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ORIGINAL PAPER

# A novel herbal formulation against dengue vector mosquitoes *Aedes aegypti* and *Aedes albopictus*

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**Abstract** The objective of this study was to develop a herbal formulation to control dengue vector mosquitoes. PONNEEM, a novel herbal formulation prepared using the oils of neem (*Azadirachta indica*), karanj (*Pongamia glabra*) and their extracts, was tested for larvicidal, ovicidal and oviposition deterrent activities against *Aedes aegypti* and *Aedes albopictus* at 1, 0.5, 0.3 and 0.1 ppm concentrations. Cent percent larvicidal and ovicidal activities were observed at 0.1 ppm in the two mosquito species under laboratory and sunlight-exposed conditions up to 12 months from the date of manufacture. Oviposition deterrent activity of 69.97% and 71.05% was observed at 1 ppm concentration of PONNEEM against *A. aegypti* and *A. albopictus*, respectively. Reduction in enzyme levels for α-esterase was 0.089±0.008 and 0.099±0.140 μg napthol produced/min/mg larval protein; for β-esterase, it was 0.004±0.009 and 0.001±0.028 μg napthol produced/min/mg larval protein; for glutathione S-transferase, it was 10.4814±0.23 and 11.4811±0.21 μmol/min/mg larval protein and for total protein, it was 0.177±0.010 and 0.008±0.005 mg/individual larva in treated groups of *A. aegypti* and *A. albopictus*, respectively. The nontarget organisms such as *Gambusia affinis* and *Diplonychus indicus* were not affected. No mortality was observed in control. PONNEEM can be used effectively for the management of human vector mosquitoes.

## Introduction

The control of mosquitoes is an important public health concern around the world. Mosquito abatement is primarily dependent on continued applications of organophosphates like temephos, malathion and fenthion, insect growth regulators like diflubenzuron and methoprene, and bacterial larvicides like *Bacillus thuringiensis* H14 and *Bacillus sphaericus*, which are still the most effective larvicides (Rozendaal 1997). Their repeated use has disrupted natural biological control systems and led to outbreaks of mosquitoes (DeBach and Rosen 1991), often resulting in the widespread development of resistance, undesirable effects on nontarget organisms, and eliciting environmental and human health concerns (Hayes and Laws 1991). These problems have highlighted the need for the development of new strategies for selective mosquito control.

*Aedes aegypti* and *Aedes albopictus* act as a vector for the arboviruses responsible for yellow fever and also for dengue fever (Figueiredo and Fonseca 1996; Halstead 2007; World Health Organization (WHO) website 2008a). The number of these cases has increased sharply in recent years. According to the World Health Organization (WHO) website (2008a, b), there may be over 50 million dengue infections in tropical and subtropical countries annually. There is an urgent need to develop new materials for controlling mosquitoes in an environmentally safe way, using biodegradable and target-specific insecticides against them.

An approach to obtain new, efficient, safe and selective insecticides from natural resources is gaining momentum. The number of plant species that can provide essential oils is high. Nevertheless, only a part of them can be successfully cultivated to provide sufficient quantities of biologically active compounds and for relatively favourable production prices. Plant essential oils in general have been recognized as important natural resources of insecticides (Gbolade et al. 2000; Adebayo et al. 1999). Their lipophilic nature facilitates them to interfere with basic metabolic, biochemical, physiological and behavioural functions of

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insects (Nishimura 2001). They have the potential to be ovicidal, fumigant, insect growth regulator and insecticidal against various insect species (Tsao et al. 1995) and ecologically sensitive pesticides (Isman 2000). Generally they are safe to humans and other mammals (Templeton 1969; Tripathi et al. 2000, 2002).

Neem tree (*Azadirachta indica*), native to India, belonging to family Meliaceae is a fast-growing evergreen tree ranging in height from 12–24 m. They are widespread in tropical and subtropical regions of the world, including semiarid and wet tropical regions (National Research Council 1992). Neem seeds contain approximately 99 biologically active compounds of which azadirachtin, nimbin, nimbidin and nimbolides are major molecules. Many of these derived products have antifeedancy, ovicidal activity, fecundity suppression besides insect growth regulation and repellency against insects (Schmutterer 1990, 2002; Locantoni et al. 2006; Su and Mulla 1998a; Sharma and Dhiman 1993).

*Pongamia glabra* Vent belonging to the family Fabaceae (Papilionaceae) is a small evergreen tree, which is widely distributed in India, China, Bangladesh and Australia. It has been recognized in different systems of traditional medicine for the treatment of various diseases and ailments of human beings (Ghani 1998; Kirtikar and Basu 1994). It contains several phytoconstituents belonging to the category of flavonoids. Dried leaves are used as an insect repellent in stored grains and also used as a pesticide (Warrier and Nambiar 1995).

Not much work has been done on the combined effect of neem and karanj oils as mosquito control agent. Hence the present work was undertaken to assess the larvicidal, ovicidal and oviposition deterrent and enzymatic activities of PONNEEM, a newly developed herbal formulation, against the human vector mosquitoes *A. aegypti* and *A. albopictus*.

## Materials and methods

### PONNEEM

PONNEEM was formulated and patented (Indian Patent No. 204381) by the Entomology Research Institute, Loyola College, Chennai, Tamil Nadu, India. *P. glabra* (karanj) and *A. indica* (neem) seeds were collected from Theni district, Western Ghats of Tamil Nadu, India. Oils were derived from the seeds by steam distillation method. Karanj oil 42.5% + 42.5% neem oil were mixed thoroughly using electric stirrer for 15 min. DMA-NE 15% (emulsifier) was added to the oil and stirred for 15 min. Crude azadirachtin extract 0.125% (using hexane) and 0.125% crude karanjin extract (using hexane) were mixed thoroughly using electric stirrer for 15 min. Finally, oil formulation was stored in brown-coloured glass containers and kept at room temperature.

### Chemicals

Fast blue RR salt [4-benzoylamine-2,5-dimethoxybenzedene-diazonium chloride hemi (zinc chloride) salt], α and β esterase, chlorodinitrobenzene (CDNB) and reduced glutathione (GSH) were procured from Sigma chemicals (USA). DMA-NE from Unitop (Emulsifier) was procured from Unitop Chemicals Private Ltd., Mumbai, India.

### Instruments

GC-MS was used to prolife the various compounds present in PONNEEM. The biochemical assay was carried out using HITACHI 2010 (Japan) UV spectrophotometer and HITACHI Ultra centrifuge was used for centrifugation.

