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Toxicological properties of several medicinal plants from the Himalayas (India) against vectors of malaria, filariasis and dengue

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Abstract. The leaves of five plants namely *Nyctanthes arbortistis* (Oleaceae), *Catharanthus roseus* (Apocynaceae), *Boeninginghusenia albiflora* (Rutaceae), *Valeriana hardwickii* (Valerianaceae) and *Eupatorium odoratum* (*Asteraceae*) were selected for the first time from the Garhwal region of north west Himalaya to investigation its toxicological properties against mosquito vectors of malaria, filariasis and dengue. In a laboratory study, using different polarity solvents (petroleum ether, chloroform and methanol) were tested against important larvae of malaria, filariasis and dengue vectors in India. It was observed that petroleum ether fraction of all selected plant possess good larvicidal properties than other solvent fraction. The LC_{50} values of isolates from *Nyctanthes arbortistis* (HAR-1), *C. roseus* (CAT-1), *B. albiflora* (BOA-1), *V. hardwickii* (SUG-1) and *E. odoratum* (EUP-1) against *Anopheles stephensi* were 185 ppm, 150 ppm, 105 ppm, 225 ppm and 135 ppm, respectively. The results therefore suggest that the fraction code BOA-1 has excellent larvicidal properties and could be incorporated as botanical insecticides against mosquito vectors with high safety to non-target organisms. The same fraction was tested against adult vectors of malaria, filariasis and dengue, but no mortality was observed.

INTRODUCTION

Mosquito bites transmit deadly diseases such as malaria, filaria, yellow fever, dengue and Japanese encephalitis, which contribute significantly to poverty and social debility in tropical countries (Jang *et al.*, 2002). Eradication of these vectors has been considered using chemical insecticides. However these synthetic insecticides not only affect the non target population, but also constantly increase resistance in the vectors (Wattal *et al.*,1981). The search for natural insecticides which do not have any ill effects on the non-target population and are easily degradable remains the top priority (Redwane, *et al.*, 2002). A large number of plant extracts have been reported to have mosquitocidal activity against mosquito vectors (Sukumar *et al.*, 1991). Dua *et al.*, (2008) have reported the insecticidal properties of *Valeriana jatamansi* (Valerianaceae) against mosquitoes. Recently Alam *et al.* (2010), reported on the toxicity of *Vernonia anthelmintica* Linn (Asteracea) seeds against mosquitoes vectors.

North West Himalaya in India represents one of the most important mega centres of bio diversity, showing fifty percent of the vegetation wealth of the Indian subcontinent. *Eupatorium odoratum* is a coarse, often straggling shrub bearing exceedingly small, numerous, fragrant flowers and abundant seeds which are easily dispersed by wind. It is an obnoxious weed in the sub Himalayan

plains and foothills, covering extensive areas in Assam and Bengal and interfering with the natural regeneration of timber trees in plantations. *Catharnthus roseaes* is an erect, much branched, annual or perennial herb, 60-90 cm in height, probably native to Madagascar occurring up to 1300 m. It is distributed / cultivated as an ornamental plant in gardens throughout the world and are commercialy bred in Malagasy, Israel, India and the United States. *Nyctanthes arbortristis* is a hardy large shrub or small tree, up to 10 m in height, with grey or greenish white rough bark. *Nyctanthes arbortristis* is a native of India occurring wild in the sub Himalayan region, from Chenab to Nepal, up to 1500 m height in Chota Nagpur, Rajasthan, Madhay Pradesh and southwards to Godawari. It is cultivated in gardens almost throughout India for its fragrant flowers (Gaur, 1999; Wealth of India, 1966). *Boenninghausenia albiflora* is a slender, erect, glaucescent, gland dotted, glabrous to somewhat pubescent, perennial herb, 15-60 cm in height. It is distributed from Kashmir to Arunachal Pradesh, Meghalaya and Mizoram, between 660-2,640 m height (Wealth of India 1988; Gaur, 1999). *Valeriana hardwickii* is a perennial, erect, branched herb, with thick rootstock; stems are simple or branched, pubescent below, glabrescent above, and 50-60 cm long. It is a pubescent herb distributed from Kashmir to Bhutan in open grassy slopes of the mountain zone at an altitude of 1200-3600 m, but in the Khasi and Jaintia hills it is found in between the attitude of 1500-1800 m. Literatures indicated that the plants mentioned above have not been reported for its toxicological properties against public health vectors.

