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# Effects of neem limonoids on the malaria vector *Anopheles stephensi* Liston (Diptera: Culicidae)

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**Abstract**

The effects of the neem (*Azadirachta indica* A. Juss) limonoids azadirachtin, salannin, deacetylgedunin, gedunin, 17-hydroxyazadiradione and deacetylnimbin on *Anopheles stephensi* Liston (Diptera: Culicidae) were investigated. In exploring advantages of pure neem limonoids, we studied the larvicidal, pupicidal, adulticidal and antiovipositional activity of neem limonoids. Azadirachtin, salannin and deacetylgedunin showed high bioactivity at all doses, while the rest of the neem limonoids were less active, and were only biologically active at high doses. Azadirachtin was the most potent in all experiments and produced almost 100% larval mortality at 1 ppm concentration. In general, first to third larval instars were more susceptible to the neem limonoids. Neem products may have benefits in mosquito control programs.


*Keywords:* Malaria vector; *Anopheles stephensi*; Neem limonoids; Larvicide; Mortality; Longevity

## 1. Introduction

In recent years, the use of environment-friendly and easily biodegradable natural insecticides of plant origin has received much attention for control of medically important arthropods. Vector borne diseases, such as malaria, still cause thousands of deaths per year (Collins and Paskewitz, 1995). Malaria is by far the most important insect transmitted disease (Gilles and Warrell, 1993), remaining a major health problem in many parts of the world and is responsible for high childhood mortality and morbidity in Africa and Asia (Kleinschmidt et al., 2000; Pates and Curtis, 2005; Senthil Nathan et al., 2005a). *Anopheles stephensi* Liston (Diptera: Culicidae), a major malaria vector, breeds in wells, overhead or ground level water tanks, cisterns, coolers, roof gulters and artificial containers (Herrel et al., 2001). Management of this disease vector using synthetic chemicals has failed because of insecticide resistance, vector resurgence and environmental pollution. Consequently, an intensive effort

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has been made to find alternative methods of control (Service, 1983). Plant-derived materials are usually safer and more ecologically acceptable. They must be tested, however, to judge their efficacy against the target hosts.

Members of the Meliaceae plant family possess insect growth regulating properties against many insect pests (Saxena et al., 1984; Jacobson, 1987; Schmutterer, 1990). It is because of these properties that the family Meliaceae has emerged as a potent source of insecticides. The Indian neem tree, *Azadirachta indica* A. Juss (Meliaceae), has been found to be a promising source of natural pesticides, several constituents of its leaves and seed showing marked insect control potential. Neem seed kernel extract suppresses the feeding, growth and reproduction of insects (Schmutterer, 1990). Butterworth and Morgan (1971) first isolated the tetranortriterpenoid azadirachtin from *A. indica* seeds, which primarily showed antifeedant activity and later, regulatory effects on larval development and metamorphosis. Due to their relative selectivity, neem products can be recommended for many integrated pest management (IPM) programs (Schmutterer, 1990; Tanzubil and McCaffery, 1990; Mordue and Blackwell, 1993; Morgan, 2004; Senthil Nathan et al., 2004). Neem leaves contain organic compounds that have insecticidal and medicinal properties (Tewari, 1992). Neem is perhaps the most useful traditional medicinal plant in India. Each part of the neem tree has some biological activity and is thus commercially exploitable.

Limonoids are tetranortriterpenes and secondary metabolites produced in plants of the order Rutales. Within this order, limonoids are most often found in the family Meliaceae and less frequently in the families Rutaceae and Cneoraceae (Champagne et al., 1989, 1992). Limonoids are described as modified triterpenes, having a 4,4,8 trimethyl-17 furanyl steroid skeleton (Fig. 1). Arrangements of sub-groups and ring structures within this basic building block provide a host of characteristics that have generated interest in this plant product (Connolly, 1983). These characteristics include insecticidal, insect growth regulation and insect antifeedant properties. It is generally believed that bioactivity of neem is due to the azadirachtin (complex limonoids) content in them (Butterworth and Morgan, 1971). In the past, approaches to the qualitative and quantitative analysis of azadirachtin and other neem triterpenoids from different sources mostly included reversed-phase high-performance liquid chromatography (RP-HPLC), because of the polarity of the neem compounds (Sundaram and Curry, 1993; Schaaf et al., 2000).

