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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**Objective:** To evaluate the toxicity of *Annona squamosa* (A. squamosa) aqueous (physiological saline) seed soluble extract and its control of mosquito population.

**Methods:** Ovicultural, 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 exposed 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 *Cx. quinquefasciatus*, and the LC50 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 LC50 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]. *Cx. quinquefasciatus* (*C. 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*, 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.
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 2000 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 °C) 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 °C and then the sample was stirred and centrifuged at 15000 r/min for 30 min at 4 °C. Supernatant was lyophilized for further bioassay experiment[8].

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 millilitre 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) °C] 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 exposed 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

| 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 (3.14–4.94)             | 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 larval 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 exposed (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](image1.png)

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

![Figure 3](image2.png)

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

![Figure 4](image3.png)

**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 shaken 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 *C. 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 mosquito species tested.

Leaf extracts of *Gliciridia 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 kernel 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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