Effect of Azadirachta indica A. Juss (Meliaceae) Seed Oil and Extract Against Culex quinquefasciatus Say (Diptera: Culicidae) Larval Susceptibility of Indian Subcontinent

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Abstract
Background. Development of resistance to chemical pesticides among the mosquito vectors leads researchers to investigate the potential mosquito control efficacy of various plant extracts.

Aim. To assess the larvicidal activity of neem (Azadirachta indica A. Juss; Meliaceae) seed extract and oil against Culex quinquefasciatus Say (Family: Culicidae).

Materials and Methods. In order to determine the median lethal concentration (LC50), following probit analysis the fourth instar larvae of C. quinquefasciatus were exposed to A. indica seed extract and oil in different concentrations ranging from 2 to 64 μg/ml, and larval mortality was observed for each concentration after 24 hours of treatment. The median lethal time (LT50) values for a single concentration (2 x LC50) of oil and extract were determined based upon probit analysis.

Results. The 100 % larval mortality was observed due to both A. indica seed oil and extract at concentrations 32 μg/ml and 64 μg/ml, respectively. The A. indica oil and extract had LC50 values 8.041 and 15.495 μg/ml, respectively, and the LT50 values were 8.328 and 15.322 min, respectively.

Conclusion. The A. indica seed oil and extract showed excellent larvicidal activity against C. quinquefasciatus, and thus the products can be used effectively as indigenous mosquito control agents, alternative to conventional chemical mosquito larvicides.

Introduction
The mosquitoes constitute a world wide public health problem as vectors of serious human diseases. The mosquito Culex quinquefasciatus Say (Family: Culicidae) is the potential vector of bancroftian filariasis throughout the world including India. The high C. quinquefasciatus population density in the cosmotropical area has triggered several interventions by the public health authorities using wide synthetic insecticide as the main means of combat and control. The conventional organophosphate, carbamate insecticides and pyrethroids that are generally used for mosquito control are known to cause the problem of environmental pollution, residual effects and resistance by their indiscriminate use [1-3]. The control of C. quinquefasciatus borne diseases are thus becoming increasingly difficult, and the mosquitoes contribute significantly to poverty and social debility in developing countries, like India. This dictates the need to develop environmentally safe, cost effective and preferably locally available agents for mosquito control.

One alternative approach is the use of natural...
products from plant origin, and modern research thus focuses on botanicals having larvicidal, oviposition inhibiting, repellent as well as insect growth regulatory effects [4-6]. These agents possessing multiple active ingredients with various modes of action reduce the chance of resistance development among mosquito populations, and in addition the botanical insecticides are generally pest specific, biodegradable and relatively harmless to non-target organisms [7].

The mosquito control at the larval stage of development with phytochemicals that occur in the oils, leaves and roots of plants is one of the techniques which affords a cheap, easy to use, and environment friendly method of filaria control. Studies have shown the potential of plants for use in C. quinquefasciatus larvae control: Agave americana Linn. (Family: Agavaceae) and Kaempferia galanga Linn. (Family: Zingiberaceae) [8, 9], Centella asiatica Linn. (Family: Apiceae) [10], Vitex negundo Linn. (Family: Lamiaceae), Nerium oleander Linn. (Family: Apocynaceae) and seeds of Syzygium jambolanum Linn. (Family: Myrtaceae) [11], Nerium indicum Mill. (Family: Apocynaceae) and Euphoria royleana Boiss. (Family: Euphorbiaceae) [12] as well as Azadirachta indica A. Juss (Family: Meliaceae) [13].

Among the most commonly plants studied in controlling mosquitoes, A. indica that contains azadiracthin as the predominant insecticide in seeds, leaves and other parts [7], was found very important, and an excellent review of the activity of A. indica with proven mosquito control potential has been made [14]. But no scientific documentation has been made on larvicidal potential of A. indica seed oil and extract from Kolkata, India against mosquitoes including C. quinquefasciatus. Herein, A. indica seed extract and oil were evaluated as a potential means of control for C. quinquefasciatus larvae.