The present study was undertaken to investigate the toxicological properties of different fractions of five medicinal plants (leaves) selected from the Himalayas (India) against different species of mosquitoes.

MATERIAL AND METHODS

Plant Materials

The leaves of five plants namely *C. roseus*, *N. arboristis*, *B. albiflora*, *E. odoratum* and *V. hardwickii* were collected from Dehradun and locally from Haridwar while the leaves of *B. albiflora* were collected from the Chakrauta (Uttranchal) India. Extraction of plant materials were conducted in the laboratory and tested for larval toxicity.

Extraction of plant materials

The collected materials (leaves) from the different plant species were thoroughly washed with distilled water and dried under a shed. The dried plant materials were ground into a powdered form using an electrical grinder. Plant materials were extracted with the following extraction methods using different polarity solvent. A known quantity of plant material was extracted for 7 hours with petroleum ether using a soxhlet apparatus. Petroleum fraction was separated and dried under rotatory evaporator at 60°C. Dried material was coded specially for each plant material. The residue was re-extracted with chloroform for seven hours using a soxhlet apparatus. Chloroform fraction was coded accordingly. After removal of the chloroform extract the residue was again extracted with methanol for seven hours. Methanol fraction was dried and coded for each plant material. All the extracts were stored at 4°C. The isolation of various fraction of each of the plant material along with their code and yields are given in Table 1.

Bioassay for larval toxicity

Bioassay test of all plant fractions were evaluated in two phases, preliminary and quantitative phase, against *Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus* in the laboratory. Bioassay were performed according to standard method as described earlier (WHO, 1996) under laboratory conditions at a temperature of 27 ± 2°C and relative humidity of 70-80%. Preliminary larvicidal activity of the different fractions of the different plant mentioned above was carried out against early fourth instar larvae of *An. stephensi*, *Ae. aegypti and Cx. quinquefasciatus* at 250ppm concentration. Twenty larvae of each mosquito species were placed in a 500 ml capacity glass beaker containing 250 ml water. A mixture of dog biscuits and yeast

Table 1. Yield of different plant fractions isolated using different polarity solvents

Plant species	Plant part (gm)	Solvent used	Fraction code	Percent yield (w/w)
Catharanthus roseus	Leaves (100gm)	Petroleum ether	CAT-1	0.07
		Chloroform	CAT-2	0.85
		Methanol	CAT-3	1.15
Eupatorium odoratum	Leaves (100gm)	Petroleum ether	EUP-1	4.28
		Chloroform	EUP-2	2.05
		Methanol	EUP-3	3.17
Nyctanthes arbortristis	Leaves (80gm)	Petroleum ether	HAR-1	0.85
		Chloroform	HAR-2	1.54
		Methanol	HAR-3	2.35
Boenninghausenia albiflora	Leaves (80gm)	Petroleum ether	BOA-1	0.98
		Chloroform	BOA-2	1.23
		Methanol	BOA-3	1.85
Valeriana hardwickii	Roots (100gm)	Petroleum ether	SUG-1	0.17
		Chloroform	SUG-2	0.25
		Methanol	SUG-3	0.75

gm= gram, w/w: weight/weight

powder in to ratio of 3: 2 were provided as nutrients. Larval mortality was monitored after 24 hours. All tests were carried out in four replicates along with untreated control to determine the larval toxicity.

Quantitative bioassay was carried out against early 4th instar larvae of the different mosquitoes species. Twenty larvae of each mosquito species were placed in a 500 ml beaker capacity containing 250 ml water. The mosquito larvae were exposed to at least four different concentrations used for the bioassay. Four replicates with parallel control were carried out. Larval mortality was monitored within 24 hours. The mortality was plotted on a log probit paper to obtain regression line from which the lethal concentration (LC_{50} and LC_{90} values) was calculated (Finney, 1971).

Bioassay for adult toxicity

The WHO method was used to determine the adulticidal activity of different plant fraction against *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus*. Whatman no. 1 filter paper was impregnated with 10% concentration of the plant extracts (250 mg plant extract / 2.5 ml acetone spread on 180 cm^2 area which is equivalent to a dose of 1.38mg/cm^2) for preliminary adulticidal activity test. Twenty 2-5 days old glucose fed adult mosquitoes were exposed on the treated paper for one hour and knockdown was counted. After one-hour exposure mosquitoes were transferred into a holding test tube for 24 hours post treatment observation. During this period the mosquitoes were kept at a room temperature of 27 ± 2°C and 70-80% relative humidity. All tests were carried out in four replicates along with untreated control (2.5 ml acetone /180 cm^2) to determine the adulticidal activity. The plant fractions were considered to have an adulticidal activity if it showed more than 70% mortality at 10% concentration on impregnated paper within 24 hours (Dua *et al.*, 2008).

