Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm

Document heading doi:

Spinosad and neem seed kernel extract as bio-controlling agents for malarial vector, *Anopheles stephensi* and non-biting midge, *Chironomus circumdatus*

Kumar AN*, Murugan K, Madhiyazhagan P, Prabhu K

Department of Zoology, Bharathiar University, Coimbatore – 641 046, India

ARTICLE INFO

Article history:
Received 6 April 2011
Received in revised form 16 May 2011
Accepted 15 June 2011
Available online 20 August 2011

Keywords:
Mosquitoes
Chironomids
Bioassay
Lethal concentrations
Larval durations

ABSTRACT

Objective: Midge egg masses are reported to support non-pathogenic strains of the cholera pathogen, *Vibrio cholera (V. cholera)*. Mosquito born diseases have been reported to cause millions of death worldwide. The present research reveals the toxicity effect of spinosad and neem seed kernel extract (NSKE) against different larval stages of *Anopheles stephensi* (*An. stephensi*) and *Chironomus circumdatus (Ch. circumdatus)*. **Methods:** The neem seeds were collected from Marudamalai hills, Bharathiar University, Coimbatore, India. Neem seed kernels were powdered, extracted and diluted for different concentrations (2 ppm to 10 ppm). Spinosad was purchased from Kalpatharu pesticide Limited, Coimbatore, Tamil Nadu, India and thoroughly mixed with distilled water to prepare various concentrations (0.01 to 0.08 ppm) and used for bioassay. **Results:** The results depict that spinosad is more toxic in lower concentrations when compared to NSKE and mosquitoes are more susceptible than chironomids. Lethal concentrations were evaluated using the observed mortality. The lowest LC_{50} value obtained from spinosad against *An. stephensi* and *Ch. circumdatus* were 0.002 05 ppm and 0.008 91 ppm. This study investigated on effect of Spinosad and NSKE on the biology of mosquito. The immature stages of both species were susceptible to Spinosad and NSKE. Spinosad and NSKE in individual as well as combined treatment provided additional days in development for mosquitoes. **Conclusions:** The results conclude that Spinosad and NSKE are potential larvicides against *An. stephensi* and *Ch. circumdatus*.

1. Introduction

Malaria and other vector-borne diseases contribute to the major disease burden in India. *Anopheles stephensi* (*An. stephensi*) is recognized as a major vector of urban malaria in India[1]. It breeds in pools, wells, overhead or ground level water tanks, running water, fountains, roof gulters and artificial containers[2].

Chironomidae midges that emerge from suburban and urban aquatic habitats in some situations result in severe nuisance and traffic hazards; accumulations of dead midges have the potential to make roads and airport runways slippery and dangerous to be operational[3]. Midge egg masses have been reported to support non-pathogenic strains of the cholera pathogen, *Vibrio cholerae* (*V. cholerae*), which utilize the gelatinous sheath of the egg biomass as a carbon source[4,5]. A bacterial strain, designated T3944DT, was isolated from a chironomid egg mass sampled from a waste-stabilization pond in northern Israel and was found to be Gram-positive, motile by peritrichous flagella, endospore-forming, halotolerant and facultatively alkaliphilic[6].

Mosquito control is a difficult task and is becoming even more so due to a variety of factors including the development of insecticide resistance and concern over environmental pollution[7]. To avoid the propensity of bioaccumulation and induction of malignancy in non-target animals, a safe and more congenial method of vector control by natural and cheaper means of using plants and microbes as insecticides became popular[8].

Spinosad is an insect control product derived from a soil bacterium, *Saccharopolyspora spinosa* (*S. spinosa*) that combines the advantages of synthetic insecticides with the advantages of traditional biological insecticides on pest species in several orders including Diptera[9]. Spinosad has an excellent human safety profile with low risk to the

*Corresponding author: Kumar AN, Department of Zoology, Bharathiar University, Coimbatore – 641 046, India.
Tel: +91-9787412379
E-mail: naresharjun06@yahoo.co.in

applicator and beneficial insects. There have been over 30 metabolites characterized from this organism and the two most active compounds have been identified as Spinosyn A and Spinosyn D. Spinosad liquid formulation showed good control of *Culex pipiens* molesttus in both laboratory and field evaluation in Ismailia Governorate[10].