Materials and Methods

Neem seed extract and oil

The method of preparation of ethanolic extract of neem (A. indica) seeds has been described in our earlier publication [15], and 50 % ethanol was used to obtain a stock solution of 5 mg/ml. The A. indica seed oil was obtained from the village residents, who use to extract oil from the seeds by indigenous method, of the district Purulia, West Bengal (India).

Sampling container and station

Plastic containers and burnt clay pots, which were able to hold up to 3 litre of water, were bought from the market, and placed in areas with vegetation like flower hedges, mango trees (Magnifera indica Linn.; Anacardiaceae), grasses that provide shade, for adult mosquitoes resting positions and breeding activities, in front and sides of house at Naihati (suburb Kolkata), India. The containers were filled with pond water in order to allow the wild strains of female mosquitoes to lay eggs, and the containers were then examined for mosquito larvae, in between the months January and March, 2007.

Collection of mosquito larvae

The fourth instar larvae were collected and transferred into a glass beaker of 1 litre capacity containing clean water, and the larvae after sorting were identified as C. quinquefasciatus larvae. The larvae were then distributed in to seven glass jars, each containing 25 larvae in 25 ml of pond water.

Larvicidal activity of neem seed extract and oil

Six different concentrations (2, 4, 8, 16, 32 and 64 μg/ml) of A. indica seed oil were taken in 6 pre-labeled 250 ml capacity beakers, each containing 75 ml of water. The contents in the beakers were stirred well to obtain oil-water emulsion. Twenty five larvae in 25 ml water, as mentioned above, were introduced in each beaker, and the larval mortality in each beaker was recorded at 2, 4, 6 and 24 hours; the moribund larvae in all cases were counted as dead.

The similar studies were followed with nimyle (a commercial neem based product of Arpita Agro Products Private Limited, South 24 Paganas, India) for C. quinquefasciatus larvae, in order to assess its larvicidal activity against the mosquito species considered in the study. The concentrations used in the study for nimyle were 2, 4, 8, 16, 32 and 64 μl/ml.
**Probit regression and statistical analysis**

The median lethal concentration (LC_{50}) values and median lethal time (LT_{50}) values of A. indica seed extract and oil were calculated using probit analysis as described by Finney [16]. The percentages of dead larvae, after 24 hours of treatment with various concentrations of A. indica seed extract and oil (for the determination of LC_{50} values), and at different time periods using a single concentration (2×LC_{50}) of A. indica seed extract and oil (for the determination of LT_{50} values), were converted into probit, and the values thus obtained were plotted against log dose of A. indica seed extract and oil. The χ^2 test was used to compare the larval mortality of A. indica seed extract and oil against C. quinquefasciatus.

**Results**

The larvicidal activities of different concentrations of A. indica seed oil and extract against C. quinquefasciatus are represented in Table 1 and Table 2. The A. indica seed oil started to show larvicidal activity at concentration 2 μg/ml, which showed larval mortality of 8 % (n=2); the A. indica seed extract was found to initiate larvae killing activity at 8 μg/ml, and at this concentration the larval mortality was 12 %. The A. indica seed oil and extract were highly larvicidal at higher concentrations; 100 % larval mortality was achieved with oil and extract at concentrations 32 and 64 μg/ml, respectively. No larval mortality was found in control experimental set up.

The time-kill activities of A. indica seed oil and extract (2×LC_{50} for each) are represented in Table 3 and Table 4. The oil and the extract started to show killing activity at 2 hours and 6 hours, respectively with 12 % and 16 % killing of C. quinquefasciatus larvae. The killing was increased up to 84 % and 68 %, respectively due to A. indica seed oil and extract, with the increment of exposure period up to 24 h.

The LC_{50} values of A. indica seed oil and extract as determined by log-probit analysis were 8.041 and 9.394 respectively.

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**Table 1:** Toxicity test results of C. quinquefasciatus larvae (n=25) exposed to A. indica seed oil for 24 hours.