### Mosquito culture

*A. aegypti* and *A. albopictus* larvae were collected from stagnant water bodies in various places within Chennai, India. They were colonized and maintained continuously for generations in the laboratory free of exposure to pathogens, insecticides or repellents. They were maintained at 27±2°C, 75–85% RH under a photoperiod of 14:10 h (light/dark) in the insectary. Larvae were fed on finely ground dog biscuit and yeast extract in the ratio of 3:1. Water was changed everyday to avoid scum formation which might create toxicity. Pupae were transferred from the trays to a cup containing tap water and placed in screened cages (30×30×30 cm dimension) where the adults emerged. The adult mosquitoes were reared in the glass cages of 30×30×30 cm dimension. The adult colony was provided with 10% sucrose solution and was periodically blood fed on restrained rats. After 3 days, the ovitrap was kept in the cages and the eggs were collected and transferred to the enamel trays. Two developmental stages, larvae and adult females, were continuously available for the experiments and were maintained at the same condition as above.

### Larvicidal activity

Larvicidal activity was evaluated following WHO method (1996) with slight modifications. Twenty-five early fourth instar larvae of *A. aegypti* and *A. albopictus* were released separately in a 500-ml glass beaker containing 249 ml of dechlorinated water and 1 ml of the desired PONNEEM concentration. Four replicates of 1, 0.5, 0.3 and 0.1 ppm concentrations were run at a time. Water was used as

negative control. NeemAzal and temephos (1 ppm) were used as positive controls. The experiment was carried out up to 96 h without changing the treated solution. At every 24 h interval, the dead larvae were removed and fresh 25 early fourth instar larvae were released into the same treated solution and the larval mortality was recorded. The experiments were carried out both in the laboratory and in sunlight. No food was offered during treatment. The moribund and dead larvae in five replicates were combined and expressed as percentage of larval mortality for each concentration. Dead larvae were identified when they failed to move after probing with a needle in the siphon or cervical region. The percentage of mortality was calculated by Abbott's formula (1925) and statistically analysed by Tukey's test using SPSS 11.5 software.

### Ovicidal activity

Ovicidal activity was evaluated by following the method of Su and Mulla (1998b) with slight modifications. Ten freshly laid (0–6, 6–12 and 12–18 h old) eggs of *A. aegypti* and *A. albopictus* were treated separately with PONNEEM at 1, 0.5, 0.3 and 0.1 ppm concentrations. Each treatment was replicated five times. Water was used as negative control. Temephos and NeemAzal (1 ppm) were used as positive controls. Ovicidal activity was observed under the microscope. The ovicidal activity was assessed up to 120 h posttreatment, and the results were calculated and analysed with Duncan's multiple range test (DMRT) using software of SPSS 11.5 version. The following formula was used

$$\text{Ovicidal activity} = \frac{\text{Number of unhatched eggs}}{\text{Total number of eggs in treated water}} \times 100$$

### Oviposition deterrent activity

The oviposition deterrent activity was assessed using the method of Rajkumar and Jebanesan (2002) with slight modifications. Ten gravid females of *A. aegypti* and *A. albopictus* (10 days old, 4 days after blood feeding) were transferred to each mosquito cage (45×45×45 cm) covered with a plastic screen, with a glass top and a muslin sleeve for access separately. A 10% sucrose solution was available at all times. Serial dilutions of PONNEEM were tested at 1, 0.5, 0.3 and 0.1 ppm; Temephos (1 ppm) and NeemAzal (1 ppm) were used as positive control. Two enamel bowls holding 100 ml of tap water for *A. aegypti* and *A. albopictus* were placed in opposite corners of each cage; one bowl was treated with the test material and the other bowl was without treatment. Four replicates were run for each treatment, with cages placed side by side for each bioassay. All experiments were at ambient temperature (27 ±2°C) with relative humidity of 75–85%. After 24 h, the number of eggs laid in treated and control bowls was recorded.

The percent effective repellency for each concentration was calculated using the following formula and statistically analysed by Tukey's test of multiple comparison using software of SPSS 11.5 version.

$$\text{ER}(\%) = \frac{\text{NC} - \text{NT}}{\text{NC}} \times 100(\%)$$

where ER is the percent effective repellency, NC number of eggs in control and NT number of eggs in treatment.

### Effect on nontarget organisms

The effect of PONNEEM was assayed against nontarget organisms of mosquito predators, such as *Gambusia affinis* (predatory fish) and *Diplonychus indicus* (predatory water bug) collected from pond of Fishery Research Institute, Chetpet, Chennai, and acclimatized at laboratory conditions for 3 days. One predator was released into 500-ml disposable bowl containing 250 ml tap water. Only one predator was used in one bowl so as to avoid cannibalism. The predators were exposed to test concentrations at 1, 0.5, 0.3 and 0.1 ppm with ten replicates along with ten untreated controls. Temephos and NeemAzal (1 ppm) were used as positive controls. The mortality of predators and other abnormalities such as sluggishness and reduced swimming activity were observed after 24 h exposure. The exposed predators were also observed continuously for 10 days to understand the posttreatment effect of PONNEEM on survival and swimming activity. The $LC_{50}$ and $LC_{90}$ values were obtained by probit analysis. Suitability index (SI) or predator safety factor (PSF) was calculated for each species of predator using Deo and colleagues' (1988) formula.

$$\text{SI/PSF} = \frac{\text{LC}_{50} \text{ of nontarget organism}}{\text{LC}_{50} \text{ of target vector species}}$$

### Stability test

Larvicidal, oviposition deterrent and ovicidal activities of PONNEEM stored at 27±2°C for up to 1 year were evaluated against *A. aegypti* and *A. albopictus* at different concentrations of 1, 0.5, 0.3 and 0.1 ppm as per the methods mentioned above.

### Sample preparation for enzyme assay

Batches of 25 early fourth instar larvae were homogenized individually in 200 μl of double distilled water using a glass homogeniser immersed in ice cubes. The homogenates were transferred to 1-ml Eppendorf tubes and spun at 10,000×*g* for 3 min at 4°C in an ultracentrifuge. The

supernatant was used as crude enzyme extract for esterase assay. However, for GST assay, 100 μl homogenate was transferred separately into two Eppendorf tubes at 10,000×*g* for 3 min and at 860 for 30 min, respectively at 4°C. The supernatant was used as enzyme samples. Five replicates were used for each enzyme assay.

