Larvicidal & emergence inhibitory activities of NeemAzal T/S 1.2 per cent EC against vectors of malaria, filariasis & dengue

K. Gunasekaran, T. Vijayakumar & M. Kalyanasundaram

Vector Control Research Centre (ICMR), Puducherry, India

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Background & objectives: Vector control, using agents of chemical origin, continues to be practiced in the control of vector borne diseases. However, due to some drawbacks including lack of selectivity, environmental contamination, and emergence and spread of vector resistance, development of natural products for vector control has been a priority in this area. In the present study we evaluated the larvicidal and emergence inhibitory activities of a neem based formulation Neem Azal T/S 1.2 per cent EC against the vectors of malaria, filariasis and dengue.

Method: Larvicidal and emergence inhibition (EI) activity of a neem formulation, NeemAzal T/S 1.2 per cent EC, was studied in the laboratory respectively against early 4th and early 3rd instar larvae of Anopheles stephensi, Culex quinquefasciatus and Aedes aegypti following standard procedures.

Results: Among the three vector species studied, An. stephensi was highly susceptible to NeemAzal T/S as revealed by the LC50 and LC90 values (1.92 and 2.76 ppm). The formulation produced an overall mortality or inhibition of emergence of 90 per cent (EI90 when 3rd instar larvae were treated) at 0.046, 0.208 and 0.866 ppm in An. stephensi, Cx. quinquefasciatus and Ae. aegypti, respectively. The corresponding EI50 values were 0.006, 0.048 and 0.249 ppm. On treatment, NeemAzal T/S induced certain morphogenetic abnormalities, broadly characterized in five types, in larvae, pupae and adults of all the three vector species. The percentage of dead specimens of any stage showing morphogenetic abnormalities was the maximum in Cx. quinquefasciatus (14.4%; n=2113) followed by Ae. aegypti.

Interpretation & conclusions: Our results indicated that because of its emergence inhibition activity, NeemAzal T/S 1.2 per cent EC could be a promising candidate for the use in integrated vector management programme and replace chemical insecticides.

Key words: Aedes aegypti - Anopheles stephensi - Culex quinquefasciatus - emergence inhibition - NeemAzal T/S
(Azadirachta indica A. Juss) as bioactive compounds contained in its seed kernel and other parts, have been found to show insecticidal activities. These activities include anti-feedancy, growth regulation, fecundity suppression, male sterility, oviposition repellency, and changes in biological fitness such as loss of flying ability, immunodepression, enzyme inhibition and splitting of biological rhythms. Various neem products have been tested against different mosquito species in many parts of the world for repellent action, larvicidal effect, growth regulating activity and biological fitness changes. The formulations tested include neem oil in kerosene, neem oil water emulsion, Neem Azal, neem cream, aqueous extract of deoiled neem, margsan-O (a product of neem seed), wettable powder Azad WP10, emulsifiable concentrate Azad EC 4.5, etc. All the formulations contain various neem limonoids, which have insecticidal property, and are described as modified triterpenes, having a 4,4,8 trimethyl-17 furanyl steroid skeleton. The neem formulation, NeemAzal T/S 1.2 per cent EC, tested during the present study contains azadirachtin, the most bioactive limonoid of neem. This study was undertaken to evaluate the larvicidal and emergence inhibition activities of the azadirachtin based formulation against Anopheles stephensi, Culex quinquefasciatus and Aedes aegypti, the vectors of malaria, filariasis and dengue, respectively with the perspective of its potential use in integrated vector management (IVM) programme to reduce the impact of synthetic chemical insecticides in the aquatic environment.

Material & Methods

Formulation: NeemAzal T/S 1.2 per cent EC is a botanical insecticide made from neem seed kernels. The formulation contains 1.2 per cent (12,000 ppm) Azadirachtin A, the most bioactive compound of neem, 2.8 per cent other limonoids and 96 per cent other ingredients. NeemAzal T/S 1.2 per cent, an emulsifiable concentrate (EC), was received from M/s. E.I.D. Parry (India) Ltd. Bio-Product Division, 234, N.S.C. Bose Road, Chennai, India for the study.