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>Log Concentration</th>
<th>Dead/Total</th>
<th>Dead %</th>
<th>Corrected %</th>
<th>Probil</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
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<td>8</td>
<td>8</td>
<td>3.59</td>
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<tr>
<td>4</td>
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</tr>
<tr>
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<td>84</td>
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<td>25/25</td>
<td>100</td>
<td>99</td>
<td>7.33</td>
</tr>
</tbody>
</table>

*Corrected formula for the dead% of larvae is mentioned in Table 1.

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**Table 2:** Toxicity test results of C. quinquefasciatus larvae (n=25) exposed to A. indica seed extract for 24 hours.

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>Log Concentration</th>
<th>Dead/Total</th>
<th>Dead %</th>
<th>Corrected %</th>
<th>Probil</th>
</tr>
</thead>
<tbody>
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<td>1</td>
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<td>3/25</td>
<td>12</td>
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<td>25/25</td>
<td>100</td>
<td>99</td>
<td>7.33</td>
</tr>
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</table>

*Corrected formula for the dead% of larvae is mentioned in Table 1.

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**Table 3:** Time-kill activity of A. indica seed oil (2×LC_{50}) against C. quinquefasciatus larvae (n=25).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Log time</th>
<th>Dead/Total</th>
<th>Dead %</th>
<th>Corrected %</th>
<th>Probil</th>
</tr>
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<td>84</td>
<td>5.99</td>
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</table>

*Corrected formula for the dead% of larvae is mentioned in Table 1.

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**Table 4:** Time-kill activity of A. indica seed extract (2×LC_{50}) against C. quinquefasciatus larvae (n=25).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Log time</th>
<th>Dead/Total</th>
<th>Dead %</th>
<th>Corrected %</th>
<th>Probil</th>
</tr>
</thead>
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<td>0</td>
<td>1</td>
<td>2.67</td>
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<tr>
<td>4</td>
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<td>0/25</td>
<td>0</td>
<td>1</td>
<td>2.67</td>
</tr>
<tr>
<td>6</td>
<td>0.778</td>
<td>4/25</td>
<td>16</td>
<td>16</td>
<td>4.01</td>
</tr>
<tr>
<td>8</td>
<td>0.903</td>
<td>8/25</td>
<td>32</td>
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<td>4.53</td>
</tr>
<tr>
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<td>17/25</td>
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<td>68</td>
<td>5.47</td>
</tr>
</tbody>
</table>

*Corrected formula for the dead% of larvae is mentioned in Table 1.

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**Figure 1:** Probit regression line for the determination of LC_{50} of A. indica seed oil (ASO) and extract (ASE) against C. quinquefasciatus larvae (n=25).
15.495 μg/ml, respectively (Figure 1), and the LT_{50} values for C. quinquefasciatus larvae treated with the agents (A. indica seed oil and extract) at concentration 2 x LC_{50} for each, were 8.328 and 15.322 min, respectively (Figure 2). The larvicidal activity of nimyle against C. quinquefasciatus mosquito vector has been represented in Figure 3.

Sharma et al. [20] concluded from their studies that Ajuga remota can be applied as an ideal larvicidal agent against A. stephensi and C. quinquefasciatus. It has been reported that the leaf extract of C. asiatica possess a remarkable larvicidal and adult emergence inhibition activity against C. quinquefasciatus [10]. The larval mortality of various products of A. indica against different mosquito species including C. quinquefasciatus has been reported earlier by many authors from different parts of the world including India [21]. In the present investigation, the A. indica seed oil and extract showed excellent larvicidal activity against C. quinquefasciatus, and this is the first report on biological control of C. quinquefasciatus using A. indica from our part of the globe. The neem tree, A. indica, is one of the most commonly studied plants for the control of mosquitoes [14]; it contains several biologically active principles, and azadirachtin being the predominant insecticide [7] produced 100 % mortality in A. stephensi at 1 ppm [22]. The larval mortality of culicids with 30 μg/ml of Margosan-O (an oil based neem seed extract) were reported as 100 % after 15 days exposure in pool water [23]. RajMohan & Ramaswamy [18] reported larval mortality up to 100 % for fourth instar larvae of C. quinquefasciatus exposed 24 hours to the leaf extract of Ageratina adenophora, and the mortality was up to 70 % for A. aegypti with the same plant. In the present communication, an increased percent mortality was recorded against fourth instar C. quinquefasciatus larvae with the increment of A. indica seed extract and oil concentration; a respective increase of 12 to 100 % and 8 to 100 % mortalities were observed with A. indica seed extract and oil, using increasing concentrations of the extract (8 to 64 %) and the oil (2 to 32 %), respectively. The percent killing activity of nimyle against larvae of the test mosquito vector was found to be concentration depended as has been recorded in our study; the 100 % larvae killing has been achieved in 24 h at concentration 64 μl/ml (Figure 3).