The neem tree [*Azadirachta indica* (*A. indica*)] has long been recognized for its properties both against insects. The biologically active molecules, azadirachtin are found mainly in the seed kernels. The neem plant (*A. indica*) and its derived products have shown a variety of insecticidal properties on a broad range of insect species[11]. Fruit extracts of *A. indica* elicit a variety of effects in insects such as antifeedant, growth retardation, reduced fecundity, moulting disorders, morphogenetic defects, and changes of behavior[12].

During the last decade, various studies on natural plant products to control mosquito vectors indicate them as possible alternatives to synthetic chemical insecticides[13]. In the present study an attempt was been made to evaluate the toxicity of spinosad and neem seed kernel extract (NSKE) on larvicidal, pupicidal and development activity on the *An. stepshensi* and the non-biting midge, *Chironomus circumdatus* (*Ch. circumdatus*).

2. Materials and methods

2.1. Collection of eggs of mosquitoes and chironomids

The eggs of *An. stephensi* and *Ch. circumdatus* were collected from drinking water sources and water storage containers. These eggs were brought to the laboratory and were transferred to 18 cm × 13 cm × 4 cm size enamel trays containing 500 mL of water and kept for larval hatching.

2.2. Maintenance of larvae

An. stephensi and *Ch. circumdatus* larval cultures were maintained in laboratory at (27±2) ℃, 75%-85% RH, under 14 L: 10 D photoperiod cycles. The mosquito larvae were fed with 0.5 g of dog biscuits and yeast at a ratio of 3:2 (wt: wt). The chironomid larvae were fed with alga. The feeding was continued till the larvae were transformed into the pupa stage.

2.3. Maintenance of pupae and adult

The pupae were collected from the culture trays and were transferred to plastic containers (12 cm × 12 cm) containing 500 mL of water. The plastic jars were kept in 90 cm × 90 cm ×90 cm size mosquito cage for adult emergence. The adults were fed with 10% sugar solution for a period of three days before they were provided an animal for blood feeding.

2.4. Blood feeding of adult An. stephensi

The adult female mosquitoes were allowed to feed on the blood of a rabbit for two days. After blood feeding, enamel trays with water from the culture trays were placed in the cage for the adults to lay eggs. Both females and males were provided with 10% glucose solution on cotton wicks.

2.5. Preparation of NSKE extract

The Neem Seeds were collected from Marudamalai hills, Bharathiar University, Coimbatore, India. Neem Seed Kernels were removed from the Pods and powdered using an electric blender. 500 g powder was extracted with two liters of distilled water in a Soxhlet apparatus (extraction period 72 h) at room temperature (27±2) ℃. The extract was stirred thoroughly and filtered through whatman filter paper and the extracts were evaporated to dryness by placing the container in rattling water bath (40 ℃). The extracts were further dried by placement near an electric fan[14]. The extract was stored in labeled specimen bottles for bioassay.

2.6. Collection of spinosad and preparation of concentration

Spinosad was purchased from Kalpatharu pesticide Limited, Coimbatore, Tamil Nadu, India. Required quantity of Spinosad was thoroughly mixed with distilled water to prepare various concentrations, ranging from 0.01 to 0.08 ppm.

2.7. Larval/pupal toxicity test of NSKE and spinosad

A laboratory colony of mosquito larvae/pupae and chironomid larvae/pupa were used for larvicidal/pupicidal activity. The NSKE was evaluated at 2, 4, 6, 8 and 10 ppm, (v/v)mL concentrations and spinosad was evaluated at 0.01 to 0.08 ppm (v/v) mL concentrations. Untreated distilled water treatment served as control. Each treatment was replicated five times. Twenty actively swimming *An. stephensi* and *Ch. circumdatus* larvae were sieved out from different rearing trays to maintain uniformity of batches of larvae and exposed to 100 mL of each concentration of NSKE, spinosad and untreated control held in separate 250 mL capacity plastic containers. Larval mortality was assessed after 24 h of exposure by probing the larvae with needle and moribund larvae were counted as dead.

2.8. Statistical analysis

Mortality was corrected using the following formula[15].

$$\text{Corrected mortality} = \frac{\text{Observed mortality in treatment}-\text{Observed mortality in control}}{100-\text{Control mortality}} \times 100$$

$$\text{Percentage mortality} = \frac{\text{Number of dead larvas/pupae}}{\text{Number of larvae/pupae introduced}} \times 100$$

The LC_{50}, LC_{90} and regression equation were calculated from toxicity data using SPSS (version 17) software package.

