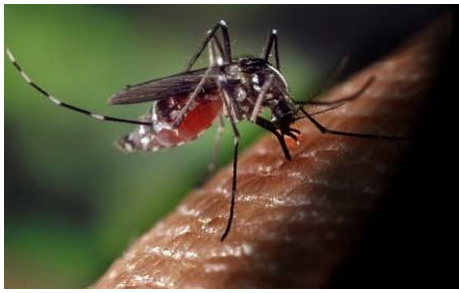

International Journal of Mosquito Research

ISSN: **2348-5906**
CODEN: **IJMRK2**
IJMR 2015; 2 (2): 45-49

Received: 01-03-2015
Accepted: 22-05-2015

T.P.N. Hariprasad
Yenepoya Research Centre,
Yenepoya University Mangalore,
Karnataka 575018, India.

A.B. Arun
Yenepoya Research Centre,
Yenepoya University Mangalore,
Karnataka 575018, India.

Correspondence:
A.B. Arun
Yenepoya Research Centre,
Yenepoya University Mangalore,
Karnataka 575018, India.
bhagwatharun@hotmail.com

Resistance status to six insecticides and efficacy of two plant extracts on *Anopheles stephensi* from Mangalore

T.P.N. Hariprasad, A.B. Arun

Abstract
Anopheles stephensi, an important Indian urban malaria vector was collected from Thokottu locality of Mangalore, India. Larval bioassays were carried out according to the procedure of WHO for evaluating the resistance levels to six insecticides and two plant extracts. The insecticides include alphamethrin, cypermethrin, fenvalerate lambda cyhalothrin, chlorpyrifos and propoxur. The plant extracts were methanol and hexane extracts of *Eucalyptus globulus* and *Calotropis gigantea*. *Anopheles stephensi* was found to be susceptible to alphamethrin (LC_{50} = 0.042 mg/L) and resistant to lambda cyhalothrin (LC_{50} = 1.99 mg/L). Among the plant extracts, hexane extracts of *Eucalyptus globulus* and *C. gigantea* were found to be effective (LC_{50} = 314.26 and 311.67 mg/L respectively) than methanol extracts. Additionally egg morphometry was carried out. The mean (M±SE) egg length and width were 488.01± 6.28µm and 169.9µm ± 3.30µm respectively. Based on the egg float ridge number, Thokottu strain was classified as Type form with 19-21 ridges.

Keywords: *Anopheles stephensi*, egg float ridge number, insecticides, plant extracts, resistance.

1. Introduction
Anopheles stephensi Liston (Diptera: Culicidae) is an urban malaria vector in the Indian Sub-continent. According to the latest estimates, about 0.62 million deaths occurred globally in 2012 [1]. In India, annually 0.2 million deaths occur due to malaria [2]. Malaria is re-emerging and causing an unacceptably higher burden of disease. About 455 formally named species of *Anopheles* have been identified [3]. There are about 58 species in India of which six are primary and four are secondary vectors of malaria [4]. *Anopheles stephensi*, a primary vector accounts for about 15% of malaria incidence in India [5]. Studies revealed three ecological variants in *Anopheles stephensi* based on the egg float ridge number *viz.*, type (14-22 ridges), intermediate (12-17 ridges) and *mysorensis* (9-15 ridges). The type and intermediate forms are found in urban and semi-urban areas and are reported to be vectors while *mysorensis* form is predominant in rural areas and is reported to be a non-vector [6-8].

Due to development and urbanization, there is a boost in the construction activity, which creates pockets for mosquito breeding. Supplementing urbanization, migration of construction workers from endemic areas is also leading to higher incidence of the disease. Chemical control is the main method employed in vector control. The control strategy usually adopted in urban settings is chiefly through anti-larval operations [9]. Haphazard application of insecticides leads to the development of insecticides and also environmental pollution. Application of insecticides to which the insects have developed/developing resistance will be futile.

Determination of the resistance spectra is the first stage in the investigation of any insecticide resistant population [10]. The indigenous mosquito species should be collected and their minimum effective dosages like LC_{50} and LC_{90} values be evaluated. Based on the results obtained in the laboratory an effective optimum dosage for field applications can be determined. Vector control operations require monitoring the insecticide susceptibility for dose determination and to establish baseline levels for future resistance work, disease incidence to evaluate effect of insecticides and vector behaviour to know the effect of insecticides on mosquito behaviour [11, 12].