### Esterase activity

The carboxyl esterase assay was carried out following the method of Ganesh et al. (2003). To 200 μl of each replicate of the homogenized sample, 2 ml of the α/β naphthyl acetate solution was added. The enzyme reaction was allowed to run for 30 min at room temperature. To this reaction mixture 50 μl of the fast blue stain solution containing 22.5 mg fast blue salt in 2.25 ml distilled water and 5% SDS in 0.2 M phosphate buffer (pH 7.2) were added. The fast blue helps to stain the mixture and SDS in it stops the reaction. Replicate blanks contained 200 μl of distilled water, 2 ml of α/β naphthyl acetate solution and 500 μl of the fast blue stain solution. Enzyme activity was read at 570 nm. Absorbance level for individual larvae was compared with the help of a standard curve of absorbance for known concentration of α and β naphthol, respectively. The enzyme activity was expressed as microgram of α/β naphthol produced/minute/milligram larval protein.

### Glutathione S-transferase activity

Glutathione S-transferase (GST) activity was estimated following the method of Ganesh et al. (2003). To 100 μl of the larval homogenate 0.1 ml of 30 mM CDNB was added and the volume was adjusted to 2.9 ml with distilled water. After preincubation of the reaction mixture for 5 min at 37°C 0.1 ml of 30 mM reduced GSH was added. The change in the absorbance level was noted at 340 nm for 5 min after every 30 s in the spectrometer. Reaction mixture without enzyme was used as blank.