The values were expressed as mean±standard deviation of five replicates. Differences between the treatments were determined by Duncan's multiple range test.

3. Results

The percentage of mortality values were calculated for the immature stages of *An. stephensi* treated with various concentrations of spinosad and presented in Table 1. The mortality range was between 62% to 100% in 1st instar and 26% to 62% in the 4th instar at different concentrations ranging from 0.01 ppm to 0.08 ppm. The LC_{50} values of the 1st, 2nd, 3rd and 4th larval instars and pupae of *An. stephensi* when exposed to different concentrations of spinosad were, 0.002%, 0.003%, 0.028%, 0.049% and 0.030% respectively. Table 2, illustrates the larval (Ⅰ to Ⅳ) and pupal mortality data of Chironomid, *Ch. circumdatus* after the treatment of spinosad at different concentrations. The maximum mortality observed was 98% at 0.08 ppm concentration in the 1st instar larvae. LC_{50} values of Ⅰ, Ⅱ, Ⅲ and Ⅳ instar larva were 0.009%, 0.015%, 0.032% and 0.053%, respectively and for pupae was 0.049%. The larval mortality values and lethal concentrations of *An. stephensi* after the treatment of biopesticide NSKE is given in the Table 3. The LC_{50} and LC_{90} values were 1.705% and 7.580% for the 1st instar, 3.005% and 10.963 for 2nd instar, 5.502 and 13.676 for 3rd instar, 7.975 and 17.714 for 4th instar and 8.984 and 18.997 for pupae respectively. The percentage mortality values of *Ch. circumdatus* after the treatment of NSKE is given the Table 4. The range of mortality was between 6% (pupa) to 90% (1st instar larvae). The LC_{50} and LC_{90} values obtained were 2.388 and 10.817 for 1st instar, 4.024 and 13.247 for 2nd instar larvae, 8.585 and 21.741 for 3rd instar larvae, 9.818 and 20.451 for 4th instar and 13.092 and 22.311 for pupae respectively.

Table 5 shows the effect of spinosad and NSKE in individual and in combined treatment on the larval duration of *An. stephensi*. Larval durations (Ⅰ to Ⅳ) were increased after the treatment. The larval duration of *An. stephensi* (1st instar) after treating with spinosad was 2.9 d at 0.01 ppm and it was been increased to 3.7 days at 0.03 ppm. Similarly, NSKE also showed considerable effect on the larval duration of *An. stephensi*. It was 2.7 d at 2% concentration and it was been increased to 3.5 d at 8% concentration. The combined treatment of spinosad (0.001 ppm) and NSKE (0.2%) drastically increased the larval durations. 5.2 d, 5.0 d, 5.5 d and 3.3 d were recorded for Ⅰ, Ⅱ, Ⅲ and Ⅳ instar larvae respectively.

Table 1
Larvicidal and pupicidal mortality of spinosad on *An. stephensi*.

Larval &pupal stage	Concentration (ppm)					LC_{50}	LC_{90}	Regression coefficient	Chi-square value (χ^2)
	0.01	0.02	0.04	0.06	0.08				
Ⅰ	62.00±0.84	76.00±0.55	96.00±0.55	100.00±0.00	100.00±0.00	0.002	0.034	40.717	1.829
Ⅱ	58.00±0.84	74.00±1.14	86.00±0.55	96.00±0.55	100.00±0.00	0.003	0.043	31.872	1.803
Ⅲ	38.00±0.84	48.00±0.45	56.00±0.55	66.00±0.89	80.00±0.71	0.028	0.113	15.103	0.899
Ⅳ	26.00±0.55	40.00±0.71	48.00±1.30	58.00±0.45	62.00±0.45	0.049	0.151	12.524	2.973
Pupa	40.00±0.71	46.00±0.89	56.00±0.89	62.00±0.45	72.00±0.84	0.030	0.142	11.472	0.228

Table 2
Larvicidal and pupicidal mortality of spinosad on chironomid, *Ch. circumdatus*.