Laboratory assay for larvicidal activity: Bioassays, following standard methods of testing larval susceptibility, were conducted in the laboratory (28 to 31°C and 73% RH) to determine LC$_{50}$ and LC$_{90}$ of NeemAzal T/S 1.2 per cent against larvae of An. stephensi, Cx. quinquefasciatus and Ae. aegypti. Younger (early) IV instar larvae of the three vector species, obtained from the cyclic colony of the Vector Control Research Centre (VCRC), Puducherry, India, were used for the bioassays. Fifty larvae were placed in 300 ml disposable wax coated paper cups containing 250 ml tap water. For treatments, the desired concentration of test solution was achieved by adding 1 ml of the appropriate stock solution, prepared in acetone, to 249 ml of tap water taken in the paper cup. For each test concentration, four replicates were set up while four cups were left untreated to serve as controls. After the treatment, larval food (dog biscuit and yeast powder mixed in 6:4 ratios) was added to both treated and control cups (90-100 mg per cup). For each vector species, the formulation was tested on three different occasions at 6-8 test concentrations. The serial dilutions were freshly prepared on each occasion. The number of larvae surviving was counted at 24 h after treatment. Death or lack of reaction to gentle prodding with a glass pipette was considered as mortality.

Laboratory assay for emergence inhibition (EI): Younger III instar larvae of An. stephensi, Cx. quinquefasciatus and Ae. aegypti also obtained from the VCRC cyclic colony were used for the tests conducted under laboratory conditions (28 to 31°C and 73% RH). Stock solutions of various concentrations (5000, 500, 100 ppm) of the formulation were prepared in acetone. Serial dilutions were subsequently made in the same solvent from the stock solutions. To obtain appropriate test dosages, 1 ml of the serially diluted solution was added to 249 ml of water in 500 ml glass beakers and stirred vigorously to ensure complete mixing. To each beaker, 50 younger III instar larvae were introduced. The material was tested on three different occasions, at six different concentrations. In each test, each concentration was run in quadruplicate, and 4 beakers were left untreated as controls.

In control runs, 1 ml of acetone was added to 249 ml of water.

Soon after treatment, larvae were given larval food (dog biscuit and yeast powder mixed in 6:4 ratios); 70-100 mg food per 50 larvae. All larvae were exposed till pupation and mortality was recorded at 24 h intervals. Larval food was given every day until the larvae reached pupal stage. Dead larvae, pupae and partially emerged and/or deformed adults were regularly removed and counted. Live pupae were collected and observed till emergence. Total mortality, for all stages including adults, which underwent incomplete emergence, was recorded. Morphogenetic abnormalities, if any, among the dead specimens were also recorded.
Results of each concentration obtained on each occasion were subjected to computer log-probit analysis; EL_{50} and EL_{90} values (in ppm or mg/l) were estimated by linear regression analysis. Overall activity of the formulation was assessed as percentage inhibition of emergence (EI) considering mortality of all stages based on the starting population.

Laboratory assay for larval duration: To determine the effect of NeemAzal T/S on the length of the larval stage, test solutions of two sub-lethal concentrations (equivalent to EL_{20} and EL_{40}, respectively) for each of the three vector species (An. stephensi: 0.006 and 0.0096 ppm; Cx. quinquefasciatus: 0.048 and 0.064 ppm; Ae. Aegypti: 0.249 and 0.32 ppm) were prepared in an enamel tray of 30cm x 25cm x 5cm dimension. Fifty eggs were released in the treated water and allowed them to hatch and the total larval duration in days was calculated from hatching to pupation. In parallel, the duration of larval stage was calculated for the larvae reared in untreated water for comparison.

Data analysis: Mortality in the treated cups in a test was corrected against any mortality in the controls. The corrected mortality was subjected to log-probit regression analysis and median lethal concentration (LC_{50}) and 90 per cent lethal concentration (LC_{90}) and associated 95 per cent confidence intervals were calculated. Similarly, emergence inhibition concentration 50 and 90 per cent were determined from the data on total mortality considering all stages including adults. Data on lethal concentration and emergence inhibition, larval duration and mortality at different stages were subjected to analysis of variance (ANOVA of arcsine and square root transformed percentages) followed by Student-Newman-Keuls (SNK) Post Hoc test. The proportion of morphogenetic abnormalities was compared between the mosquito species using χ^2 analysis.

Results

Larvicidal activity: Among the three species tested, the 50 per cent lethal concentration (LC_{50}) of NeemAzal T/S was significantly (P<0.05) lower for An. stephensi compared to that for Ae. aegypti and Cx. quinquefasciatus. The LC_{50} of NeemAzal T/S against An. stephensi (1.92 ppm) was about 4 and 8 times lesser compared to the LC_{50} against Ae. aegypti and Cx. quinquefasciatus, respectively. The Post Hoc test further indicated that the concentration that caused 50 per cent mortality of Ae. aegypti larvae was significantly (P<0.05) lower than that of Cx. quinquefasciatus. The trend in susceptibility of the three vector species to the formulation, in terms of larval mortality, was same when LC_{90} values were considered (Table I).