The larval toxicity to mosquito vectors including C. quinquefasciatus of different plants has been reported in

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**Figure 2:** Probit regression line for the determination of LT_{50} of A. indica seed oil (ASO) and extract (ASE) against C. quinquefasciatus larvae (n=25).

**Figure 3:** The percent killing of C. quinquefasciatus larvae exposed to various concentrations (2-32 μl/ml) of nimyle (v/v) for 24 h; (n=25). Percentages within the parentheses indicate the larvae killing rates.

**Discussion**

In light of the emergence of mosquito vectors of diseases showing resistance to conventional chemical pesticides, several authors reported earlier the potential larvicidal activities of different plant species against mosquitoes like Anopheles stephensi Liston (Family: Culicidae), Aedes aegypti Linn. (Family: Culicidae) as well as C. quinquefasciatus. Jayaprakasha et al. [17] studied larvicidal activity of the isolated main ingredient, lemonine, from the Citrus reticulate Blan (Family: Rutaceae) seed. The larvicidal effect of the leaf extract of a weed plant, Ageratina adenophora Spreng (Family: Compositae), on mosquito species including C. quinquefasciatus has been reported by RajMohan & Ramaswamy [18]. Fresh leaf extract of milkweed, Calotropis procera Aiton (Family: Asclepiadaceae) showed larvicidal activity against three mosquitoes, A. stephensi, C. quinquefasciatus and A. aegypti [19].
terms of LC_{50} values. The LC_{50} values of methanol, benzene and acetone extract of *Pemphis acidula* Forst. (Family: Lythraceae) were respectively 10.81, 41.07 and 53.22 ppm for *C. quinquefasciatus* and 22.10, 43.99 and 57.66 ppm for *Ae. aegypti* [6]. The LC_{50} values of ethyl acetate extract of *Swertia chirata* Buch.-Hams. ex Wall. (Family: Gentianaceae) against first, second, third and fourth instar larvae of *C. quinquefasciatus* were 164.91, 220.10, 284.05 and 326.46 ppm, and against *Ae. aegypti* 192.67, 237.30, 339.06 and 329.29 ppm, respectively [24]. In the present study, the *A. indica* seed oil and extract were highly toxic to the fourth instar *C. quinquefasciatus* larvae; the low 24 hours LC_{50} values, 8.041 and 15.495 μg/ml, respectively fort the oil and the extract supported this view. The calculated χ^{2} value between LC_{50} of *A. indica* seed oil (8.041 μg/ml) and that of *A. indica* seed extract (15.495 μg/ml) was 3.5885, and it was less than the table value of χ^{2} (3.841) at 0.05 probability, and thus there was no significance difference between the activities of *A. indica* seed oil and that of *A. indica* seed extract. Previous studies have shown that neem extracts possess significant larvicidal activity against mosquito vectors. Dua et al [5] recorded mean LC_{50} values of a neem oil formulation 1.6, 1.8 and 1.7 ppm against three mosquito vectors *An. stephensi*, *C. quinquefasciatus* and *Ae. aegypti*. Tandon and Sirohi [25] demonstrated the potency of neem seed extract as an effective larvical agent against *C. quinquefasciatus* with LC_{50} of 0.53 ppm. The LC_{50} of NeemAzal T/S against *An. stephensi* (1.92 ppm) was about 4 and 8 times lesser when compared to the LC_{50} against *Ae. aegypti* and *Cx. quinquefasciatus*, respectively, as has been reported by Gunasekaran et al [21]. Vatandoost and Vaziri [26] reported the LC_{50} of 0.36 ppm for *A. stephensi* and 0.69 ppm for *C. quinquefasciatus* using neemarin, a commercial preparation of neem extract. The LC_{50} values of many other plants having larvicidal activities against different mosquitoes, in addition to *C. quinquefasciatus*, have also been reported earlier. The Copaifera reticulata Ducke (Family: Leguminosae) oil-resin demonstrated larvicidal activity for all the *C. quinquefasciatus* instars, and the LC_{50} values for first, second, third and fourth larval instars were reported as 0.4, 0.9, 39 and 80 ppm, respectively [27]. Cetin et al. [28] reported *Teucrum divaricatum* Sieber (Family: Lamiaceae) as the most toxic to *C. pipientis*, followed by *Mentha longifolia* Linn. (Family: Lamiaceae), *Melissa officinalis* Linn. (Family: Lamiaceae), *Salvia sclarea* Linn. (Family: Lamiaceae) and *Mentha pulegium* Linn. (Family: Lamiaceae), with LC_{50} values 18.6, 26.8, 39.1, 62.7 and 81.0 ppm, respectively. In the case of *C. quinquefasciatus* larvae, the *Ajuga remotae* Benth (Family: Labiatae) extract exhibited maximum efficacy with LC_{50} values of 0.043 % after 24 hours and 0.026 % after 48 hours of exposure, as reported by Sharma et al [20]. The LC_{50} of the leaf extract of *A. adenophora* for *A. aegypti* was reported to be 356.70 ppm and that for *C. quinquefasciatus* was 227.20 ppm [18]. The *C. reticulata* oil-resin LC_{50} for the fourth instar larvae of *C. quinquefasciatus* was 80 ppm [27]. Thus, the findings of the present study are comparable with the findings reported by other researchers, but the variation in LC_{50} values is due to mosquito species, larval instars, and formulation of plant extracts, climate and method of application.