Insecticides are the main stay in mosquito control. Apart from their effectiveness, over dependence and excessive use of these insecticides primarily attribute to the development of resistance, environment pollution and also effects non-target organisms. Plants are rich source

of alternative agents for control of mosquitoes, because they possess bioactive chemicals, which act against number of species including specific target-insects and are eco-friendly [13].

Members of the plant families - Solanaceae, Asteraceae, Cladophoraceae, Labiatae, Meliaceae, Oocystaceae and Rutaceae have various types of larval, adulticidal or repellent activities against different species of mosquitoes [14]. Conventional insecticides which are based on a single active ingredient, plant derived insecticides comprise botanical blends of chemical compounds which act collectively on both behavioural and physiological processes. Thus there is very little chance of pests developing resistance to such substances. Identifying bio-insecticides that are efficient, as well as being suitable and adaptive to ecological conditions, is imperative for continued effective vector control management [15]. About 1,200 plant species with potential insecticidal activity [16] and about 344 plant species with mosquitocidal activity [13] have been reported.

The aim of the present work was to study the resistance status of *Anopheles stephensi* from Mangalore to various insecticides and plant extracts.

2. Materials and methods

2.1 *Anopheles stephensi* maintenance.

Anopheles stephensi was collected from Thokottu locality of Mangalore, India (12.8177 °N, 74.8591°E) as larvae from construction sites (Fig. 1A and B). The colony was maintained in the insectary according to the procedure of Shetty [17]. The larvae were reared in white enamel pans containing filtered tap water and were fed with powdered yeast tablets on regular schedule throughout the larval period. To avoid scum formation, water in the pan was changed every day. Following pupation, the pupae were transferred into wide mouthed bottles and emerged adults were released into cages. The adult mosquitoes were maintained in cages made of iron frames and covered with mosquito net. Adults were fed with 10% sucrose solution soaked in sterilized cotton. The females were provided blood meal on restrained mice five days after their emergence. Plastic cups filled with water and lined with filter paper were placed inside the cage for oviposition. The gravid females laid eggs 48 hours after taking blood meal. The eggs were kept for 72 hours to ensure complete hatching. The larvae hatched were reared in enamel pans and fed with powdered yeast tablets. These stocks were maintained at 25˚±1 °C with 75 ± 5% relative humidity and 10:14 hours light and dark periods per day.

2.2 Insecticides and Plant extracts

Six insecticides were used in the present study. One organophosphate - chlorpyrifos (94% TC), a carbamate – propoxur (Baygon- 2% E.C) and four synthetic pyrethroids alphamethrin (97.6% TC), cypermethrin (93.3% TC), fenvalerate (94% TC) and lambda cyhalothrin (88.9% TC) procured from Tata Rallis India Limited were used. The different test concentrations (mg/L) for five insecticides were prepared in denatured alcohol (98 mL of absolute alcohol + 2 mL ethyl methyl ketone) except for propoxur which were prepared in water.

Plant extracts used were methanol and hexane extracts of *Eucalyptus globulus* Labill, 1799 (Family: Myrtaceae) and *Calotropis gigantea* (L.) W.T. Aiton 1811 (Family: Apocynaceae). The leaves of the plants were collected from Mangalore University campus (12.8158° N, 74.9241° E). The leaves were thoroughly cleaned and shade dried. The dry leaves were powdered using electrical blender. The plant material was extracted in methanol and hexane (60 to 70 °C) for 24 hours using soxhlet apparatus. The residue was collected and stock solution was prepared by dissolving 1gm in 100 ml of acetone and dimethyl sulfoxide DMSO (1:1). Various concentrations for larval bioassay were prepared from the stock by serial dilution.

2.3 Larval Bioassays

Susceptibility tests were carried out according to the procedure of WHO [18]. Twenty-five late third instar larvae were transferred into a glass bottle containing the test concentration (249 mL of dechlorinated tap water + 1 mL stock concentration) each, with four replicates. A control was setup with 25 larvae and 1 mL of denatured alcohol/water/1:1 acetone and DMSO in 249 mL water.