Emergence inhibition (EI): The concentration of NeemAzal T/S that inhibited 50 per cent emergence (EI_{50}) of An. stephensi (mortality of all stages included) was significantly (P<0.05) lower in comparison to that of the other two species. When the 50 per cent emergence inhibition concentration for the other two species were compared (SNK test), it was significantly (P<0.05) lower for Cx. quinquefasciatus compared to that for Ae. aegypti. Comparison of EI_{90} of the formulation obtained for the three species also showed a similar trend, significantly (P<0.05 by one way ANOVA) lower for An. stephensi confirming its higher susceptibility to NeemAzal T/S (Table II). The lethal concentrations

Table I. Probit regression equation parameters and 50 and 90 per cent lethal concentration of NeemAzal T/S against IV instar larvae of the three vector species

<table>
<thead>
<tr>
<th>Mosquito species</th>
<th>Constant</th>
<th>Slope</th>
<th>LC_{50} (ppm) (SE)</th>
<th>95% CI</th>
<th>LC_{90} (ppm) (SE)</th>
<th>95% CI</th>
<th>χ^2 (df)</th>
<th>Fold*</th>
</tr>
</thead>
<tbody>
<tr>
<td>An. stephensi</td>
<td>2.692</td>
<td>3.529</td>
<td>1.923 (0.026)</td>
<td>1.828 - 2.023</td>
<td>2.764 (0.039)</td>
<td>2.507 - 2.989</td>
<td>2.027 (6)</td>
<td>-</td>
</tr>
<tr>
<td>Ae. aegypti</td>
<td>1.419</td>
<td>1.681</td>
<td>8.416 (0.044)</td>
<td>7.716 - 9.178</td>
<td>18.019 (0.081)</td>
<td>15.386 - 21.101</td>
<td>15.476 (6)</td>
<td>4.4</td>
</tr>
<tr>
<td>Cx. quinquefasciatus</td>
<td>0.986</td>
<td>1.452</td>
<td>15.866 (0.049)</td>
<td>14.386 - 17.498</td>
<td>38.305 (0.128)</td>
<td>29.802 - 49.235</td>
<td>1.375 (6)</td>
<td>8.3</td>
</tr>
</tbody>
</table>

*LC_{50} of Ae. aegypti or Cx. quinquefasciatus / LC_{50} of An. stephensi

Table II. Probit regression equation parameters and 50 and 90 per cent emergence inhibition (EI) concentration of NeemAzal T/S against the three mosquito vectors

<table>
<thead>
<tr>
<th>Mosquito species</th>
<th>Constant</th>
<th>Slope</th>
<th>EI_{50} (ppm) (SE)</th>
<th>95% CI</th>
<th>EI_{90} (ppm) (SE)</th>
<th>95% CI</th>
<th>χ^2 (df)</th>
<th>Fold*</th>
</tr>
</thead>
<tbody>
<tr>
<td>An. stephensi</td>
<td>8.223</td>
<td>0.631</td>
<td>0.006 (0.067)</td>
<td>0.005 - 0.007</td>
<td>0.046 (0.121)</td>
<td>0.036 - 0.058</td>
<td>12.539 (6)</td>
<td>-</td>
</tr>
<tr>
<td>Cx. quinquefasciatus</td>
<td>7.652</td>
<td>0.874</td>
<td>0.048 (0.046)</td>
<td>0.044 - 0.053</td>
<td>0.208 (0.104)</td>
<td>0.170 - 0.256</td>
<td>16.274 (6)</td>
<td>8</td>
</tr>
<tr>
<td>Ae. aegypti</td>
<td>6.428</td>
<td>1.029</td>
<td>0.249 (0.039)</td>
<td>0.231 - 0.270</td>
<td>0.866 (0.079)</td>
<td>0.741 - 1.012</td>
<td>25.764 (6)</td>
<td>41.5</td>
</tr>
</tbody>
</table>

* EI_{50} of Cx. quinquefasciatus or Ae. aegypti / EI_{50} of An. stephensi
**Effect on stages:** During the tests for emergence inhibition, most of the mortality was in larval as well as in pupal stage and only a very few were dead at adult stage (Fig. 1). In the case of *An. stephensi*, at all concentrations, the average per cent mortality obtained for larvae (29.7 ± 5.1) and pupae (30.3 ± 5.5) was almost equal without significant difference. While in *Cx. quinquefasciatus* the average per cent larval mortality (39.5 ± 8.4) was significantly (*P*<0.05) higher compared to its average per cent pupal mortality (18.9 ± 1.9), in *Ae. aegypti* the average per cent mortality of larvae (21.3 ± 2.98) was lower than the average pupal mortality (36.9 ± 7.30), but the difference was not significant.