Studies in recent years have revealed insecticidal effects of many different limonoids. The specific effects studied include growth inhibition, feeding inhibition, molt inhibition and insect growth regulation. Most studies have focused on the insect orders Coleoptera, Diptera, Heteroptera, Lepidoptera and Orthoptera. Azadirachtin or azadirachtin containing extracts have been shown to affect over 400 species of insects and mites (Schmutterer, 1990; Mordue and Blackwell, 1993). Azadirachtin possessing antifeeding and repellent or masking action against harmful insect were studied enough earlier (Mordue and Blackwell, 1993; Mordue and Nisbet, 2000; Huang et al., 2004; Senthil Nathan et al., 2005a,b,c,d,e; Senthil Nathan and Kalaivani, 2005), but their efficacy against malarial vectors such as *A. stephensi* has not been tested. This present investigation was undertaken to study the effect of neem limonoids against the larvae and adults of the important malaria vector *A. stephensi* (Liston).

## 2. Materials and methods

### 2.1. Mosquito culture

*A. stephensi* eggs were collected from in and around Bharathiar University Campus, Coimbatore, Tamil Nadu, India, and larvae were reared in plastic and enamel trays in tap water. They were maintained at $27 \pm 2\,^{\circ}C$, 75–85% RH with 14:10 L/D photo period. Larvae were fed with Brewers yeast, dog biscuits and algae collected from ponds in the ratio of 3 (Brewers yeast):1 (dog biscuits):1 (algae). Pupae were transferred from the trays to a cup containing tap water and placed in screened cages (23 cm × 23 cm × 32 cm) where adults emerged. Adults of *A. stephensi* were reared in 30 cm × 30 cm × 30 cm glass cages. Adults were continuously provided with 10% sucrose solution in a jar with a cotton wick. On day 5 post-emergence, adult females were deprived of sugar from 12 h and then provided with a mouse placed in resting cages overnight for blood feeding. Adult mosquitoes were