2.4 Data analysis

LC_{50} and LC_{90} were calculated by subjecting dosage mortality data to probit analysis [19]. The number of dead larvae was counted 24 hours post exposure. Percent mortality was calculated for each test. Mortality data from bioassays were corrected by natural control mortality using Abbott's formula [20] if more than 5-20% larval mortality occurred in the control.

$$\%\ mortality = \frac{No.\ of\ dead\ larvae}{Total\ number\ of\ larvae} X\ 100$$

Abbott's formula for corrected mortality,

$$Corrected\ mortality = \frac{\%Test\ mortality - \%\ Control\ mortality}{100 - \%\ Control\ mortality}$$

2.5 Egg float ridge number

The fresh eggs (un-hatched) laid by the blood fed adult female were placed under the microscope along with filter paper, which was lined for oviposition, for counting the egg float ridge number. The ridges on the egg float were counted under 10X magnification using Motic BA310 Trinocular microscope and analyzed by using Motic Image Plus 2.0 software. Based on the number of ridges on the egg floats, the strain would be grouped either as type (14-22 ridges), intermediate (12-17 ridges) or *mysorensis* (9-15 ridges) [7, 8].

3. Results and Discussion

The collection sites of *Anopheles stephensi* from Thokottu, Mangalore are presented in Fig 1A and B. The results of the resistance levels to chemical insecticides (Table 1 and Fig 2) and plant extracts (Table 1 and Fig 3) are presented.

The LC_{50} values for different insecticides ranged from 0.0421 mg/L to 1.995 mg/L and LC_{90} values ranged from 0.2296 to 4.4218 mg/L. The least LC_{50} (0.0421 mg/L) and LC_{90} (0.2296) were found against alphamethrin and the highest LC_{50} (1.995 mg/L) and LC_{90} (4.4218 mg/L) were found against lambda cyhalothrin. The LC_{50} and LC_{90} values for methanol extract of *Eucalyptus globulus* were 372.2 mg/L and 944.2 mg/L while for hexane extract were 314.26 mg/L and 504.54 mg/L respectively. Similarly the LC_{50} and LC_{90} values for methanol extract of *Calotropis gigantea* were 508.04 mg/L and 823.75 mg/L while for hexane extract were 311.67 mg/L and 784.15 mg/L. Among the studied insecticides, *Anopheles stephensi* was found susceptible to alphamethrin, followed by chlorpyrifos and propoxur while resistant to lambda cyhalothrin followed by fenvelarate and cypermethrin. Among

the plant extracts, *Anopheles stephensi* was susceptible to hexane extracts of both *Eucalyptus globulus* and *Calotropis gigantea* and while tolerable to both the methanol extracts. Chi-squire values (P<0.05) for all the insecticides and plant extracts were found to be non-significant.

Resistance studies on *Anopheles stephensi* have been carried out for various insecticides including fenthion, temephos, propoxur, deltamethrin and lambdacyhalothrin [21] and for different solvent extracts of *Eucalyptus globulus* and *Centella asiatica* [22]. Their results indicated high resistance to propoxur and susceptible to hexane extracts. Susceptibility status of *Anopheles stephensi* from Mangalore showed resistance to malathion, tolerance to deltamethrin, cyfluthrin, alphacypermethrin but was susceptible to DDT, lambdacyhalothrin and permethrin [9]. Studies on resistance to various insecticides on *Anopheles stephensi* from different parts of India showed mixed results owing to geographical variations [23-27]. Larvae of *Anopheles stephensi* and *Anopheles subpictus* were found to be tolerant to chlorpyrifos (0.025 mg/l) from Gujarat and Rajasthan, India [28].

Insecticide treatment has been in wide use and is currently indispensable for its control in almost all crops and public health programmes, especially in vector control programmes. The rational use of insecticides largely depends on a broad knowledge of the susceptibility and irritability levels of malaria vectors. This knowledge enables us to take all necessary precautions to prevent the occurrence of resistance and to prepare in advance a plan for coping with it at the early stages of its development in the field [29]. The first step is to asses trends in frequency of resistance gene/s by means of susceptibility tests and to investigate the efficacy of insecticides by bioassays. The most effective insecticides possible giving 100% kill should be used in rotation in vector control programmes [30]. Application of inappropriate insecticides without understanding the prevailing resistance mechanisms may lead to control failure. Hence, periodic monitoring of insecticide resistance status is an important criterion in vector control programmes [21].