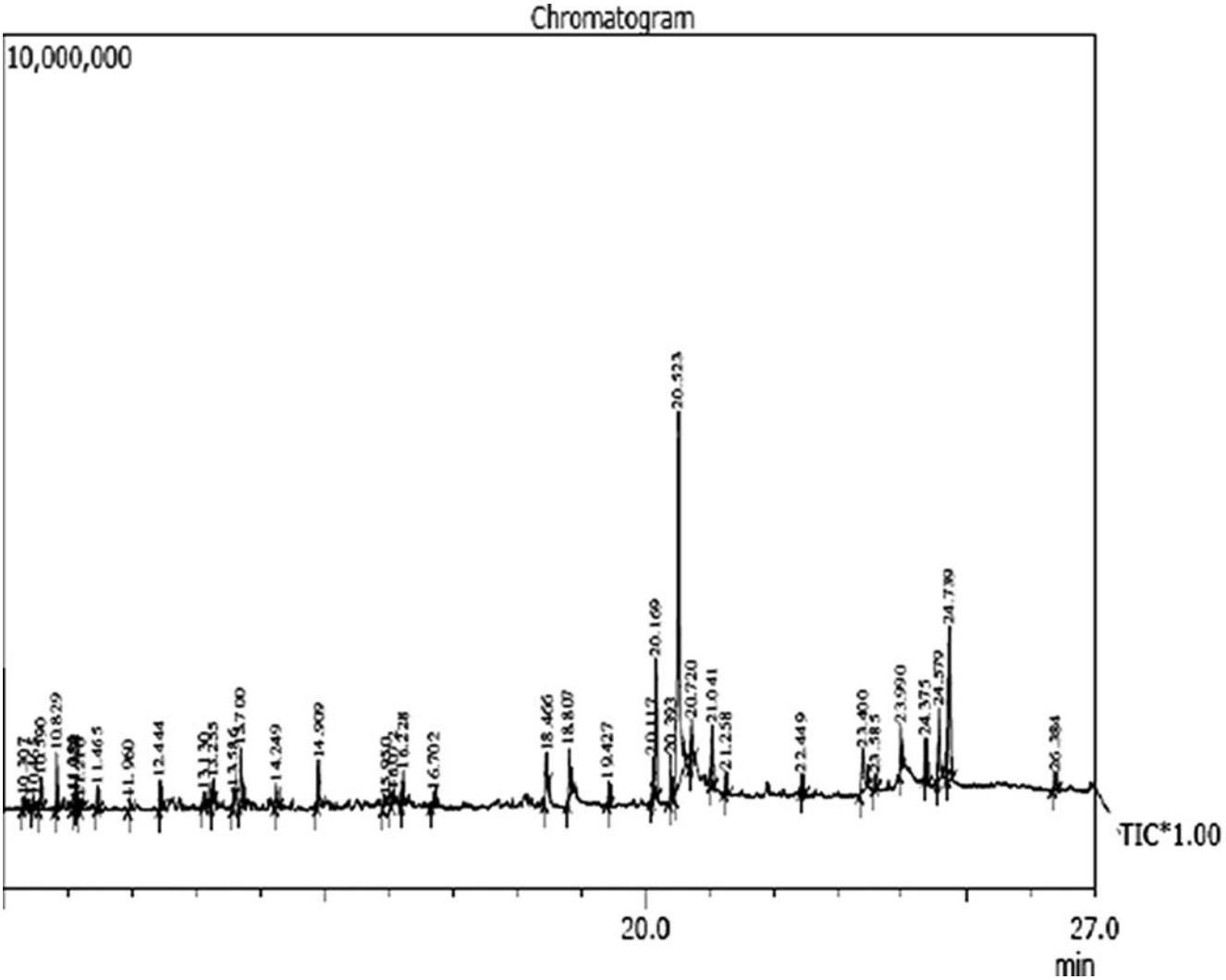


**Fig. 1** GC-MS analysis of PONNEEM

**Table 1** Larvicidal activity of PONNEEM against *A. aegypti*

| Under laboratory | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Hours | PN | Concentrations (ppm) | | | | | | | | | | | | | | | | | | |
| | | 1st month | | | | 3rd month | | | | 6th month | | | | 9th month | | | | 12th month | | | |
| | | 1 | 0.5 | 0.3 | 0.1 | 1 | 0.5 | 0.3 | 0.1 | 1 | 0.5 | 0.3 | 0.1 | 1 | 0.5 | 0.3 | 0.1 | 1 | 0.5 | 0.3 | 0.1 |
| 24 | Pon | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100b | 100b | 100b | 100b |
| | Tem | 92.00±0.81b | | | | 89.00±0.95b | | | | 92.00±1.15b | | | | 94.00±0.57b | | | | 95.00±0.50b | | | |
| | Nee | 85.00±0.95a | | | | 82.00±0.57a | | | | 82.00±0.57a | | | | 86.00±0.57a | | | | 87.00±2.06a | | | |
| 48 | Pon | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c |
| | Tem | 88.00±0.81b | | | | 85.00±0.95b | | | | 88.00±0.81b | | | | 91.00±0.95b | | | | 91.00±0.95b | | | |
| | Nee | 82.00±0.57a | | | | 79.00±0.50a | | | | 77.00±0.95a | | | | 80.00±0.81a | | | | 84.00±0.81a | | | |
| 72 | Pon | 100b | 100b | 100b | 100b | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100b | 100b | 100b | 100b |
| | Tem | 81.00±0.50a | | | | 85.00±0.50b | | | | 87.00±0.50b | | | | 89.00±1.25b | | | | 87.00±0.50a | | | |
| | Nee | 76.00±1.41a | | | | 76.00±2.00a | | | | 79.00±1.89a | | | | 76.00±0.81a | | | | 86.00±0.57a | | | |
| 96 | Pon | 100c | 100c | 100c | 100c | 100b | 100b | 100b | 100b | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c |
| | Tem | 78.00±1.00b | | | | 86.00±0.57a | | | | 89.00±1.25b | | | | 87.00±0.95b | | | | 93.00±0.95b | | | |
| | Nee | 70.00±1.29a | | | | 85.00±1.89a | | | | 81.00±0.50a | | | | 71.00±1.50a | | | | 85.00±0.95a | | | |
| Under sunlight | | | | | | | | | | | | | | | | | | | | | |
| 24 | Pon | 100b | 100b | 100b | 100b | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c |
| | Tem | 93.00±0.95a | | | | 93.00±0.95b | | | | 92.00±2.00b | | | | 96.00±0.00b | | | | 95.00±0.50b | | | |
| | Nee | 88.00±1.41a | | | | 87.00±0.95a | | | | 83.00±0.50a | | | | 88.00±0.81a | | | | 89.00±1.25a | | | |
| 48 | Pon | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100b | 100b | 100b | 100b | 100c | 100c | 100c | 100c |
| | Tem | 91.00±0.95b | | | | 91.00±0.50b | | | | 93.00±0.95b | | | | 92.00±2.16a | | | | 94.00±0.57b | | | |
| | Nee | 84.00±1.15a | | | | 83.00±1.50a | | | | 83.00±0.95a | | | | 85.00±1.89a | | | | 87.00±0.50a | | | |
| 72 | Pon | 100b | 100b | 100b | 100b | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100b | 100b | 100b | 100b |
| | Tem | 91.00±1.89a | | | | 88.00±0.81b | | | | 91.00±0.95b | | | | 92.00±1.15b | | | | 89.00±1.50a | | | |
| | Nee | 85.00±0.50a | | | | 81.00±0.81a | | | | 79.00±0.50a | | | | 82.00±1.73a | | | | 87.00±1.50a | | | |
| 96 | Pon | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100b | 100b | 100b | 100b | 100b | 100b | 100b | 100b | 100c | 100c | 100c | 100c |
| | Tem | 84.00±0.81b | | | | 92.00±0.81b | | | | 88.00±1.41a | | | | 93.00±2.21a | | | | 94.00±1.00b | | | |
| | Nee | 78.00±1.00a | | | | 78.00±0.57a | | | | 84.00±1.41a | | | | 82.00±0.57a | | | | 86.00±0.57a | | | |

Values with different letters are significantly different at $P<0.05$ level (Tukey's test)

*PN* product name, *Pon* PONNEEM, *Tem* temephos, *Nee* NeemAzal

**Table 2** Larvicidal activity of PONNEEM against *A. albopictus*

| Under laboratory | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Hours | PN | Concentrations (ppm) | | | | | | | | | | | | | | | | | | |
| | | 1st month | | | | 3rd month | | | | 6th month | | | | 9th month | | | | 12th month | | | |
| | | 1 | 0.5 | 0.3 | 0.1 | 1 | 0.5 | 0.3 | 0.1 | 1 | 0.5 | 0.3 | 0.1 | 1 | 0.5 | 0.3 | 0.1 | 1 | 0.5 | 0.3 | 0.1 |
| 24 | Pon | 100b | 100b | 100b | 100b | 100b | 100b | 100b | 100b | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100b | 100b | 100b | 100b |
| | Tem | 97.00±0.50b | | | | 96.00±0.81b | | | | 95.00±0.50b | | | | 95.00±0.50b | | | | 98.00±0.57b | | | |
| | Nee | 91.00±0.95a | | | | 89.00±1.25a | | | | 83.00±0.95a | | | | 89.00±0.95a | | | | 84.00±2.00a | | | |
| 48 | Pon | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100b | 100b | 100b | 100b | 100b | 100b | 100b | 100b | 100c | 100c | 100c | 100c |
| | Tem | 95.00±0.50b | | | | 94.00±1.29b | | | | 95.00±0.95b | | | | 92.00±0.81a | | | | 92.00±1.41b | | | |
| | Nee | 89.00±0.95a | | | | 88.00±0.81a | | | | 82.00±1.00a | | | | 88.00±0.81a | | | | 73.00±0.95a | | | |
| 72 | Pon | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100b | 100b | 100b | 100b | 100b | 100b | 100b | 100b | 100c | 100c | 100c | 100c |
| | Tem | 91.00±1.50b | | | | 93.00±0.50b | | | | 94.00±1.73b | | | | 89.00±0.95a | | | | 83.00±1.25b | | | |
| | Nee | 81.00±0.50a | | | | 85.00±0.95a | | | | 79.00±0.50a | | | | 84.00±2.44a | | | | 74.00±1.73a | | | |
| 96 | Pon | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100b | 100b | 100b | 100b | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c |
| | Tem | 94.00±0.57b | | | | 91.00±0.50b | | | | 98.00±0.57b | | | | 91.00±1.25b | | | | 88.00±0.81b | | | |
| | Nee | 85.00±0.95a | | | | 82.00±1.00a | | | | 87.00±0.50a | | | | 81.00±1.50a | | | | 75.00±0.50a | | | |
| Under sunlight | | | | | | | | | | | | | | | | | | | | | |
| 24 | Pon | 100b | 100b | 100b | 100b | 100b | 100b | 100b | 100b | 100b | 100b | 100b | 100b | 100c | 100c | 100c | 100c | 100b | 100b | 100b | 100b |
| | Tem | 95.00±0.50b | | | | 96.00±0.81b | | | | 95.00±1.25b | | | | 95.00±0.50b | | | | 95.00±0.50b | | | |
| | Nee | 84.00±1.41a | | | | 84.00±0.81a | | | | 86.00±1.00a | | | | 91.00±0.50a | | | | 88.00±1.41a | | | |
| 48 | Pon | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100b | 100b | 100b | 100b | 100b | 100b | 100b | 100b | 100c | 100c | 100c | 100c |
| | Tem | 93.00±1.70b | | | | 93.00±1.50b | | | | 96.00±0.81b | | | | 96.00±0.89a | | | | 95.00±0.50b | | | |
| | Nee | 79.00±0.50a | | | | 81.00±0.50a | | | | 86.00±1.29a | | | | 94.00±0.57a | | | | 85.00±1.25a | | | |
| 72 | Pon | 100c | 100c | 100c | 100c | 100b | 100b | 100b | 100b | 100c | 100c | 100c | 100c | 100b | 100b | 100b | 100b | 100c | 100c | 100c | 100c |
| | Tem | 89.00±2.06b | | | | 92.00±2.00b | | | | 95.00±0.50b | | | | 96.00±0.81b | | | | 91.00±1.25b | | | |
| | Nee | 77.00±0.95a | | | | 81.00±2.06a | | | | 91.00±0.95a | | | | 89.00±1.50a | | | | 79.00±0.50a | | | |
| 96 | Pon | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100b | 100b | 100b | 100b | 100c | 100c | 100c | 100c | 100c | 100c | 100c | 100c |
| | Tem | 89.00±1.50b | | | | 93.00±0.50b | | | | 96.00±0.81a | | | | 95.00±0.50b | | | | 95.00±0.50b | | | |
| | Nee | 75.00±1.25a | | | | 82.00±0.57a | | | | 92.00±0.81a | | | | 85.00±2.50a | | | | 78.00±1.00a | | | |