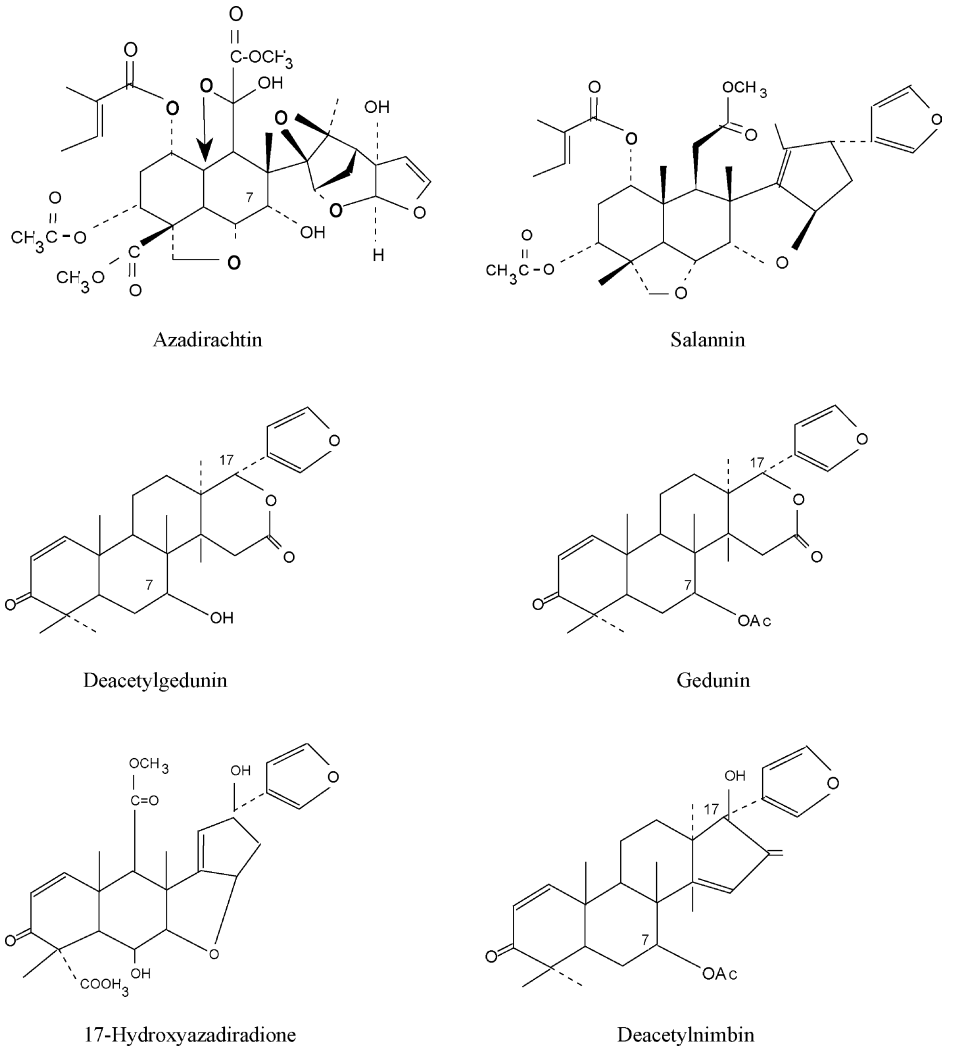


Fig. 1. Structure of neem limonoids tested against *A. stephensi*.

maintained at the same environmental conditions as the larvae.

### 2.2. Neem limonoids

Six neem limonoids (Fig. 1) (purity > 99%), namely azadirachtin, salannin, deacetylgedunin, gedunin, 17-hydroxyazadiradione and deacetylnimbin (Fig. 1), were sent from Dr. M. Ishida, Central Research Laboratories, Taiyo Kagaku Co. Ltd., Japan. They were dissolved in isopropanol and different concentrations were prepared by dilution with isopropanol.

### 2.3. Bioassays and larval mortality

Bioassays were performed with first to fifth instars of *A. stephensi* using concentration from 0.25 to 1 ppm. Isopropanol served as a control. A minimum of 20 larvae/concentration were used for all the experiments, which were replicated five times. The effective concentration ($EC_{50}$) was calculated using Probit analysis (Finney, 1971).

For mortality studies, 20 larvae each of first, second, third and fourth instars and pupae were introduced in 250 ml glass beakers containing various concentration

(0.25, 0.5 and 1 ppm) of the neem limonoids supplemented with 50 mg/l of yeast extract. A control was maintained. The treatments were replicated five times and each replicate set contained one control. The percentage mortality was calculated by using the formula (1) and corrections for mortality when necessary were done by using Abbot's (1925) formula (2):

$$\text{Percentage of mortality} = \frac{\text{number of dead larvae}}{\text{number of larvae introduced}} \times 100 \quad (1)$$

$$\text{Corrected percentage of mortality} = \left(1 - \frac{n \text{ in T after treatment}}{n \text{ in C after treatment}}\right) \times 100 \quad (2)$$

where $n$ is the number of larvae, T the treated and C is the control.

### 2.4. Adulticidal assay

The newly emerged *A. stephensi* fresh adults (10 numbers) were exposed to filter paper (90 mm, Advantec Toyo, Japan) treated with various concentrations of the neem limonoids. The paper was kept inside the beaker. Muslin cloth covering the beaker was also treated. Control insects were exposed only to isopropanol treated paper and muslin cloth. Mortality count was taken after 24 h.

### 2.5. Oviposition assay

Different concentrations (0.1–0.5 ppm) of the neem limonoids were mixed thoroughly with 200 ml of rearing food in 250 ml glass jars to obtain the desired concentration for the tests with *A. stephensi*. Ten gravid females were given a choice between treated and control jars. Isopropanol acted as a control. During the tests, the groups of 10 females each were kept separate for 48 h in cages measuring 25 cm × 25 cm × 30 cm. After the eggs were counted, the oviposition deterrence index (ODI) (Hwang et al., 1982) was calculated by using the formula (3):

$$\text{ODI} = \frac{N_t - N_s}{N_t + N_s} \times 100 \quad (3)$$

where $N_t$ is the total number of egg rafts in test solution and $N_s$ is the total number of egg rafts in control.

### 2.6. Ovicidal assay

In order to determine ovicidal activity, a total of 250 eggs were released in water. The test extracts were added in desired quantities (0.1–0.5 ppm) and hatching was observed for one week. The eggs were then exposed to deoxygenated water and the number of first stage larvae was recorded. Percentage hatching was compared with the control in which only isopropanol was used (Sharma et al., 1992).