sIn the present study, hexane extracts of the plants, *Eucalyptus globules* and *Calotropis gigantea* were more effective than methanol extracts. The hexane extracts of *Eucalyptus globules* (LC_{50}: 192.8 mg/L and LC_{90}: 827 mg/L) was found to be effective over methanol extract (889.6 and 2655.1 mg/L) on *Anopheles stephensi* from Bangalore [22]. Larvicidal potential of essential oils extracted from the *Eucalyptus* species on mosquito vectors have been carried out [31-36]. The larvicidal activity *Calotropis procera* latex was reported for the first time by Giridhar *et al.* [37]. In the present study, the observed LC_{50} for methanol extract of *Calotropis gigantea* was 508.04 mg/L while some studies reported much less LC_{50} values; 155.49 mg/L on *Anopheles stephensi* [38], 351.43 mg/L on *Aedes aegypti* [39] from India and 109.71 mg/L of *Calotropis procera* from Iran [40]. The observed variations in tolerance may to plant extracts may be by the intervention of biological and genetic factors [22].

It has been shown that the extraction of active biochemical from plants depends upon the polarity of the solvents used. The insects feed on the secondary metabolites (plant extracts) potentially encountering toxic substances with relatively non-specific effects on a wide range of molecular targets. These targets range from proteins (enzymes, receptors, signaling molecules, ion-channels and structural proteins), nucleic acids, biomembranes, and other cellular components [41]. This in turn, affects insect physiology in many different ways and at various receptor sites such as inhibition of acetylcholinesterase (by essential oils), GABA-gated chloride channel (by thymol), sodium and potassium ion exchange disruption (by pyrethrin) and inhibition of cellular respiration (by rotenone), inhibition of acetylcholinesterase activity (AChE) [41, 42].

The egg morphometry of the Thokottu strain of *Anopheles stephensi* showed the following details. The mean (M±SE) egg length and width were 488.01± 6.28 µm and 169.9 µm ± 3.30 µm respectively. Based on the egg float ridge number, the strain was classified as Type form with 19-21 ridges (20.13 ± 0.137).

Anopheles stephensi was classified as Type form and *mysorensis* basis on the differences in egg length, width and number of ridges on the egg-float [43]. Later *Anopheles stephensi* was classified under three ecological variants, type form, intermediate and *mysorensis* based on the egg float ridge number. Type form and intermediate forms were predominant in urban and semi-urban areas which were reported to be vectors while *mysorensis* form is predominant in rural areas and was determined as a non-vector [43, 7, 8]. Classification of the vector into ecological variants has a propounding effect on disease transmission [8]. The result of the present study is in accordance with the earlier reports where type form was found from semi-urban area.

3.1 Tables and Figures

Fig 1: A and B. Collection sites of *Anopheles stephensi* from Thokottu, Mangalore

A: Construction site where water was poured for curing

B: Cement well rings where water was filled for storing/curing

Table 1: LC_{50}, LC_{90} and Regression co-efficient of different insecticides on Thokottu strain of *Anopheles stephensi*

Insecticides / Plant extract	Class of the insecticide / Extract	LC_{50}	LC_{90}	Regression Equation	r	χ^{2*}	d.f.
Chlorpyrifos (CLP)	Organophosphate	0.1921	1.0216	y = 1.763x + 0.972	0.963	2.306	6
Propoxur (PPX)	Carbamate	0.2395	0.4166	y = 5.324x - 2.344	0.914	4.761	6
Alphamethrin (APM)	Synthetic pyrethroids	0.0421	0.2296	y = 1.737x + 0.441	0.991	1.102	9
Cypermethrin (CPM)		0.2543	2.8999	y = 1.210x + 2.087	0.965	2.386	6
Fenvelarate (FVR)		1.2984	3.6453	y = 2.855x - 1.034	0.964	0.974	6
Lambda cyhalothrin (LCT)		1.9950	4.4218	y = 3.703x + 0.186	0.978	0.926	7
Eucalyptus globulus (EGM)	Methanol extract	372.20	944.20	y = 3.166x - 3.140	0.986	0.494	5
Eucalyptus globulus (EGH)	Hexane extract	314.26	504.54	y = 6.226x - 10.54	0.989	0.183	5
Calotropis gigantea (CGM)	Methanol extract	508.04	823.75	y = 6.098x - 11.50	0.995	0.137	5
Calotropis gigantea (CGH)	Hexane extract	311.67	784.15	y = 3.194x - 2.965	0.966	0.867	5

* = non significant at P<0.05.