Values with different letters are significantly different at $P<0.05$ level (Tukey's test)

*PN* product name, *Pon* PONNEEM, *Tem* temephos, *Nee* NeemAzal

**Table 3** Ovicidal activity PONNEEM against *A. aegypti*

| Under laboratory | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Age of eggs | PN | Concentrations (ppm) | | | | | | | | | | | |
| | | 1st month study | | | | 6th month study | | | | 12th month study | | | |
| | | 1 | 0.5 | 0.3 | 0.1 | 1 | 0.5 | 0.3 | 0.1 | 1 | 0.5 | 0.3 | 0.1 |
| 0–6 h | Pon | 100a | 92.00±1.09ab | 78.00±1.78bc | 66.00±1.14c | 100a | 84.00±1.14ab | 68.00±1.30bc | 58.00±1.48c | 100a | 100a | 78.00±1.09b | 64.00±1.14c |
| | Tem | 72.00±1.64c | | | | 64.00±1.51c | | | | 64.00±0.89c | | | |
| | Nee | 60.00±1.73c | | | | 54.00±1.34c | | | | 54.00±1.67c | | | |
| 6–12 h | Pon | 94.00±0.89a | 80.00±1.58ab | 72.00±1.30bc | 60.00±1.87c | 94.00±1.34a | 82.00±1.48ab | 60.00±1.41cd | 56.00±1.34cd | 86.00±1.14a | 78.00±1.09a | 62.00±1.09b | 56.00±1.14b |
| | Tem | 56.00±1.14c | | | | 68.00±0.83bc | | | | 56.00±1.14b | | | |
| | Nee | 32.00±1.48d | | | | 42.00±1.48d | | | | 38.00±1.48c | | | |
| 12–18 h | Pon | 80.00±1.58a | 62.00±1.48ab | 56.00±1.14bc | 48.00±1.78bc | 80.00±1.22a | 66.00±1.51ab | 54.00±1.51bc | 42.00±1.30cd | 74.00±1.67a | 66.00±2.19ab | 52.00±1.92abc | 40.00±0.70cd |
| | Tem | 38.00±1.30cd | | | | 46.00±1.67bcd | | | | 48.00±1.30bc | | | |
| | Nee | 28.00±0.83d | | | | 26.00±1.51d | | | | 20.00±2.12d | | | |
| Under sunlight | | | | | | | | | | | | | |
| 0–6 h | Pon | 100a | 80.00±1.00b | 68.00±1.64bc | 58.00±1.48c | 98.00±0.44a | 92.00±0.83a | 78.00±1.09b | 64.00±1.67c | 100a | 92.00±0.83ab | 78.00±0.83bc | 66.00±1.51cd |
| | Tem | 52.00±2.16cd | | | | 56.00±0.89c | | | | 60.00±1.22d | | | |
| | Nee | 36.00±0.54d | | | | 40.00±1.00d | | | | 32.00±1.78e | | | |
| 6–12 h | Pon | 82.00±1.48a | 70.00±1.73ab | 62.00±2.16abc | 46.00±1.14cd | 80.00±1.00a | 74.00±1.94ab | 60.00±1.00bc | 48.00±1.48c | 92.00±1.30a | 76.00±0.89ab | 66.00±0.54bc | 52.00±2.16cd |
| | Tem | 50.00±1.58bc | | | | 52.00±1.09c | | | | 46.00±1.81cd | | | |
| | Nee | 28.00±1.30d | | | | 28.00±1.78d | | | | 34.00±1.67d | | | |
| 12–18 h | Pon | 70.00±1.22a | 66.00±1.67a | 52.00±2.04ab | 34.00±1.14bc | 72.00±1.48a | 62.00±2.04ab | 54.00±1.14abc | 40.00±1.4c | 86.00±1.14a | 72.00±1.64ab | 52.00±1.09bc | 36.00±2.19cd |
| | Tem | 44.00±2.07b | | | | 46.00±1.14bc | | | | 32.00±1.64cd | | | |
| | Nee | 14.00±1.14c | | | | 14.00±1.14d | | | | 24.00±1.51d | | | |

Values in a column with different letters are significantly different at $P<0.05$ level (DMRT test)