RESULTS AND DISCUSSION

The leaves of five plants namely *N. arbortistis* (Oleaceae), *C. roseus* (Apocynaceae), *B. albiflora* (Rutaceae), *V. hardwickii* (Valerianaceae) and *E. odoratum* (Asteraceae) were sequentially extracted using petroleum ether, chloroform and methanol. The percent yields of the different fractions with different solvents are given in Table 1.

Preliminary larvicidal activity of all the five plant fractions were carried out at 250 ppm concentration with four replicates against *An. stephensi*, *Ae. aegypti and Cx. quinquefasciatus* mosquito larvae and mortality was recorded after 24 hours. Preliminary larvicidal study clearly showed that petroleum extracts of all plant fractions possessed good larvicidal properties against all three important vectors viz. *An. stephensi* (malaria), *Cx. quinquefasciatus* (filariasis) and *Ae. aegypti* (dengue) at 250 ppm concentration. It was observed that the other solvent (chloroform and methanol) fractions gave less than 50% mortality at same concentration against all three vectors (Table 2).

Table 2. Preliminary screening of plant fractions for larvicidal activity against mosquitoes at 250 ppm concentration

Plant species	Fraction code	Mosquito larvae	No. of Larvae	% Mortality (24 hrs)				% Mortality (mean ± sd)
				R_1	R_2	R_3	R_4	
Catharanthus roseus (Apocynaceae)	CAT-1	*An. stephensi*	20	48	50	55	60	53.6 ± 5.3
		Ae. aegypti	20	55	45	49	50	49.8 ± 4.1
		Cx. quinquefasciatus	20	60	52	55	51	54.5 ± 4.0
		Control	20	0	0	0	0	0.0
	CAT-2	*An. stephensi*	20	25	20	30	25	25.0 ± 4.0
		Ae. aegypti	20	20	15	25	20	20.0 ± 4.0
		Cx. quinquefasciatus	20	15	20	15	20	17.5 ± 2.8
		Control	20	0	0	0	0	0.0 ± 0.0
	CAT-3	*An. stephensi*	20	15	10	15	10	12.5 ± 2.8
		Ae. aegypti	20	10	05	10	05	07.5 ± 2.8
		Cx. quinquefasciatus	20	05	05	10	05	06.2 ± 2.5
		Control	20	0	0	0	0	0.0
Eupatorium odoratum (*Asteraceae*)	EUP-1	*An. stephensi*	20	60	55	50	50	53.7 ± 4.7
		Ae. aegypti	20	55	50	45	50	50.0 ± 4.0
		Cx. quinquefasciatus	20	50	53	55	59	54.3 ± 3.8
		Control	20	0	0	0	0	0.0
	EUP-2	*An. stephensi*	20	25	20	15	20	20.0 ± 4.0
		Ae. aegypti	20	15	20	20	15	17.5 ± 2.8
		Cx. quinquefasciatus	20	10	15	10	15	12.5 ± 2.8
		Control	20	0	0	0	0	0.0 ± 0.0
	EUP-3	*An. stephensi*	20	05	0	10	0	3.7 ± 4.7
		Ae. aegypti	20	0	0	05	0	1.2 ± 2.5
		Cx. quinquefasciatus	20	0	05	0	05	2.5 ± 2.8
		Control	20	0	0	0	0	0.0
Nyctanthes arbortristis (Oleaceae)	HAR-1	*An. stephensi*	20	52	48	55	49	51.0 ± 3.2
		Ae. aegypti	20	55	50	49	51	51.2 ± 2.6
		Cx. quinquefasciatus	20	51	49	50	53	50.7 ± 1.7
		Control	20	0	0	0	0	0.0
	HAR-2	*An. stephensi*	20	10	10	05	10	08.7 ± 2.5
		Ae. aegypti	20	05	05	10	05	06.2 ± 2.5
		Cx. quinquefasciatus	20	05	05	0	10	05.0 ± 4.0
		Control	20	0	0	0	0	0.0
	HAR-3	*An. stephensi*	20	0	0	05	0	01.2 ± 2.5
		Ae. aegypti	20	0	0	0	0	0.0 ± 0.0
		Cx. quinquefasciatus	20	0	0	0	0	0.0 ± 0.0
		Control	20	0	0	0	0	0.0