### 2.7. Larval and pupal duration assay

To determine if neem limonoids affected the length of the larval and pupal stages, test solutions of sublethal concentrations (0.1, 0.25 and 0.5 ppm) were prepared in an enamel tray of 30 cm × 25 cm × 5 cm dimension. Fifty eggs were released in treated water and allowed to hatch and the total larval duration (days) was calculated from hatching to pupation. Pupae were placed in a small container closed with a transparent mesh. The pupal duration (days) was calculated from the pupal molt to adult emergence.

### 2.8. Larval and pupal duration assay

The fecundity experiments were conducted by taking 10 each of male and female *A. stephensi*, which had emerged from the control and treated sets of each concentration. They were mated in the cages of 30 cm × 30 cm × 30 cm dimension individually to each concentration. Three days after the blood meal, eggs were collected daily from the small plastic bowls containing water kept in oviposition traps in the cages. The fecundity was calculated by the number of eggs laid in the oviposition traps divided by the number of females allowed to mate. Male and female *A. stephensi* adult longevity were recorded.

### 2.9. Statistical analysis

The analysis program Probit (Finney, 1971) was used for the determination of $EC_{50}$. Data from biology, mortality, oviposition deterrence and effective concentrations were subjected to analysis of variance (ANOVA of arcsine, logarithmic and square root trans-

formed percentages). Differences between the treatments were determined by Tukey's multiple range test ($P \leq 0.05$) (Snedecor and Cochran, 1989; SAS Institute, 2001).

## 3. Results

Exposure of neem limonoids in the mosquito larval diet reduced larval longevity and increased mortality in all larval instars. Tables 1 and 2 demonstrate the efficacy of neem limonoids against *A. stephensi*. The effect on larval mortality and longevity was concentration-dependent (Tables 1 and 2). $EC_{50}$ values of neem limonoids against the larvae are shown in Table 3. The $EC_{50}$ for first, second, third and fourth instars was 0.014, 0.021, 0.028 and 0.034 ppm, respectively (Table 3).

### 3.1. Effect of neem limonoids on biological parameters and mortality of A. stephensi

The highest concentration, 1 ppm of the neem limonoid azadirachtin, produced almost 100% mortality in first to second instars (Table 1). Duration of larval instars and the total developmental time was prolonged at all doses (Table 2). Percent mortality in the fourth instar at 0.5 ppm treatment of salannin was 65% and it was further increased to 85% at 1 ppm treatment (Table 1). When *A. stephensi* larvae were treated by neem limonoids, they exhibited slower movement in the water.

Table 1
Percentage mortality of *A. stephensi* after treatment with neem limonoids