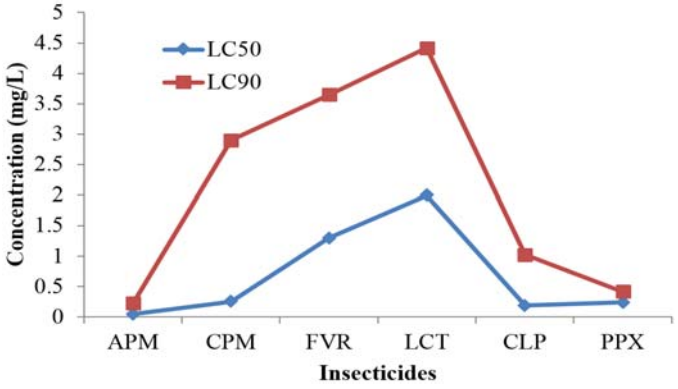

Fig 2: Resistance levels *of Anopheles stephensi* to different insecticides

APM = Alphamethrin; CPM = Cypermethrin; FVR = Fenvalerate
LCT = Lambda cyhalothrin; CLP = Chlorpyrifos; PPX = Propoxur

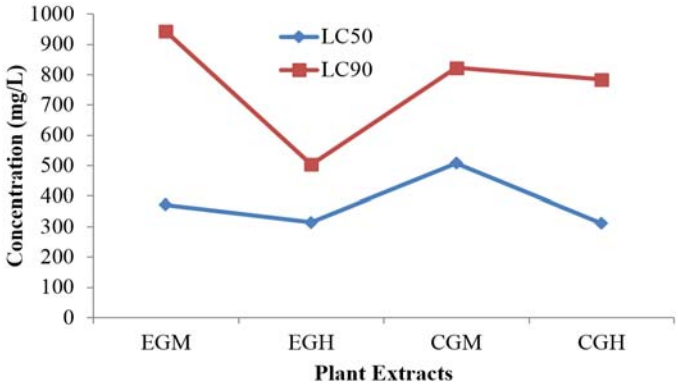

Fig 3: Resistance levels *of Anopheles stephensi* to different plant extracts

EGM = *Eucalyptus globulus* methanol extract; EGH =*Eucalyptus globulus* hexane extract
CGM = *Calotropis gigantea* methanol extract; CGH =*Calotropis gigantea* hexane extract

4. Conclusions

Anopheles stephensi from Thokottu, Mangalore was found to be susceptible to alphamethrin and resistant to lambda cyhalothrin. The hexane extracts of *Eucalyptus globulus* and *Calotropis gigantea* showed potential for control. Rotation of insecticides or sub-lethal doses which do not kill the vectors but reduce the fertility substantially can also be considered for effective control. This study provides preliminary information regarding insecticide resistance status of *Anopheles stephensi* subsequent biochemical and molecular investigations into the mechanisms of resistance will be carried out.

5. Acknowledgments

The authors HP and A.B.A thank Yenepoya University for financial support and providing facilities for the present study.