	BOA-1	*An. stephensi*	20	65	70	55	60	62.5 ± 6.4
		Ae. aegypti	20	60	65	55	60	60.0 ± 4.0
		Cu. quinquefasciatus	20	55	60	50	65	57.5 ± 6.4
		Control	20	00	00	00	00	0.0
Boenninghausenia albiflora (Rutaceae)	BOA-2	*An. stephensi*	20	55	45	50	50	50.0 ± 4.0
		Ae. aegypti	20	40	55	45	40	45.0 ± 7.0
		Cx. quinquefasciatus	20	40	50	50	40	45.0 ± 5.7
		Control	20	00	00	00	00	0.0
	BOA-3	*An. stephensi*	20	05	10	05	05	06.2 ± 2.5
		Ae. aegypti	20	05	00	05	00	02.5 ± 2.8
		Cx. quinquefasciatus	20	05	05	00	05	03.7 ± 2.5
		Control	20	00	00	00	00	0.0
	SUG-1	*An. stephensi*	20	50	55	57	54	54.0 ± 2.9
		Ae. aegypti	20	65	60	59	55	59.7 ± 4.1
		Cx. quinquefasciatus	20	55	52	61	55	55.7 ± 3.7
		Control	20	00	00	00	00	0.0
Valeriana hardwickii (*Valerianaceae*)	SUG-2	*An. stephensi*	20	10	10	15	10	11.2 ± 2.5
		Ae. aegypti	20	10	10	10	10	10.0 ± 0.0
		Cx. quinquefasciatus	20	05	10	05	10	07.5 ± 2.8
		Control	20	00	00	00	00	0.0
	SUG-3	*An. stephensi*	20	25	20	20	20	21.2 ± 2.5
		Ae. aegypti	20	20	20	25	20	18.7 ± 2.5
		Cx. quinquefasciatus	20	15	15	20	15	16.2 ± 2.5
		Control	20	0	0	0	0	0.0

R= Replicate; *An. = Anopheles; Ae. = Aedes* and *Cx. = Culex*

Therefore, further quantitative estimation of the active fractions (petroleum ether) was carried out to determine the lethal concentration of a particular fraction. Thus active fractions CAT-1 (*C. roseus*), EUP-1 (*E. odoratum*), BOA-1 (*B. albiflora*), HAR-1 (*N. arbortristis*) and SUG-1 (*V. hardwickii*) were studied for the quantitative bio assay. The LC_{50} and LC_{90} values of the active fractions are given in Table 3.

Mean LC_{50} and LC_{90} values of active fraction code CAT-1 was 150 and 280 ppm; 145 and 225 ppm; 160 and 270 ppm against *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus* respectively. Mean LC_{50} and LC_{90} values of active fraction code EUP-1 was 135 and 250 ppm; 155 and 290 ppm and 145 and 300 ppm against *An. stephensi*, *Ae. aegypti and Cx. quinquefasciatus* respectively. While the mean LC_{50} and LC_{90} values of active fraction code HAR-1 were 185 and 320 ppm; 180 and 340 ppm and 160 and 335 ppm against *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus* respectively. Mean LC_{50} and LC_{90} values of active fraction code BOA-1 were 105 and 180 ppm; 125 and 190 ppm; 115 and 200 ppm against *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus* respectively. While the mean LC_{50} and LC_{90} values of active fraction code BOA-1 were 225 and 390 ppm; 235 and 415 ppm; 180 and 385 ppm against *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus* respectively. The finding indicates that petroleum fraction of all plants are more effective against malaria, filariasis and dengue vectors (Fig. 1). Results revealed that the fraction code BOA-1 is more active against all test species in comparison to other isolated fractions. Thus the plant species *B. albiflroa* was found to be more effective for mosquito larval control.