| Concentration (ppm) | Larval mortality (%) | | | | Pupal mortality (%) | Adult mortality (%) |
|---|---|---|---|---|---|---|
| | I instar | II instar | III instar | IV instar | | |
| Control | 2.5 ± 0.2$^{f*}$ | 1.8 ± 0.1$^{f*}$ | 1.2 ± 0.1$^{f*}$ | 1.0 ± 0.1$^{f*}$ | 0.8 ± 0.06$^{e*}$ | 0.6 ± 0.04$^{f*}$ |
| Azadirachtin | | | | | | |
| 0.025 | 69.4 ± 7.2$^{c}$ | 68.2 ± 5.8$^{c}$ | 68.0 ± 6.2$^{bc}$ | 66.5 ± 6.1$^{c}$ | 70.3 ± 6.7$^{c}$ | 69.3 ± 6.5$^{c}$ |
| 0.050 | 81.5 ± 7.6$^{b}$ | 79.5 ± 7.2$^{b}$ | 76.4 ± 6.9$^{b}$ | 75.5 ± 6.9$^{bc}$ | 83.6 ± 7.9$^{b}$ | 80.4 ± 8.1$^{b}$ |
| 0.100 | 96.7 ± 4.3$^{a}$ | 96.4 ± 4.6$^{a}$ | 96.2 ± 4.8$^{a}$ | 95.6 ± 4.2$^{a}$ | 96.2 ± 3.7$^{a}$ | 95.4 ± 4.3$^{a}$ |
| Salannin | | | | | | |
| 0.025 | 61.2 ± 5.6$^{cd}$ | 61.0 ± 5.8$^{cd}$ | 60.5 ± 5.8$^{c}$ | 55.3 ± 5.2$^{cd}$ | 60.1 ± 5.6$^{cd}$ | 55.0 ± 5.2$^{d}$ |
| 0.050 | 74.2 ± 7.1$^{c}$ | 74.0 ± 7.1$^{c}$ | 73.6 ± 6.9$^{b}$ | 65.4 ± 6.7$^{c}$ | 75.4 ± 6.3$^{bc}$ | 63.2 ± 5.9$^{c}$ |
| 0.100 | 94.6 ± 5.6$^{a}$ | 94.2 ± 6.3$^{a}$ | 94.0 ± 5.5$^{a}$ | 84.6 ± 7.9$^{b}$ | 86.9 ± 8.1$^{ab}$ | 89.3 ± 9.0$^{a}$ |
| Deacetylgedunin | | | | | | |
| 0.025 | 58.3 ± 6.1$^{d}$ | 57.0 ± 5.2$^{d}$ | 56.3 ± 6.1$^{d}$ | 50.1 ± 5.3$^{d}$ | 56.2 ± 6.1$^{d}$ | 52.3 ± 5.1$^{d}$ |
| 0.050 | 70.2 ± 9.6$^{c}$ | 70.0 ± 6.5$^{c}$ | 68.5 ± 6.3$^{bc}$ | 62.7 ± 6.1$^{c}$ | 70.2 ± 6.9$^{c}$ | 62.9 ± 5.9$^{c}$ |
| 0.100 | 93.2 ± 6.1$^{a}$ | 92.7 ± 7.1$^{a}$ | 91.0 ± 8.0$^{a}$ | 78.6 ± 8.1$^{b}$ | 84.3 ± 7.6$^{b}$ | 87.5 ± 8.5$^{ab}$ |
| Gedunin | | | | | | |
| 0.025 | 39.5 ± 4.5$^{f}$ | 37.5 ± 4.1$^{f}$ | 35.2 ± 4.0$^{f}$ | 33.5 ± 3.9$^{f}$ | 45.2 ± 5.0$^{e}$ | 42.0 ± 4.5$^{f}$ |
| 0.050 | 48.6 ± 5.2$^{e}$ | 47.3 ± 5.3$^{e}$ | 45.2 ± 5.1$^{e}$ | 40.3 ± 5.0$^{e}$ | 52.2 ± 5.0$^{d}$ | 50.8 ± 4.9$^{d}$ |
| 0.100 | 66.2 ± 6.1$^{c}$ | 64.0 ± 7.2$^{c}$ | 62.8 ± 6.1$^{c}$ | 55.6 ± 6.1$^{cd}$ | 61.6 ± 5.9$^{cd}$ | 61.0 ± 5.9$^{cd}$ |
| 17-Hydroxyazadiradione | | | | | | |
| 0.025 | 42.3 ± 4.7$^{f}$ | 39.5 ± 4.3$^{f}$ | 37.7 ± 4.3$^{f}$ | 34.7 ± 3.9$^{f}$ | 46.3 ± 5.1$^{e}$ | 43.7 ± 5.1$^{f}$ |
| 0.050 | 50.7 ± 5.2$^{e}$ | 48.2 ± 4.2$^{e}$ | 46.1 ± 5.2$^{e}$ | 45.2 ± 5.2$^{de}$ | 54.2 ± 4.8$^{d}$ | 51.0 ± 4.9$^{d}$ |
| 0.100 | 67.3 ± 6.5$^{c}$ | 66.2 ± 5.6$^{c}$ | 64.3 ± 5.9$^{c}$ | 59.3 ± 6.1$^{c}$ | 64.3 ± 6.2$^{c}$ | 61.7 ± 5.9$^{cd}$ |
| Deacetylnimbin | | | | | | |
| 0.025 | 41.5 ± 4.9$^{f}$ | 38.2 ± 4.6$^{f}$ | 35.5 ± 8.1$^{f}$ | 33.8 ± 3.7$^{f}$ | 45.7 ± 5.1$^{e}$ | 42.3 ± 4.5$^{f}$ |
| 0.050 | 50.3 ± 4.5$^{e}$ | 47.5 ± 5.1$^{e}$ | 45.9 ± 5.1$^{e}$ | 41.2 ± 5.2$^{e}$ | 52.0 ± 5.4$^{d}$ | 51.2 ± 4.9$^{d}$ |
| 0.100 | 63.5 ± 5.3$^{cd}$ | 62.2 ± 5.3$^{cd}$ | 61.5 ± 5.9$^{c}$ | 55.9 ± 5.9$^{cd}$ | 63.0 ± 6.5$^{cd}$ | 61.0 ± 5.9$^{cd}$ |

±S.E.: standard error. Means (±S.E.) followed by the same letters (a–f) within columns indicate no significant difference in a Tukey test.
$^{*}$ $P \geq 0.05$.