There is scanty report on LT_{50} values of plant extracts or their products against mosquito vectors. Obomanu et al. [13] reported that the mortality of larvae of mosquitoes including *C. quinquefasciatus* exposed to the plant extracts (Lepidagathis alopecuroides Family: Acanthaceae; and *A. indica*) increased with time of exposure as well as concentration of extracts, and they recorded LT_{50} values of *A. indica* extract, using increasing concentration starting from 100 to 500 ppm, as 152.3 - 181.19 min and that of *L. alopecuroides* 6.98 - 15.44 min for *C. quinquefasciatus* larvae. We studied, using a single concentration of *A. indica* seed oil (2 x LC_{50}) and extract (2 x LC_{50}), the killing rate of *C. quinquefasciatus* larvae, and recorded similar observations as reported by Obomanu et al. [13]. Based on the probit analysis, in the present investigation, the LT_{50} values of the agents were recorded as 8.328 and 15.322 min, respectively, and no significance difference was observed between the two (p > 0.05).

Currently environmental friendly and easily biodegradable insecticides have gained renewed importance. Neem products are relatively safe towards non-target biota, with only minimal risk of direct adverse effects on aquatic macro invertebrates due to contamination of water bodies with neem-based insecticides [29-30], and in addition, the products are less likely to induce resistance due to their multiple modes of action on insects [7]. But, an important factor in relation to the use of neem-based products as larvicides is high decay rate of its active ingredients, such as azadiracthin, on exposure to sunlight, and changes in pH [31], and hence the advantage of minimal residual activity and possible side effects are gained, but short term and repeated application may be necessary in field trials. Nevertheless, the use of neem products will be cost effective as it works at a very low dose and with high rate as suggested by the present findings, and it is indigenously available. Moreover, the variety of components
and different mechanisms of action, mosquito resistance to neem compounds seems likely to be low [4-6]. Thus, it is concluded that A. indica seed oil and extract can be used effectively as cheap alternative to conventional larvicidal agents against the bancroftian filariasis disease vector C. quinquefasciatus.

References

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