Preliminary adulticidal toxicity of all five plant fraction (*C. roseus*, *N. arboristis*, *B. albiflora*, *E. odoratum* and *V. hardwickii*) were carried out at 10% concentration (250 mg plant extract / 2.5 ml acetone spread on 180 cm^2 area which is equivalent to a dose of 1.38mg/cm^2) with four replicates against *An. stephensi*, *Ae. aegypti and Cx. quinquefasciatus*. Results showed that

Table 3. Quantitative study of larvicidal properties of active fraction isolated from different plants species

Plant species	Active fraction code	Mosquito species	Larvicidal activity (ppm) (Mean ± SD) LC_{50}	LC_{90}
Catharanthus roseus	CAT-1	*An. stephensi*	150±8.6	280±5.0
		Ae. aegypti	145±6.8	255±7.3
		Cx. quinquefasciatus	160±5.7	270±4.5
Eupatorium odoratum	EUP-1	*An. stephensi*	135±7.5	250±6.2
		Ae. aegypti	155±5.5	290±7.5
		Cx. quinquefasciatus	145±6.0	300±4.0
Nyctanthes arboritis	HAR-1	*An. stephensi*	185±4.0	320±6.6
		Ae. aegypti	180±5.0	340±5.0
		Cx. quinquefasciatus	160±5.5	335±6.8
Boenninghausenia albiflora	BOA-1	*An. stephensi*	105±7.5	180±3.5
		Ae. aegypti	125±5.5	190±2.5
		Cx. quinquefasciatus	115±6.0	200±5.5
Valeriana hardwickii	SUG-1	*An. stephensi*	225±3.8	390±6.2
		Ae. aegypti	235±7.6	415±8.0
		Cx. quinquefasciatus	180±4.0	385±7.5

n=4; Standard deviation SD; part per million ppm

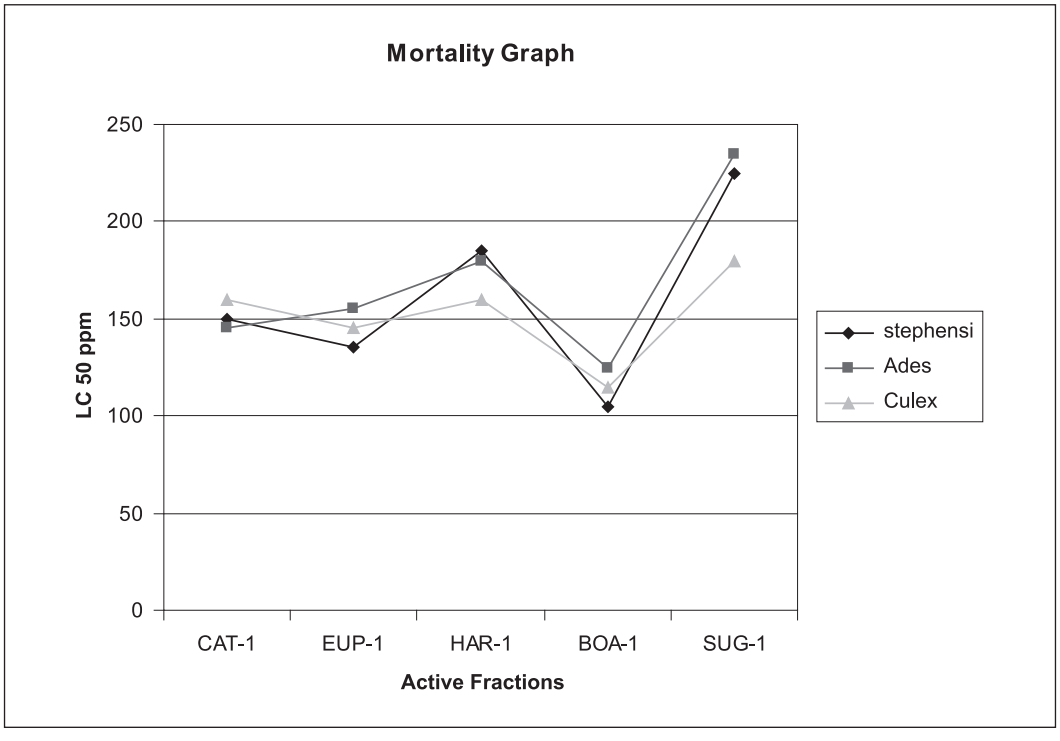

Figure 1. LC_{50} value of active fraction (coded as CAT-1, EUP-1, HAR-1, BOA-1 and SUG-1) against mosquitoes vectors

exposure to fraction BOA-1 of *B. albiflora* gave a 40% knockdown mosquitoes while the remaining plant fractions did not result in a single knockdown upon exposure. When the exposed mosquitoes were transferred to a holding tube after 1 hour all knockdown mosquitoes started to recover. Finally after 24 hours no mortality and knockdown were observed. Therefore, no further studies on the quantitative estimation of adulticidal activities were conducted.