6. References

1. World Health Organization. Fact sheet no. 94, 2014. http://www.who.int/mediacentre/factsheets/fs094/en/
2. Dhingra N, Jha P, Sharma VP, Cohen AA, Jotkar RM, Rodriguez PS *et al.* Adult and child malaria mortality in India: a nationally representative mortality survey. Lancet 2010; 376:1768-1774.
3. Harbach RE. The Culicidae (Diptera): a review of taxonomy, classification and phylogeny. Zootaxa 2007; 1668:591-638.
4. Nagpal BN, Sharma VP. Indian Anophelines. Oxford & IBH Publishing Co. Pvt. Ltd, New Delhi, 1995, 415.
5. Shetty NJ. The genetic control of *Anopheles stephensi* – a malaria mosquito, In Proceedings of Trends in malaria and vaccine research, the current Indian scenario, the second Dorabji Tata symposium. Ed Ragunath D, Nayak R, Tata McGraw Hill, New Delhi, 2002a, 44-79.
6. Rao BA, Sweet WC, Subba Rao AM. Ova measurements of *Anopheles stephensi* type and *Anopheles stephensi* var. *mysorensis*. Journal of the Malaria Institute of India 1938; 1:261-266.
7. Subbarao SK, Vasantha K, Adak T, Sharma VP, Curtis CF. Egg-float ridge number in *Anopheles stephensi*: ecological variation and genetic analysis. Medical and Veterinary Entomology 1987; 1:265-271.
8. Shetty NJ, Madhyastha AD, Ghosh C, Rajashree BH. Egg float ridge number in *Anopheles stephensi*: Ecological Variation. Journal of Parasitic Diseases 1999; 23(1):45-48.
9. Tiwari S, Gosh SK, Pojha V, Dash AP, Raghavendra K. Reduced susceptibility to selected synthetic pyrethroids in urban malaria vector *Anopheles stephensi*: a case study in Mangalore city, South India. Malaria Journal 2010; 9:179 doi: 10.1186/1475-2875-9-179.
10. Hemingway J. Genetics and biochemistry of insecticide resistance in Anophelines. Ph. D Thesis. University of London, 1981, 310.
11. World Health Organization. Instruction for determining susceptibility or resistance of mosquito larvae to insecticides. WHO/VBC 1981; 81:807.
12. National Research Council Report. Strategies and tactics for management, In Pesticide resistance. The Academy, Washington, 1986, 471.
13. Sukumar K, Perich MJ, Boobar LR. Botanical derivatives in mosquito control: a review. Journal of American Mosquito Control Association 1991; 7:210-237.
14. Shaalan EAS, Canyonb D, Younesc MWF, Abdel-Wahaba H, Mansoura AH. A review of botanical phytochemicals with mosquitocidal potential. Environment International 2005; 3:1149-1166.
15. Ghosh A, Chowdhury N, Chandra G. Plant extracts as potential mosquito larvicides. Indian Journal of Medical Research 2012; 135:581-598
16. Roark RC. Some promising insecticidal plants. Economic Botany 1947; 1:437-445.
17. Shetty NJ. Chromosomal translocations and inherited semisterility in the Malaria Vector, *Anopheles fluviatilis*, James. Indian Journal of Malariology 1983; 20:45-47.
18. World Health Organization Guidelines for laboratory and field testing of mosquito larvicides, 2005. WHO/CDS/WHOPES/GCDPP/2005.13. Geneva, Switzerland.
19. Finney DJ. Probit Analysis, Edn 3, Cambridge University Press, Cambridge, 1971, 25-235.
20. Abbot WS. Method of computing the effectiveness of an insecticide. Economic Entomology 1925; 18:265-267.
21. Shetty V, Sanil D, Shetty NJ. Insecticide susceptibility status in three medically important species of mosquitoes, *Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus*, from Bruhat Bengaluru Mahanagara Palike, Karnataka, India. Pest Management Science 2012; 69:257-267.
22. Nair SS, Shetty V, Shetty NJ. Relative toxicity of leaf extracts of *Eucalyptus globulus* and *Centella asiatica* against mosquito vectors *Aedes aegypti* and *Anopheles*