*PN* product name, *Pon* PONNEEM, *Tem* temephos, *Nee* NeemAzal

**Table 4** Ovicidal activity of PONNEEM against *A. albopictus*

| Under laboratory | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Age of eggs | PN | Concentrations (ppm) | | | | | | | | | | | |
| | | 1st month study | | | | 6th month study | | | | 12th month study | | | |
| | | 1 | 0.5 | 0.3 | 0.1 | 1 | 0.5 | 0.3 | 0.1 | 1 | 0.5 | 0.3 | 0.1 |
| 0–6 h | Pon | 100a | 94.00±1.34ab | 86.00±0.89abc | 82.00±1.09bc | 100a | 90.00±0.70ab | 80.00±0.00bc | 76.00±1.51bc | 100a | 100a | 84.00±0.54b | 76.00±1.34bc |
| | Tem | 74.00±1.67cd | | | | 66.00±1.81cd | | | | 68.00±1.48cd | | | |
| | Nee | 66.00±1.14d | | | | 60.00±1.22d | | | | 56.00±1.94d | | | |
| 6–12 h | Pon | 88.00±1.09a | 84.00±1.14a | 76.00±0.54ab | 70.00±1.58ab | 90.00a | 78.00±0.44ab | 66.00±0.54bc | 64.00±1.94bc | 88.00±1.09a | 82.00±1.09ab | 72.00±1.64ab | 66.00±1.81b |
| | Tem | 64.00±2.07b | | | | 56.00±1.34c | | | | 62.00±1.78bc | | | |
| | Nee | 58.00±1.30b | | | | 52.00±1.09c | | | | 44.00±1.67c | | | |
| 12–18 h | Pon | 79.00±1.51a | 68.00±1.78ab | 64.00±1.51ab | 58.00±1.48abc | 82.00±1.30a | 72.00±0.86ab | 58.00±1.92bc | 56.00±0.89bc | 72.00±1.30ab | 76.00±1.14a | 68.00±2.16ab | 56.00±1.51ab |
| | Tem | 48.00±2.04bc | | | | 42.00±1.48cd | | | | 52.00±1.30b | | | |
| | Nee | 36.00±1.51c | | | | 30.00±1.58d | | | | 32.00±0.83c | | | |
| Under sunlight | | | | | | | | | | | | | |
| 0–6 h | Pon | 100a | 86.00±1.34ab | 72.00±1.48bc | 66.00±0.89cd | 100a | 96.00±0.89a | 76.00±1.14b | 54.00±2.30cd | 100a | 98.00±0.44a | 84.00±1.67ab | 72.00±0.83bc |
| | Tem | 52.00±1.30d | | | | 60.00±1.00bc | | | | 58.00±2.04cd | | | |
| | Nee | 26.00±1.51e | | | | 38.00±1.30d | | | | 42.00±1.30d | | | |
| 6–12 h | Pon | 84.00±1.34a | 78.00±1.64ab | 64.00±0.54ab | 60.00±1.00bc | 82.00±1.64a | 78.00±1.30a | 68.00±0.83a | 50.00±1.41b | 88.00±1.09a | 80.00±0.00ab | 76.00±1.34ab | 64.00±2.40bc |
| | Tem | 44.00±1.94c | | | | 48.00±0.44b | | | | 48.00±0.44c | | | |
| | Nee | 18.00±1.78d | | | | 26.00±1.67c | | | | 30.00±1.22d | | | |
| 12–18 h | Pon | 74.00±1.14a | 64.00±2.07ab | 52.00±2.28bc | 48.00±1.09bc | 70.00±0.00a | 64.00±1.51ab | 50.00±1.00bc | 42.00±1.30c | 78.00±1.30a | 72.00±1.64a | 50.00±0.70b | 44.00±1.51b |
| | Tem | 36.00±1.34c | | | | 40.00±1.00c | | | | 34.00±1.81bc | | | |
| | Nee | 08.00±1.09d | | | | 14.00±1.34d | | | | 22.00±1.09c | | | |

Values in a column with different letters are significantly different at $P<0.05$ level (DMRT test)

*PN* product name, *Pon* PONNEEM, *Tem* temephos, *Nee* NeemAzal

**Table 5** Oviposition deterrent activity of PONNEEM against *A. aegypti* and *A. albopictus*

| Insect | Evaluation period | Product name | Concentrations (ppm) | | | |
|---|---|---|---|---|---|---|
| | | | 1 | 0.5 | 0.3 | 0.1 |
| *A. aegypti* | On the date of production | Pon | 69.97±6.35a | 58.00±7.11b | 52.59±5.90b | 37.96±6.24c |
| | | Tem (1 ppm) | 31.74±6.68cd | | | |
| | | Nee (1 ppm) | 28.61±6.65d | | | |
| | After 6 months from the date of production | Pon | 63.06±7.72a | 52.91±5.73b | 46.99±8.05b | 27.69±6.89c |
| | | Tem (1 ppm) | 27.17±7.32c | | | |
| | | Nee (1 ppm) | 25.20±4.57c | | | |
| | After 1 year from the date of production | Pon | 57.34±7.93a | 49.68±3.16b | 36.17±7.04c | 21.88±7.97d |
| | | Tem (1 ppm) | 11.82±8.81e | | | |
| | | Nee (1 ppm) | 09.02±7.13e | | | |
| *A. albopictus* | On the date of production | Pon | 71.05±5.71a | 65.87±6.16a | 57.12±7.50b | 44.60±3.86c |
| | | Tem (1 ppm) | 38.01±4.20d | | | |
| | | Nee (1 ppm) | 31.74±7.25e | | | |
| | After 6 months from the date of production | Pon | 67.60±5.71a | 58.53±7.02b | 49.13±3.30c | 31.42±8.53cd |
| | | Tem (1 ppm) | 36.17±8.69c | | | |
| | | Nee (1 ppm) | 25.37±8.46d | | | |
| | After 1 year from the date of production | Pon | 61.71±7.41a | 54.77±4.99b | 45.11±5.44c | 27.00±3.86d |
| | | Tem (1 ppm) | 31.23±6.02d | | | |
| | | Nee (1 ppm) | 12.68±9.28e | | | |

Each value of five replicates

*Pon* PONNEEM, *Tem* temephos, *Nee* NeemAzal

### Quantification of total protein

Quantification of the total protein of the early fourth instar larvae was done according to the standard procedure of Lowry et al. (1951). A known concentration of bovine serum albumin was used as the standard protein.

## Results

The profile of GC-MS analysis is given in Fig. 1. The major components were: azadirachtin, salanin, meliantriol, nimbin, nimbinin, azadiradione, meldenin, hexadecane, methyl oleate, oleic acid, 2-phenyl-furo[*b*]benzopyran-4(4*H*)-one, 2-[5-(2-methyl-benzooxazol-7-yl)-1*H*-pyrazol-3-yl]-phenyl, karanjin, pongamol, pogapin, pongaglabrone and pongallone.