Table 2
Total larval, pupal and adult duration of *A. stephensi* after treatment with neem limonoids

| Concentration (ppm) | Mean (±S.E.) total larval duration (days) | Mean (±S.E.) pupal duration (days) | Mean (±S.E.) adult female longevity (days) | Fecundity (no. of eggs laid by the female) |
|---|---|---|---|---|
| Control | 9.1 ± 0.4$^{bc*}$ | 2.8 ± 0.1$^{c*}$ | 32.5 ± 2.4$^{a*}$ | 95 ± 10$^{a*}$ |
| Azadirachtin | | | | |
| 0.010 | 11.6 ± 0.9$^{b}$ | 4.5 ± 0.3$^{b}$ | 25.4 ± 1.8$^{b}$ | 61 ± 7$^{cd}$ |
| 0.025 | 14.7 ± 1.3$^{ab}$ | 5.8 ± 0.5$^{ab}$ | 23.1 ± 1.9$^{b}$ | 48 ± 5$^{d}$ |
| 0.050 | 18.5 ± 1.5$^{a}$ | 7.1 ± 0.6$^{a}$ | 18.1 ± 1.6$^{cd}$ | 18 ± 3$^{e}$ |
| Salannin | | | | |
| 0.010 | 10.8 ± 1.2$^{b}$ | 4.0 ± 0.3$^{b}$ | 26.6 ± 2.3$^{ab}$ | 70 ± 8$^{c}$ |
| 0.025 | 14.1 ± 1.5$^{ab}$ | 5.1 ± 0.5$^{ab}$ | 24.2 ± 2.1$^{b}$ | 53 ± 6$^{d}$ |
| 0.050 | 16.6 ± 1.5$^{a}$ | 6.2 ± 0.5$^{a}$ | 21.3 ± 2.0$^{c}$ | 32 ± 4$^{f}$ |
| Deacetylgedunin | | | | |
| 0.010 | 10.1 ± 0.9$^{b}$ | 3.8 ± 0.3$^{bc}$ | 27.2 ± 2.5$^{ab}$ | 75 ± 8$^{bc}$ |
| 0.025 | 13.2 ± 1.0$^{ab}$ | 4.7 ± 0.5$^{b}$ | 24.8 ± 2.3$^{b}$ | 58 ± 5$^{d}$ |
| 0.050 | 15.2 ± 1.3$^{ab}$ | 6.0 ± 0.6$^{a}$ | 22.2 ± 2.1$^{bc}$ | 35 ± 5$^{f}$ |
| Gedunin | | | | |
| 0.010 | 9.4 ± 1.3$^{b}$ | 3.2 ± 0.5$^{c}$ | 31.2 ± 3.4$^{a}$ | 87 ± 10$^{ab}$ |
| 0.025 | 9.8 ± 0.9$^{b}$ | 3.5 ± 0.3$^{bc}$ | 28.0 ± 2.7$^{a}$ | 85 ± 9$^{ab}$ |
| 0.050 | 10.5 ± 0.9$^{b}$ | 4.2 ± 0.3$^{b}$ | 25.3 ± 2.4$^{b}$ | 68 ± 7$^{c}$ |
| 17-Hydroxyazadiradione | | | | |
| 0.010 | 9.6 ± 1.2$^{b}$ | 3.5 ± 0.4$^{bc}$ | 30.9 ± 2.4$^{a}$ | 84 ± 10$^{ab}$ |
| 0.025 | 9.9 ± 1.0$^{b}$ | 3.7 ± 0.3$^{bc}$ | 30.1 ± 2.8$^{a}$ | 80 ± 7$^{b}$ |
| 0.050 | 10.3 ± 1.2$^{b}$ | 4.2 ± 0.3$^{b}$ | 28.4 ± 2.5$^{a}$ | 65 ± 6$^{c}$ |
| Deacetylnimbin | | | | |
| 0.010 | 9.6 ± 1.3$^{b}$ | 3.5 ± 0.5$^{bc}$ | 29.5 ± 3.2$^{a}$ | 86 ± 10$^{ab}$ |
| 0.025 | 9.9 ± 1.0$^{b}$ | 3.1 ± 0.2$^{bc}$ | 28.0 ± 2.5$^{a}$ | 82 ± 7$^{b}$ |
| 0.050 | 11.2 ± 1.3$^{b}$ | 4.1 ± 0.2$^{b}$ | 25.8 ± 2.4$^{b}$ | 65 ± 7$^{c}$ |