stephensi. Journal of Insects, 2014. doi:10.1155/2014/985463.
23. Basker P, Shetty NJ. Susceptibility status of *Anopheles stephensi* Liston to insecticides. Journal of Communicable Diseases 1992; 24(3):188-190.
24. Shetty NJ. Evaluation of the insecticide susceptibility studies of mosquitoes of river Cauvery Basin, Karnataka State. Entomon 2002b; 27(4):375-383.
25. Ghosh C, Rajashree BH, Priyalakshmi BL, Shetty NJ. Susceptibility status of different strains of *Anopheles stephensi* Liston to fenitrothion, deltamethrin and cypermethrin. Pestology 2002c; 26(4):47-52.
26. Shetty NJ, Zin T, Hariprasad TPN, Minn MZ. Insecticide susceptibility studies in thirty stains of *Anopheles stephensi* Liston – a malaria vector to alphamethrin, bifenthrin (synthetic pyrethroids) and neem (a botanical insecticide). Pestology 2006; 30(10):21-28.
27. Shetty NJ, Vasanth SN, Sanil D. Insecticide susceptibility studies of fenthion and temephos (organophosphate) in thirty strains of *Anopheles stephensi* Liston, a malaria mosquito. Pestology 2007; 31(9):33-38.
28. Tikar SN, Mendki MJ, Sharma AK, Sukumaran D, Vee V, Prakash S *et al*. Resistance status of the malaria vector mosquitoes, *Anopheles stephensi* and *Anopheles subpictus* towards adulticides and larvicides in arid and semi-arid areas of India. Journal of Insect Science 2011; 11:85.
29. Vatandoost H, Borhani N. Susceptibility level and irritability of synthetic pyrethroids against main malaria vectors in the endemic areas of Iran. Acta Medica Iranica 2004; 42:247-255.
30. Kasap H, Kasap M, Alptekin D, Luleyap U, Herath PR. Insecticide resistance in *Anopheles sacharovi* Favre in southern Turkey. Bulletin of the World Health Organization 2000; 78:687-692.
31. Senthilnathan S. The use of *Eucalyptus tereticornis* Sm. (Myrtaceae) oil (leaf extract) as a natural larvicidal agent against the malaria vector *Anopheles stephensi* Liston (Diptera: Culicidae) Bioresource Technology 2007; 98(9):1856-1860.
32. Lucia A, Juan LW, Zerba EN, Harrand L, Marc´o M, Masuh HM. Validation of models to estimate the fumigant and larvicidal activity of *Eucalyptus* essential oils against *Aedes aegypti* (Diptera: Culicidae). Parasitology Research 2012; 110(5):1675-1686.
33. Cheng SS, Huang CG, Chen YJ, Yu JJ, Chen WJ, Chang ST. Chemical compositions and larvicidal activities of leaf essential oils from two eucalyptus species. Bioresource Technology 2009; 100(1):452-456.
34. Tesfay A, W/Yowhanns A, Niguse E, Brhane G, Brhane S, Nagappan R. Evaluation of water and ethanol extract of *Eucalyptus globules* labillardiere (Myrtaceae) leaves against immature stages of filarial vector *Culex quinquefasciatus* say (Diptera: Culicidae). Current Research Journal of Biological Sciences 2014; 4(2):220-224.
35. Vera SS, Zambrano DF, Méndez-Sanchez SC, Rodríguez-Sanabria F, Stashenko EE, Duque Luna JE. Essential oils with insecticidal activity against larvae of *Aedes aegypti* (Diptera: Culicidae). Parasitology Research 2014; 113(7):2647-2654.
36. Senthilkumar N, Varma P, Gurusubramanian G. Larvicidal and adulticidal activities of some medicinal plants against the Malarial Vector, *Anopheles stephensi* (Liston). Parasitology Research 2009; 104(2):237-244.
37. Giridhar G, Deval K, Mittal PK, Vasudevan P. Mosquito control by *Calotropis procera* latex. Pesticides 1984; 18:26-29.
38. Kovendan K, Murugan K, Kumar KP, Panneerselvam C, Kumar PM, Amerasan D *et al.* Mosquitocidal properties of *Calotropis gigantea* (Family: Asclepiadaceae) leaf extract and bacterial insecticide, *Bacillus thuringiensis*, against the mosquito vectors. Parasitology Research 2012; 111(2):531-544
39. Shreya N, Raghavendra NP, Mukherji V, Maria Vincy R, Namratha, Pradeep AS, Ghosh SK *et al.* Larvicidal activity of *Calotropis gigantea* (L.) R.Br. on dengue and chikungunya vector *Aedes aegypti*. Research Journal of Pharmaceutical, Biological and Chemical Sciences 2012; 3(3):118-121.
40. Shahi M, Hanafi-Bojd AA, Iranshahi M, Vatandoost H, Hanafi-Bojd MY. Larvicidal efficacy of latex and extract of *Calotropis procera* (Gentianales: Asclepiadaceae) against *Culex quinquefasciatus* and *Anopheles stephensi* (Diptera: Culicidae). Journal of Vector Borne Diseases 2010; 47:185-188.
41. Senthilnathan S, Choi MY, Seo HY, Paik CH, Kalaivani K, Kim JD. Effect of azadirachtin on acetylcholinesterase (AChE) activity and histology of the brown planthopper *Nilaparvata lugens* (Stål). Ecotoxicology and Environment Safety 2008; 70:244-250.
42. Rattan RS. Mechanism of action of insecticidal secondary metabolites of plant origin. Crop Protection 2010; 29:913-920.
43. Sweet WC, Rao BA. Races of *Anopheles stephensi* Liston, 1901. Indian Medical Gazette 1937; 72:665-674.