The larvicidal activities of PONNEEM against the early fourth instar larvae of *A. aegypti* and *A. albopictus* are given in Tables 1 and 4. Cent percent larval mortality was observed in lowest concentration of 0.1 ppm at 24, 48, 72 and 96 h under laboratory and sunlight-exposed conditions up to 12 months from the date of manufacture (Tables 1 and 2). However, in temephos and NeemAzal-treated groups,

**Table 6** $LC_{50}$ and $LC_{90}$ values (in parts per million) of PONNEEM for nontarget organisms of *G. affinis* and *D. indicus*

| Evaluation period | *G. affinis* | | *D. indicus* | |
|---|---|---|---|---|
| | $LC_{50}$ | $LC_{90}$ | $LC_{50}$ | $LC_{90}$ |
| Under laboratory | | | | |
| On the date of production | 6.18 | 22.12 | 19.18 | 63.47 |
| After 6 months from the date of production | 6.64 | 17.81 | 10.42 | 21.56 |
| After 1 year from the date of production | 2.28 | 6.64 | 6.23 | 14.86 |
| Under sunlight | | | | |
| On the date of production | 0.67 | 9.38 | 5.11 | 11.36 |
| After 6 months from the date of production | 1.56 | 5.74 | 4.86 | 10.31 |
| After 1 year from the date of production | 0.98 | 13.76 | 7.01 | 17.83 |

Each value of ten replicates

**Table 7** SI/PSF of different mosquito predators with respect to immature vector mosquitoes exposed to PONNEEM (in parts per million)

| Predator species | Evaluation period | *A. aegypti* *A. albopictus* |
|---|---|---|
| Under laboratory | | |
| *G. affinis* | On the date of production | 3.98 |
| | After 6 months from the date of production | 4.28 |
| | After 1 year from the date of production | 1.47 |
| *D. indicus* | On the date of production | 12.37 |
| | After 6 months from the date of production | 6.72 |
| | After 1 year from the date of production | 4.01 |
| Under sun light | | |
| *G. affinis* | On the date of production | 0.43 |
| | After 6 months from the date of production | 1.00 |
| | After 1 year from the date of production | 0.63 |
| *D. indicus* | On the date of production | 3.29 |
| | After 6 months from the date of production | 3.13 |
| | After 1 year from the date of production | 4.52 |

the larval mortality was lower up to 12 months from the date of manufacture under laboratory and sunlight-exposed conditions. Among the two positive controls, temephos was more effective compared with NeemAzal (Tables 1 and 2). No mortality was observed in negative control.

The symptamatological observations were carried out throughout the experimental period under laboratory and sunlight-exposed conditions. At the time of exposure to PONNEEM, all the larvae were active and exhibited normal movement. After 5 to 10 min of exposure, the larvae were restless and frequently sank down and floated up quickly. At 15 to 20 min the restlessness persisted, and tremor and convulsion were observed in all treated larvae at the bottom of the container. After 1 h of treatment, all the larvae were dead. After 24 h shrunken and tracheal gills were observed under the microscope in PONNEEM-treated cohorts. Temephos and NeemAzal-treated larvae also exhibited a similar pattern of behaviour.

The results of the ovicidal activity of PONNEEM against *A. aegypti* and *A. albopictus* are given in Tables 3 and 4. Ovicidal activity of PONNEEM both under laboratory and sunlight-exposed conditions was higher than chemical synthetic pesticides in the two mosquito species. Older age group of eggs showed less ovicidal activity. Highest ovicidal activity was observed in early age group of eggs (0–6 h old).

Oviposition was decreased with the increasing concentration of PONNEEM. The efficacy decreased as the duration of storage of PONNEEM increased (Table 5). The $LC_{50}$ and $LC_{90}$ values indicated that PONNEEM was not toxic to predators such as *G. affinis* and *D. indicus* both under laboratory and sunlight-exposed conditions (Table 6). Survival index/predatory safety factor indicated that PONNEEM was less harmful to predatory fish (Table 7). The survival and swimming activity of nontarget organisms were not altered during the experimental period.

The enzymatic activity of α esterase level was reduced (0.089±0.008 and 0.099±0.140 μg napthol produced/min/mg larval protein) and β esterase level was also reduced (0.004±0.009 and 0.001±0.028 temephos g napthol produced/min/mg larval protein) at 1 ppm concentration of PONNEEM against the larvae of *A. aegypti* and *A. albopictus*, respectively (Table 8). The glutathione S-transferase enzyme was reduced in *A. aegypti* and *A.*

**Table 8** Esterase activity of PONNEEM against the larvae of *A. aegypti* and *A. albopictus*

| Concentration (ppm) | α-Esterase activity | | β-Esterase activity | |
|---|---|---|---|---|
| | *A. aegypti* | *A. albopictus* | *A. aegypti* | *A. albopictus* |
| 1.0 | 0.089±0.008 | 0.099±0.140 | 0.004±0.009 | 0.001±0.028 |
| 0.5 | 0.096±0.009 | 0.107±0.007 | 0.127±0.007 | 0.015±1.562 |
| 0.3 | 0.107±0.011 | 0.118±0.015 | 0.024±0.005 | 0.031±0.006 |
| 0.1 | 0.124±0.008 | 0.128±0.010 | 0.047±0.013 | 0.048±0.005 |
| Control | 0.226±0.058 | 0.242±0.029 | 0.117±0.046 | 0.123±0.008 |
| Temephos (1 ppm) | 0.232±0.008 | 0.407±0.140 | 0.120±0.105 | 0.165±0.147 |

Values of mean±SD. Total number of larvae used for enzyme=25. Activity is expressed as micrograms napthol produced/minute/milligram larval protein

*albopictus* (10.4814±0.23 and 11.4811±0.21 μmol/min/mg larval protein, Table 9). The total body protein was also reduced in treated larvae of *A. aegypti* and *A. albopictus* (0.177±0.010 and 0.008±0.005 mg/individual larva) compared with the control (Table 10).

## Discussions

The growing resistance of *A. aegypti* populations to the current commercial pesticides has hampered the efforts to control dengue vector effectively. In addition, other serious problems such as high environmental and human toxicity and low biodegradability have been created by the continuous use of synthetic pesticides. Hence, there has been an increasing interest in the development of alternative methods of mosquito control which are less hazardous to humans and other living organisms. In this regard, plant-derived compounds have emerged as good candidates, not only as new effective tools in vector management but also as environmentally safer agents (Garcez et al. 2009; Govindarajan et al. 2011; Kalaivani et al. 2011; Prophiro et al. 2011; Kanis et al. 2011).