Larval &pupal stage	Concentration (ppm)					LC_{50}	LC_{90}	Regression coefficient	Chi-square value (χ^2)
	0.01	0.02	0.04	0.06	0.08				
Ⅰ	54.00±0.55	60.00±0.71	74.00±0.55	86.00±0.89	98.00±0.45	0.009	0.063	23.637	3.097
Ⅱ	44.00±0.55	56.00±0.89	68.00±0.84	78.00±0.45	88.00±0.84	0.015	0.086	18.038	0.560
Ⅲ	38.00±0.45	46.00±0.55	56.00±0.55	62.00±0.84	70.00±0.71	0.032	0.145	11.325	0.532
Ⅳ	22.00±0.45	34.00±0.89	46.00±0.55	54.00±0.55	64.00±0.55	0.053	0.139	14.895	1.913
Pupa	32.00±0.84	40.00±0.71	48.00±0.45	56.00±0.54	60.00±0.71	0.049	0.177	10.028	0.842

Table 3
Larvicidal and pupicidal effect of NSKE on *An. stephensi*.

Larval & pupal stage	Concentration (%)					LC_{50}	LC_{90}	Regression co-efficient	Chi-square value (χ^2)
	2	4	6	8	10				
Ⅰ	56.00±0.89	68.00±0.84	80.00±0.71	88.00±0.84	100.00±0.00	1.705	7.580	0.218	6.231
Ⅱ	48.00±0.45	56.00±0.55	62.00±0.84	76.00±0.55	92.00±0.45	3.005	10.963	0.161	5.504
Ⅲ	30.00±0.71	42.00±0.45	50.00±0.71	64.00±1.14	78.00±0.45	5.502	13.676	0.157	0.789
Ⅳ	22.00±0.45	30.00±0.71	38.00±0.45	52.00±0.84	60.00±0.71	7.975	17.714	0.132	0.288
Pupa	18.00±0.45	28.00±0.84	34.00±0.55	44.00±0.55	56.00±0.55	8.984	18.997	0.128	0.316

Table 4
Larval and pupal toxicity effect of NSKE on chironomid, *Ch. circumdatus*.

Larval & pupal stage	Concentration (%)					LC_{50}	LC_{90}	Regression co-efficient	Chi-square value (χ^2)
	2	4	6	8	10				
Ⅰ	50.00±0.71	58.00±0.84	70.00±0.71	78.00±0.45	90.00±1.00	2.388	10.817	0.152	1.229
Ⅱ	42.00±0.84	50.00±0.71	56.00±0.55	68.00±0.45	84.00±0.55	4.024	13.247	0.139	2.951
Ⅲ	28.00±0.84	32.00±0.45	38.00±0.84	46.00±0.55	58.00±0.45	8.585	21.741	0.097	0.774
Ⅳ	16.00±0.71	24.00±0.55	36.00±0.55	40.00±0.71	50.00±1.00	9.818	20.451	0.121	0.860
Pupa	6.00±0.55	10.00±0.71	18.00±0.45	22.00±0.45	34.00±0.84	13.092	22.311	0.139	0.478

Table 5
Effect of spinosad and NSKE (Biopesticide) on larval duration of *An. stephensi*.

Treatment	Concentration	Larval duration (d)			
		Ⅰ instar	Ⅱ instar	Ⅲ instar	Ⅳ instar
Control	–	2.2	2.3	3.6	1.6
Spinosad (ppm)	0.01	2.9	2.9	4.2	1.9
	0.02	3.3	3.2	4.5	2.2
	0.03	3.7	3.6	4.7	2.6
NSKE (%)	2	2.7	2.6	4.1	1.8
	4	3.0	3.0	4.3	2.0
	8	3.5	3.4	4.9	2.5
0.2% NSKE + 0.001 ppm spinosad	–	5.2	5.0	5.5	3.3

4. Discussion

In the present study NSKE and Spinosad have displayed toxicity on different larval instars of *An. stephensi* and *Ch. circumdatus*. The study showed an increase in mortality with the increase in concentration and the early instar larvae are much susceptible than the later ones. The results suggested that small volume of spinosad and NSKE is sufficient and can be directly used in the dwelling habitats of mosquitoes for effective control. The toxicity effect may be due to presence of the active compounds (spinosyns A and D in Spinosad and Azadirachtin, tetranortriterpenoid plant limonoid in NSKE). The larvae of a number of mosquito species (*Aedes* spp., *Anopheles* spp.) are sensitive to neem products and show antifeedant and growth regulating effects[16,17]. Earlier reports[18] stated that that the spinosad is known to be highly active against Dipterans. Recently work[19] reported that Azadirachtin is promising as a larvicidal agent against *Culex pipiens* and naturally ocurring biopesticide could be an alternative for chemical pesticides.