±S.E.: standard error. Means (±S.E.) followed by the same letters (a–f) within columns indicate no significant difference in a Tukey test.
$^{*}$ $P \geq 0.05$.

Adult longevity and fecundity was markedly decreased by the neem limonoids treatment. In addition to significantly lower survivorship and protracted development, larval duration was significantly increased (Table 2). Larval duration was almost 9 days in control larvae but at 0.25 ppm concentration of azadirachtin, larval development took up to 15 days. This was further increased to almost 19 days in same treatment at 0.50 ppm. These data show a gradual increase in the pupal duration and reduced adult

Table 3
Effective concentrations ($EC_{50}$) of neem limonoids against first to fourth instar larvae of *A. stephensi*

| Concentrations (ppm) | Larval instar | | | |
|---|---|---|---|---|
| | I | II | III | IV |
| Azadirachtin | 0.014 ± 0.001$^{d*}$ | 0.021 ± 0.001$^{d*}$ | 0.028 ± 0.003$^{e*}$ | 0.034 ± 0.003$^{e*}$ |
| Salannin | 0.023 ± 0.002$^{c}$ | 0.036 ± 0.004$^{c}$ | 0.047 ± 0.005$^{d}$ | 0.061 ± 0.007$^{d}$ |
| Deacetylgedunin | 0.028 ± 0.002$^{c}$ | 0.041 ± 0.004$^{c}$ | 0.0614 ± 0.007$^{c}$ | 0.078 ± 0.008$^{c}$ |
| Gedunin | 0.058 ± 0.006$^{a}$ | 0.073 ± 0.007$^{a}$ | 0.095 ± 0.010$^{a}$ | 0.117 ± 0.012$^{a}$ |
| 17-Hydroxyazadiradione | 0.047 ± 0.005$^{b}$ | 0.054 ± 0.006$^{b}$ | 0.076 ± 0.008$^{b}$ | 0.104 ± 0.010$^{b}$ |
| Deacetylnimbin | 0.055 ± 0.006$^{a}$ | 0.067 ± 0.007$^{a}$ | 0.091 ± 0.010$^{a}$ | 0.113 ± 0.010$^{a}$ |

±S.E.: standard error. Means ( ± S.E.) followed by the same letters (a–e) within columns indicate no significant difference in a Tukey test.
$^{*}$ $P \geq 0.05$.

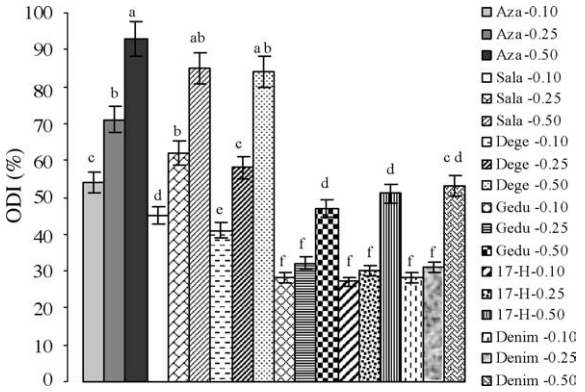


Fig. 2. Oviposition deterrence index (ODI) of *A. stephensi* after treatment with neem limonoids. Means (±standard error (S.E.)) followed by the same letters above bars indicate no significant difference ($P \geq 0.05$) in a Tukey test. Neem limonoids concentrations in ppm. *Abbreviations*: Aza, azadirachtin; Sala, salannin; Dege, deacetylgedunin; Gedu, gedunin; 17-H, hydroxyazadiradione; Denim, deacetylnimbin; ±S.E., standard error.

longevity of insects exposed to neem limonoids. Adult longevity was decreased after treatment with neem limonoids at all concentrations (Table 2).