**Larval duration:** The larval duration of *An. stephensi* was around 9 days in untreated and in treated at lower sub-lethal concentration of NeemAzal T/S, but at higher sub-lethal concentration, larval development took only about 7 days. In the case of the other two species also, treatment at higher sub-lethal concentration reduced the duration of larval stage, while larvae in untreated and treated at lower concentration took same duration for their development (Table III).

**Morphogenetic abnormalities:** The morphogenetic abnormalities induced by NeemAzal T/S after treating III instar larvae of the three vector species fell broadly in to the five categories (Table IV, Fig. 2). Among the three vector species tested, the percentage of dead specimens of any stage showing morphogenetic abnormalities was significantly (*P*<0.0001) higher in *Cx. quinquefasciatus* (14.4%; *n*=2113) than in *Ae. aegypti* (11.1%; *n*=2113) and *An. stephensi* (4.9%; *n*=2171).

**Discussion**

Among the three species tested, *An. stephensi* showed significantly higher susceptibility to NeemAzal T/S compared to *Ae. aegypti* and *Cx. quinquefasciatus*, for larvae were compared with the concentrations for emergence inhibition. The 50 or 90 per cent emergence inhibition concentrations for the three species were significantly (*P*<0.05) lower than the concentrations required to produce 50 or 90 per cent larval mortality.

![Image](https://example.com/image.png)

**Fig. 1.** Per cent mortality at different stages of *An. stephensi* (A), *Cx. quinquefasciatus* (B) and *Ae. aegypti* (C) after exposure of larval stages (from 3rd instar to adult emergence) to different concentrations of NeemAzal T/S.

<table>
<thead>
<tr>
<th>Species tested</th>
<th>Sub-lethal concentrations equivalent to</th>
<th>Untreated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EI&lt;sub&gt;50&lt;/sub&gt; (ppm)</td>
<td>EI&lt;sub&gt;80&lt;/sub&gt; (ppm)</td>
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<tr>
<td>----------------</td>
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<td>---------------------------</td>
</tr>
<tr>
<td><em>An. stephensi</em></td>
<td>Larvae treated: 522</td>
<td>9.43 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Cx. quinquefasciatus</em></td>
<td>Larvae treated: 537</td>
<td>8.66 ± 0.15&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Ae. aegypti</em></td>
<td>Larvae treated: 614</td>
<td>8.52 ± 0.12&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are mean ± SE. Values followed by the same letters (a-f) between the columns indicate no significant (*P*>0.05) difference in a Post Hoc test (SNK)
Fig. 2. Morphogenetic abnormalities induced by NeemAzal T/S: Enlarged distended IV instar larva, category I (A), de-chitinized extended pupa, category II (B), pupa and adult intermediate (partially emerged adult), category III (C), adult attached to pupal case by legs, category IV (D and E) and partially exuviated adult with part of abdomen contained within pupal case, category V (F).
Table IV. Morphogenetic abnormalities induced by NeemAzal T/S

<table>
<thead>
<tr>
<th>Mosquito species</th>
<th>Categories of morphogenetic abnormalities</th>
<th>Total (%) of the total dead</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>An. stephensi</td>
<td>34</td>
<td>28</td>
</tr>
<tr>
<td>Cx. quinquefasciatus</td>
<td>168</td>
<td>22</td>
</tr>
<tr>
<td>Ae. aegypti</td>
<td>52</td>
<td>44</td>
</tr>
</tbody>
</table>

*As given in legend of Fig. 2.

and between these two species, Ae. aegypti was more susceptible than Cx. quinquefasciatus. Also, as revealed by the EI<sub>50</sub> and EI<sub>90</sub> values, An. stephensi was more susceptible to NeemAzal T/S compared to the other two species. While there could be intrinsic differences in the susceptibility of different mosquito species, it is also possible that the observed differences might be due to the feeding behaviour of the species.

For instance, in mosquitoes, rate of ingestion plays a role in the activity and the effectiveness of bacterial toxin by affecting the response of an individual. Larvae of Cx. quinquefasciatus respond to the toxin with reduced filtration rates after an initial period of contact. This could be the reason for the lower susceptibility of this species in contrast to An. stephensi and Ae. aegypti larvae that filter toxin suspensions with unchanged rates, until the ingested amount of toxin causes mortality.