The Chironomid, *Ch. circumdatus* showed more resistibility than *An. stephensi* to both spinosad and NSKE. The mortality rate in *An. stephensi* was higher when compared with *Ch. circumdatus*. This may be due to the dwelling habitat of the *Ch. circumdatus*, where it covers itself within the soil particles. The larval abundances of two chironomid species, *Polypedilum nubifer* and *Kiefferulus intertinctus* were not affected by the pesticide application[20].

The present work reveals that the novel pesticide, spinosad seems to be more effective than the NSKE. The concentration prepared for spinosad was much lower than the NSKE. It could be that the secondary metabolites in the bacterial pesticide, the spinosad is more active than the active compounds in NSKE. The quicker mortality is also due to the bacterial fermented product, spinosyn which affects the nervous system. Earlier scientists reported that the spinosad was most effective at the lowest concentrations (0.024 to 0.025 ppm) and Spinosad effectively prevented breeding of *Culex* (Diptera: Culicidae) mosquitoes and chironomids (Diptera: Chironomidae)[21]. The results of a recent work showed that spinosad was very effective in the control of Spodoptera littoralis[22].

The low dosage of spinosad and NSKE in individual as well as combined treatment provided additional days in development for mosquitoes. The moulting was delayed in the larvae after which were subjected to treatment. As a support to the present work recently experiments were made to study the effect of biologically active plant oils on larval and pupal durations of mosquitoes[23-25]. A research team[26] clearly indicated that the neem treated female mosquito, *An. stephensi*, displayed a delay in oocyte development in the vitellogenesis. Similarly work reported that the total developmental period of *An. stephensi* was extended when treated against *Az. indica* and it remarkably influenced hatching rates, larval-pupal transformation and development, adult emergence and growth index[27].

This research shows that spinosad and NSKE pesticides can control the malarial vector, *An. stephensi*. These results show that these two biological agents could reduce the malarial incidence.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

We thank the authorities of Department of Zoology, Bharathiar University for providing all facilities for the undertaken research work.

References

[1] Mittal PK, Adak T, Subbarao SK. Inheritance of resistance to Bacillus sphaericus toxins in a laboratory selected strain of *Anopheles stephensi* (Diptera: culicidae) and its susceptibility to *Bacillus thuringiensis* var, israelensis. *Curr Sci* 2005; **89**: 442.

[2] Abelall SA, Nugud AED, Mahgoub O, Elhassan AM, Elhassan IM. The role of water coolers in breeding of anopheles mosquitoes in Khartoum, Sudan. *Sudanese J Publ Health* 2006; **1**(4): 307–308.

[3] Richard J, Lobinske, Arshad Ali. Population monitoring, ecology and control possibilities for nuisance midges (Diptera: Chironomidae). *Tech Bull Florida Mosq Control Assoc* 2006; **7**: 63–66.

[4] Halpern M, Raats D, Lavion R, Mittler S. Dependent population dynamics between chironomids (non-biting midges) and *Vibrio cholerae*. *FEMS Microbiol Ecol* 2006; **55**: 98–104.

[5] Senderovich Y, Gershtein Y, Halewa E, Halpern M. *Vibrio cholerae* and *Aeromonas*; do they share a mutual host? *ISME J* 2008; **2**: 276–283.

[6] Raats D, Halpern M. Oceanobacillus chironomi sp. nov., a halotolerant and facultatively alkaliphilic species isolated from a chironomid egg mass. *Int J Syst Evol Microbiol* 2007: **57**: 255–259.

[7] Wondji CS, Irving H, Morgan J, Lobo NF, Collins FH, Hunt RH, et al. Two duplicated P450 genes are associated with pyrethroid resistance in *Anopheles funestus*, a major malaria vector. *Genome Res* 2009; **19**: 452–459.

[8] Prabhu K, Murugan K, Naresh Kumar A, Ramasubramanian N, Bragadeeswaran S. Larvicidal and repellent potential of *Moringa oleifera* against malarial vector, *Anopheles stephensi* Listen (Insecta: Diptera: Culicidae). *Asian Pac J Tropical Biomed* 2011; 127–132.

[9] Bret BL, Larson LL, Schoonaer JR, Sparks TC, Thompson GD. Biological properties of spionosad. *Down to Earth* 1997; **52**: 6–15.

[10] Iman M, Bahgat GA, El Kady, Temerak SA, Lysandrou M. The natural bio-insecticide spinosad and its toxicity to combact some mosquito species in Ismailia Governorate, Egypt. *World J Agric Sci* 2007; **3**(4): 396–400.