There was a gradual decrease in eclosion when eggs were treated with neem limonoids in lower dose treatments but after treatment with more than 0.5 ppm, the effect was more pronounced (Fig. 2). Larvae treated at lower doses were deformed, which impeded their development.

## 4. Discussion

Changes in larval behavior, increased mortality and decreased adult life span of neem treated individuals, reduce the overall performance of the malaria vector, *A. stephensi*. Our data support this hypothesis, and we conclude that neem limonoids treatments in the larval diet reduce larval, pupal and adult survival.

### *4.1. Effect of neem limonoids on biology and adult mortality of A. stephensi*

It was also proved that neem applied in the dosage of 500 ppm had no negative effect on parasitoids of harmful insect species in glasshouses (Hoelmer et al., 1990), but synthetic pesticides were toxic to beneficial insects like natural parasitoids (Abudulai et al., 2001). Results of the mortality, biology, reproduction and ovipositional deterrence effects of above 0.1 ppm of neem limonoids on *A. stephensi* reported in the present study confirm their potential for control of the mosquito populations. In general, neem products act as larvicides, adulticides, repellents, deterrents and growth inhibitors in a variety of test insect species (Schmutterer, 1990; Mordue and Blackwell, 1993). The growth regulatory effect is the most important physiological effect of neem on insects because this property enables neem to be a potent source of insecticides. Antifeedant, insecticidal activity and inhibition of hormone and enzyme activity have been attributed to the tetranortriterpenoid azadirachtin in the extract (Ma et al., 2000; Senthil Nathan et al., 2004, 2005a,b,c,d,e).

Exposure of *A. stephensi* larvae to sub-lethal doses of neem leaf extract in the laboratory prolonged larval development and reduced pupal weight (Murugan et al., 1996). In the field, delayed phenology of surviving larvae and reduced pupal weight are common occurrence after treatment with neem. The results of this study indicate the plant-based compounds such as limonoids may be effective alternative to conventional synthetic insecticides for the control of *A. stephensi*.

The plant extracts drastically reduced the fecundity of the females and only few adults survived. Neem extracts reduced adult longevity (Table 2). These and other naturally occurring insecticides may play a more prominent role in mosquito control programs in the future (Mordue and Blackwell, 1993), because vector control is facing a threat due to the emergence of resistance in vector mosquitoes to conventional synthetic insecticides warranting either counter measures or development of newer insecticides.

### *4.2. Effect of neem limonoids on larval, pupal, adult duration and reproduction of A. stephensi*

In the present study, application of neem limonoids greatly affected the growth of *A. stephensi.* The lower dose treatments inhibited their growth and caused malformation and mortality in a dose-dependent manner. After neem limonoid treatment at a higher dose, the larvae die immediately before their pupal stage.

The highest concentration tested significantly reduced oviposition. The neem limonoids would act as

an oviposition repellent and/or deterrent to *A. stephensi* (Zebitz, 1984; Su and Mulla, 1999). In this study, the potent larvicides were azadirachtin, salannin and deacetylgedunin. These compounds have some common structural features such as furan ring and an unsaturated ketone in their A ring. Azadirachtin is by far the most potent enzyme inhibitor among all the limonoids, being more than five times as effective as the least potent enzyme inhibitor, gedunin. Azadirachtin is a highly oxidised tetranortriterpeniod with *trans*-connected A and B rings, an epoxide ring at positions 13 and 14. Three hydroxyl groups at positions 7, 11 and 20 are free in the azadirachtin molecule (Rembold, 1989). These hydroxyl groups seem to be a main factor determining antifeedent activity, since limonoids having one or more hydroxyl groups are more potent than those lacking them (Ishida et al., 1992; Murugan et al., 1998). Lee et al. (1991) reported that a hydroxyl group at C-7 reduced the activity of azadiradione, while a hydroxyl group at C-17 increased the activity of 7-deacetylazadiradione against *Heliothis virescens*.

Larval development was delayed and mortality rate increased in the neem limonoid treatment in concentrations of 0.1 ppm and above. The results of this study may contribute to a reduction in the application of synthetic insecticides, which in turn increases the opportunity for natural control of various medically important pests by botanical pesticides. Since these are often active against specific target insects, less expensive, easily biodegradable to non-toxic products and potentially suitable for use in mosquito control program (Alkofahi et al., 1989; Su and Mulla, 1999), they could lead to development of new classes of possible safer insect control agents.

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