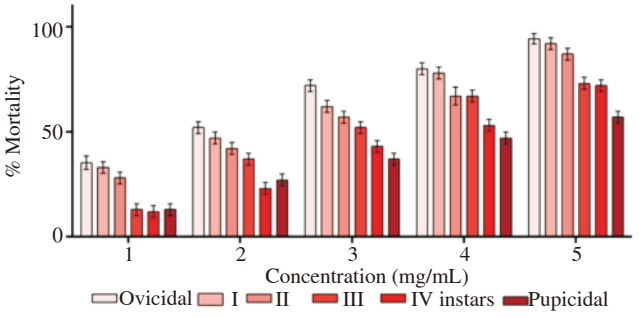

Figure 4. Percent mortality of all stages of *Cx. quinquefasciatus* mosquito for 24 h.

The pupicidal percent mortalities of *Ae. aegypti* recorded were 70, 55, 45, 35 and 25; *An. stephensi* were 85, 65, 50, 45 and 30 and *Cx. quinquefasciatus* were 55, 45, 35, 25 and 15, respectively (Figures 2–4). The pupicidal lethal effect of *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus* was shown in Table 1. The control (distilled water) showed nil mortality in the concurrent assay.

The lowest LC_{50} and LC_{90} values of *A. squamosa* extract (LC_{50} = 1.26, 1.86, 2.09 and 2.51 mg/mL; LC_{90} = 3.78, 5.67, 6.27 and 7.59 mg/mL), (LC_{50} =1.19, 1.80. 2.07 and 2.81 mg/mL; LC_{90} = 3.57, 5.4, 6.21 and 9.51 mg/mL) against all four instars (1st to 4th) of *Ae. aegypti* and *An. stephensi* laboratory strain were demonstrated (Table 1). While, the slight low concentration variations of LC_{50} and LC_{90} values of (LC_{50} = 2.12, 2.43, 2.97 and 3.41 mg/mL; LC_{90} = 6.36, 7.29, 8.91 and 10.23 mg/mL), respectively (Table 1) were obtained by *A. squamosa* extract against *Cx. quinquefasciatus* field strain (Table 1), which concluded that this plant extract was more efficient in killing mosquito larvae.

In addition, the toxicity test was studied to investigate the toxicological property of *A. squamosa* against *C. costatus* for 24 h, and no mortality was noted when cup was slowly shaked after 24 h treatment.

4. Discussion

In this study, the *A. squamosa* seed was used due to economical enhancement as well as to make perfect environment free from mosquito population. Based on this aspect, the mosquitoes have been controlled continuously by using plants dependent insecticidal source without affecting the non-target organisms in aquatic eco-systems. Plant has numerous biological active compounds and is composed of primary and secondary metabolites, which has been characterized to various biological activities including mosquito control[13]. Several researchers reported the aqueous extracts (distilled water) of different parts of indigenous plants tested against one or two species of only selected developmental stages of mosquito[14]. In a similar way, the nine different plants were screened and it is reported that the aqueous extract of *Piper retrofractum* fruits showed the highest larvicidal effect against third- and fourth-instar larvae of *Ae. aegypti* and *Cx. quinquefasciatus*[15]. Several investigators screened 36 plants using aqueous extract, among which 29 plants have potent larvicidal activity with selected mosquitoes stage and especially the *Azadirachta indica* caused 100% mortality against *Aedes*, *Mansonia* and *Culex* sp., at 0.5 mL for 24 h[16]. Besides, the toxicity of *A. squamosa* aqueous soluble extract has been revealed with LC_{50} and LC_{90} values, which were more susceptible to the extract against three tested species of all stages, but in our investigation, these extracts were also more susceptible and elicited maximum mortality (100%) against *Cx. quinquefasciatus* like other species. In contrast with the pupal stage of *Culex* sp., it was less susceptible to the extract. In a similar study, the aqueous extracts of *Sapindus emarginatus* showed effective ovicidal, larvicidal and pupicidal activity against two vectors of *Ae. aegypti* and *An. stephensi*, and were less susceptible to the *Cx. quinquefasciatus* with all stages like egg and first to fourth instars of mosquito species especially to pupae stage[6]. More than one study have reported that *Ae. aegypti* was more susceptible than *Cx. quinquefasciatus* when exposed to three plant extracts[17]. But in

our investigation, only the pupal stage has been shown that it is less susceptible to the extract not like other stages of egg and larvae (I to IV) of *Cx. quinquefasciatus*. For the differences in the susceptibility of different mosquito species to the plant extract, it may be attributed to inherent differences in physiological mechanisms among various mosquitoes species tested.

Leaf extracts of *Gliricidia sepium* showed potent larvicidal activity and did not show any lethal effect against freshly moulted tadpole and guppy fry[18]. In our study, the potent antimosquito activity of aqueous soluble extracts of *A. squamosa* did not cause any mortality against non-target aquatic insects of *C. costatus*, even with preferable concentration exposed for 24 h. In a similar study the *Sapindus emarginatus* seed kernal aqueous extracts caused mortality with LC_{50} = 5.71 mg/mL and LC_{90} = 9.29 mg/mL after 24 h against two non-target aquatic insects of *C. costatus* and *Diplonychus rusticus*[6]. All these experimental observations of *A. squamosa* seed kernel aqueous soluble extract suggested that this soluble extract is plausibly safe for aquatic non-target organisms, and the prime action of the biocidal molecules in the extract appears to be a menace towards various developmental stages of the mosquitoes. Thus, the findings of this study clearly demonstrate the potent anti-mosquito property of aqueous soluble seed kernel extract from *A. squamosa* for its ability to kill all the developmental stages of three important mosquito species. Further studies are needed to identify the bioactive molecules from this extract and evaluate its systemic effects on target mosquitoes, which would eventually enable the application of the *A. squamosa* extract as an eco-friendly biocidal agent for the effective control of vector mosquitoes.

Conflict of interest statement

We declare that we have no conflict of interest.

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