[11] Isman MB. Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world. *Annu Rev Entomol* 2006; **51**: 45–66.

[12] Ofusori DA, Falana BA, Ofusori AE, Caxton-Martins EA. Regenerative potential of aqueous extract of neem *Azadirachta indica* on the stomach and ileum following ethanol-induced mucosa lesion in adult wistar rats. *Gastroenterol Res* 2010; **3**(2): 86–90.

[13] Naresh Kumar A, Murugan K. Antimalarial and antivectoral activities of traditionally used indigenous medicinal plants. *Adv Environ Biol* 2011; **5**(2): 407–412.

[14] Ascher KRS. Some physical (solubility) properties and biological (sterilant for Epilachna varivestis females) effects of a dried methanolic neem (*Azadirachta indica*) seed kernel extract. Rottach-Egern: Proceedings of 1st international neem conference; 1980, p. 63–74.

[15] Abbott WS. A method of computing the effectiveness of insecticides. *J Econ Entomol* 1925; **18**: 265–267.

[16] Zebitz CPW. Potential of neem seed kernel extracts in mosquito control. In: Schmutterer H, Ascher KRS (eds). *Proceedings of the third international neem conference* (*Nairobi, Kenya*). Eschborn: German Technical Cooperation GTZ; 1987, p. 555–573.

[17] Murugan K, Jahanmohani P, Babu R. *Effect of neen kernal extract and neem oil on nutritive and reporactive physioliogy of Helianthus armigera Hub. Neem and Environment*. Delhi: Oxford and IBH Co., Pvt. Ltd.; 1996, p. 321–334.

[18] Vargas RI, Peck, McQuate GT, Jackson CG, Stark JD, Amstrong JW. Potential for area wide integrated management of Mediterranean fruit fly (Diptera: Tephritidae) with a braconid parasitoid and a novel bait spray. *J Econ Entomol* 2001; **94**: 817–825.

[19] Abdelouaheb Alouani, Nassima Rehimi, Noureddine Soltani. Larvicidal activity of a neem tree extract (Azadirachtin) against mosquito larvae in the Republic of Algeria. *Jordan J Biol Sci* 2009; **2**(1): 15–22.

[20] Trayler KM, Pinder AM, Davis JA. Evaluation of the juvenile hormone mimic pyriproxyfen (S-31183) against nuisance chironomids (Diptera: Chironomidae), with particular emphasis on Polypedilum nubifer (Skuse). *Aust J Entomol* 2007; **33**(2): 127–130.

[21] Bond JG, Marina CF, Williams T. The naturally derived insecticide spinosad is highly toxic to *Aedes* and *Anopheles* mosquito larvae. *Med Vet Entomol* 2004; **18**: 50–56.

[22] Aydin H, Gurkan MO. The efficacy of spinosad on different strains of *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae). *Turk J Biol* 2006; **30**: 5–9.

[23] Khater HF, Shalaby AA. Potential of biologically active plant oils to control mosquito larvae (*Culex pipiens*, Diptera: Culicidae) from an Egyptian locality. *Rev Inst Med Trop São Paulo* 2008; **50**(2): 107–112.

[24] Prabhu K, Murugan K, Nareshkumar A, Ramasubramanian N, Bragadeeswaran S. Larvicidal and repellent potential of *Moringa oleifera* against malarial vector, *Anopheles stephensi* Liston (Insecta: Diptera: Culicidae). *Asian Pac J Trop Biomed* 2011; **1**(2): 124–129.

[25] Govindarajan M, Mathivanan T, Elumalai K, Krishnappa K, Anandan A. Ovicidal and repellent activities of botanical extracts against *Culex quinquefasciatus*, *Aedes aegypti* and *Anopheles stephensi* (Diptera: Culicidae). *Asian Pac J Trop Biomed* 2011; **1**(1): 43–48.

[26] Lucantoni L, Giusti F, Cristofaro M, Pasqualini L, Esposito F, Lupetti P, et al. Effects of a neem extract on blood feeding, oviposition and oocyte ultrastructure in *Anopheles stephensi* Liston (Diptera: Culicidae). *Tissue Cell* 2006; **38**: 361–371.

[27] Preeti Shamia, Lalit Mohan, Srivastava CN. Impact analysis of neem kernel extracts on the developmental profile of *Anopheles stephensi*. *J Asia-Pac Entomol* 2006; **9**(1): 11–17.