The LC<sub>50</sub> values of NeemAzal T/S 1.2 per cent EC were compared with that of other neem based products. Two products viz., ethanolic extract of neem leaves of Azadirachta indica and NSKE (neem seed kernel extract) had higher LC<sub>50</sub> values against Cx. quinquefasciatus (390 ppm) and Ae. aegypti (18.1 ppm), respectively indicating their lower larvicidal activity compared to NeemAzal T/S 1.2 per cent. Similarly, hexane extract of neem leaves caused 100 per cent mortality (after 48 h) of third instar larvae of An. stephensi only at 250 ppm and its LC<sub>50</sub> at 72 h was 69 ppm. In contrast, compared to NeemAzal T/S, a higher larvicidal activity was shown by neutral fraction of winter neem leaves against Ae. Aegypti (LC<sub>50</sub> = 0.58 ppm) and by Neemarin (a neem tree extract) against An. stephensi and Cx. quinquefasciatus (LC<sub>50</sub> and LC<sub>90</sub> were 0.35 and 1.81 ppm, and 0.69 and 3.18 ppm, respectively). Also, larvicidal activity of the present formulation was many fold lesser compared to some chemical products (such as temephos, fenthion, etc.) as their LC<sub>50</sub> values were normally in the range of 10<sup>-3</sup>-10<sup>-4</sup> ppm.

Larvicidal activity is the function of bioactive neem limonoids with azadirachtin being most potent. Therefore, the different results obtained on efficacy with varying LC<sub>50</sub> or LC<sub>90</sub> values might be due to the technique by which the active ingredient is extracted and formulated with inert/binding materials and the mosquito species tested. Further improvement could, therefore, be made to the present formulation, NeemAzal T/S 1.2 per cent EC, in order to produce higher larvicidal activity at lower concentration, i.e., <1 ppm, the accepted LC<sub>50</sub> for any formulation to be considered for field use. However, by looking at its EI<sub>50</sub> and EI<sub>90</sub> values that were many folds lower than its LC<sub>50</sub> or LC<sub>90</sub> and were closer to the EI<sub>50</sub> and EI<sub>90</sub> values of other formulations of chemical origin, NeemAzal T/S 1.2 per cent could be a formulation for emergence inhibition as it caused considerable level of mortality at pupal stage also; in Ae. aegypti mortality at pupal stage was higher than that at larval stage. Neemarin, another neem formulation, caused a significantly higher mortality in the pupal stage than the other stages of An. stephensi and Cx. quinquefasciatus.

It was clear that the NeemAzal T/S 1.2 per cent EC formulation on treatment against larval disrupted insect growth by affecting different molting stages and thereby inhibiting emergence of adult mosquitoes. Most of the mortality was therefore recorded in larval or pupal stage and some in larval-pupal intermediary and pupal-adult developmental stages, and that was the reason why only a few died as adults. Morphogenetic abnormalities induced by NeemAzal T/S 1.2 per cent at all stages of mosquito life cycle should also be taken into account while considering and comparing its overall activity.

By its inhibition effect on adult emergence at lower concentrations, NeemAzal T/S 1.2 per cent could be a promising candidate for the use in integrated vector management (IVM) programme that envisages to reduce reliance on chemicals and slow down build up of vector resistance by introducing less harmful or non chemical vector control methods. The only
considerations are concentration and half-life. Higher concentrations have been reported to be harmful to non-target organisms; at a concentration of 20 ppm, all the tadpoles died within 9 days, while other organisms such as larvae of Bufo regularis (Amphibia), Gambusia affinis (Peciliidae), Cyclops sp., and Daphnia magna (Crustacea) died within 5 to 8 days after exposure to a concentration of 10 ppm of NeemAzal insecticide. Half-life of the active compound is considered important as it facilitates persistence of the residue and thus the pesticide’s efficacy. Field degradation kinetic studies on azadirachtin showed that the mechanism of disappearance of the pesticides was unrelated to evaporation, thermodegradation and co-distillation, but related to photodegradation as azadirachtin and related compounds are very sensitive to sunlight. However, by adding suitable surfactants or stabilizers, the rate of photodegradation was decreased and the bioactivity of azadirachtin-A was retained for a considerable period of exposure to sunlight. In a recent study the half-life of azadirachtin-A WP was reported to be 3.45 months in water. Thus, azadirachtin-A, in suitable formulation, could be adequately stable in water retaining its bioactivity for a longer period.

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References


Reprint requests: Dr K. Gunasekaran, Scientist ‘E’, Vector Control Research Centre (ICMR), Medical Complex, Indira Nagar Puducherry 605 006, India
e-mail: k_guna@yahoo.com