The present study evaluated the effect of a novel herbal formulation PONNEEM prepared from *A. indica* and *P. glabra* oils to minimize human vector mosquitoes. PONNEEM showed cent percent larvicidal activity against *A. aegypti* and *A. albopictus* at least concentration of 0.1 ppm up to 96 h in laboratory and sunlight-exposed conditions. Even after 1 year from the date manufactured, the same effect was observed at the above-mentioned conditions. The effect was due to the presence of plant molecules of *A. indica* (azadirachtin, salanin, nimbidin, nimbin, nimbolide, mahmoodin and gedunin) and *P. glabra* (karanjin, oleic acid, linoleic acid, linolenic acid, palmitic acid and stearic acid). Rao and Dhingra (1997) and Parmar and Dutta (1987) have reported that karanj oil is a good synergist.

The results of the present work compared well with previous observations of Shanmugasundaram et al. (2001, 2008) who reported that the neem and karanj oil fractions and cakes formulations showed highest larvicidal activity against *A. aegypti* and *Culex quinquefasciatus*. The PONNEEM-treated larvae exhibited restlessness, sluggishness and convulsions. The sluggish movement and peculiar coiling of treated larvae might be due to neuronal or muscular disturbance caused by active principles released into the water from PONNEEM. After exposure to PONNEEM, the treated larvae exhibited restlessness, sluggishness, tremors and convulsions followed by paralysis at the bottom of the bowl. The abnormal and irregular movement of larvae was also observed by Choochote et al. (2004) treated with *Apium graveolens*. Corbet et al. (1995) noted the susceptibility of mosquito larvae and pupae to surface materials entering the trachea. Our results are in agreement with those reported by Chaithong et al. (2006) and Islam et al. (2003) against *C. quinquefasciatus*. Hafeez et al. (2011) reported that the liminoids from citrus showed the highest larvicidal activity against *A. albopictus*.

From the present evaluation, it is obvious that the performance of neem and karanja oil formulation was better against the mosquito larvae than their individual application. No significant difference in the larvicidal activity of the formulation was observed during 12 months storage period at room temperature. The present study revealed that this oil formulation is highly effective in controlling *A. aegypti* and *A. albopictus* both at laboratory and field conditions. Under sunlight conditions PONNEEM showed very good larvicidal activity. The sunlight enhanced the larvicidal activity of insecticides against *Anopheles stephensi*, *A. aegypti* and *C. quinquefasciatus* (Dondji et al. 2005). Nicoletti et al. (2010) observed highest larvicidal activity in ethyl acetate fraction of *A. indica* against the larvae of *A. albopictus*. Methanol extract of *A. indica* showed the most potent larvicidal effect against *C. quinquefasciatus* (Batabyal et al. 2009).

The ovicidal activity of PONNEEM was 100% at 1 ppm concentration against the two species of mosquitoes compared to temephos and NeemAzal which showed less

**Table 9** Glutathione S-transferase activity of PONNEEM against the larvae of *A. aegypti* and *A. albopictus*

| Concentration (ppm) | *A. aegypti* | *A. albopictus* |
|---|---|---|
| 1.0 | 10.4814±0.23 | 11.4811±0.21 |
| 0.5 | 11.7990±0.25 | 12.5805±0.27 |
| 0.3 | 13.6096±0.33 | 14.4150±0.67 |
| 0.1 | 15.2770±0.90 | 15.5210±0.25 |
| Control | 18.0475±0.51 | 17.5452±0.30 |
| Temphos (1 ppm) | 18.9460±0.18 | 18.4169±0.32 |

Values of mean±SD. Total number of larvae used for enzyme=25. Activity is expressed as micromoles/minute/milligram larval protein

**Table 10** Quantitative analysis of protein in the larvae of *A. aegypti* and *A. albopictus* treated with PONNEEM

| Concentration (ppm) | *A. aegypti* | *A. albopictus* |
|---|---|---|
| 1.0 | 0.177±0.010 | 0.008±0.005 |
| 0.5 | 0.144±0.013 | 0.033±0.007 |
| 0.3 | 0.116±0.010 | 0.070±0.018 |
| 0.1 | 0.097±0.007 | 0.138±0.011 |
| Control | 0.181±0.004 | 0.199±0.010 |
| Temphos (1 ppm) | 0.303±0.013 | 0.949±0.311 |

Values of mean±SD. Total number of larvae used for enzyme=25. In milligrams/individual larva

activity. The oviposition deterrent activity of PONNEEM was lower than that of larvicidal and ovicidal activities at 1 ppm concentration. Zebitz (1984, 1986) reported that the *A. indica* seed kernel extract showed ovicidal and oviposition deterrent against *A. aegypti*, and Mohsen et al. (1995) reported similar activity against *A. albopictus*. Rajkumar and Jebanesan (2008) reported that the isolated compounds from *Ponicrus trifoliata* showed remarkable ovicidal and oviposition deterrent activity against *A. aegypti*.

The effect on nontarget organisms revealed that PONNEEM was harmless to predatory fish *G. affinis* and predatory insect *D. indicus*. The safe index suggested that PONNEEM could be used along with the predatory fish and beneficial insect in integrated vector control programmes. The results were highly correlated with earlier findings of Sivagnaname and Kalyanasundaram (2004), who reported that the methanolic extract of *Atalantia monophylla* was safe to nontarget aquatic organisms. Neem products exhibit little residual persistence in the environment and are less hazardous to nontarget organisms than conventional chemical insecticides. Crude preparations of neem appear to have a low risk of resistance development because of the complexity of components and multiple actions (Ascher 1993).

The activities of acetylcholine esterase, GST and α and β esterases showed a reduction due to treatment with PONNEEM. The present results positively correlated with the findings of Mouches et al. (1987) who noticed reduction of protein in organophosphate resistant strains of *A. aegypti*, *Myzus persicae* and *Musca domestica* species. Kady et al. (2008) reported similar biochemical results.

The mosquitocidal activity of PONNEEM may be due to various compounds existing in plant oils; these compounds may jointly or independently contribute to produce 100% ovicidal, larvicidal, oviposition deterrent activities and reduced levels of enzymes against the *A. aegypti* and *A. albopictus*.

## Conclusion

PONNEEM offers great potential for the control of vectors such as *A. aegypti* and *A. albopictus*. PONNEEM showed marked larvicidal, ovicidal and oviposition deterrent activities but did not harm the nontarget organisms. Since oil formulations like PONNEEM are relatively less toxic, ecofriendly and limit the risk of resistance development, they may be used as alternatives to chemical pesticides for control of vectors to reduce vector-borne diseases.

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