Pizzarro, *et al.* (1999) studied the activity of the saponin fraction of *Agave sisalana* and estimated that the LC_{50} and LC_{90} value against 3rd instar larvae of *Cx. quinquefasciatus*, were 183 and 408 ppm, respectively. Markouk *et al.* (2000) evaluated 16 extracts of four Moroccan medicinal plants for larvicidal properties against *Anopheles labranchiae* mosquito larvae and reported nine extracts gave high larvicidal activity with LC_{50} values (in the range of 28 to 325 ppm). Recently, Ansari *et al.* (2005) reported the larvicidal activity of pine oil against *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti* with LC_{50} values of 112.6, 85.7 and 82.1 ppm, respectively. The leaves extract of *N. arbortristis* had been earlier reported by Mathew *et al.* (2009). These concentrations were equivalent to those reported in the present study. More recently, Patil *et al.* (2010) reported on the larvicidal activity of six plants (including *N. arbortristis*) extracts prepared with dichloromethane and methanol against two mosquito species *Ae. aegypti* and *An. stephensi.* However, as evident from the present results, petroleum faction of *Nyctanthes arbortristis* leaves was more effective (LC_{50} 180.50 ppm) as compared to its extract prepared with dichloromethane (LC_{50} 260.72 ppm) against *Ae. aegypti*.

The findings of the present study indicate that the leaves of *Boenninghausenia albiflora* possess excellent larvicidal properties against mosquito vectors. Further investigations are needed to elucidate the pure compounds and test against a wide range of mosquito species.

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REFERENCES

Alam, M.F., Chopra, A.K., Shafi, M. Mohd & Dua, V.K. (2010). Toxicity of *Vernoni anthelmintica* Linn(Asteracea) against mosquitoes vectors. *Journal of Applied and Natural Science* **2**(2): 190-193.

Ansari, M.A., Mittal, P.K., Razdan, R.K. & Sreehari, U. (2005). Larvicidal and repellent activities of Pine (*Pinus longifolia*, Family : Pinaceae) oil. *Journal of Vector Borne Diseases* **42**: 95-99.

Dua, V.K., Alam, M.F., Pandey, A.C., Rai, S., Chopra, A.K., Kaul, V.K. & Dash, A.P. (2008). Insecticidal activity of *Valeriana jatamansi* (Valerianaceae) against mosquitoes. *Journal of American Mosquito Control Association* **24**(2): 315-318.

Finney, D.J. (1971). *Probit Analysis*, third ed. Cambrideg University Press, Cambridge, 9-158.

Jang, Y.S., Kim, M.K., Ahn, Y.J. & Lee, H.S. (2002). Larvicidal activity of Brazilian plants against *Aedesaegypti* and *Culex pipiens* (Diptera: Culicidae) *Agricultural Chemistry and Biotechnology* **45**(3): 131-134.

Markouk, M., Bekkouche, K., Larhsini, M., Bousaid, Lazrek, H.B. & Jana, M. (2000). Evaluation of some Moroccan medicinal plant extracts for larvicidal activity. *Journal of Ethnopharmacology* **73**(1-2): 293-297.

Mathew, N., Anitha, M.G., Bala, T.S.L., Sivakumar, S.M., Narmadha, R. & Kalyanasundaram, M. (2009). Larvicidal activity of saraca indica, Nyctanthes arbor-tristis and clitoria ternatea extracts against three mosquito vector species. *Parasitology Research* **104**: 1017-1025.

Pizzarro, A.P.B., Oliveira-filho, A.M., Parente, J.P., Melo, M.T.V., Santos, C.E. & Lima, P.R. (1999). Oaproveitamento do residuo da industria do sisal no controle de larvas de mosquitos. *Revista da Sociedade Brasileira de Medicina Tropical* **32**: 23-29.

Rajkumar, S. & Jebanesan, A. (2005). Larvicidal and Adult emergence inhibition effects of *Centella asiatica* Brahmi (Umbelliferae) against mosquito *Culex quinquefasciatus* Say (Diptera : Culicidae) *African Journal of Biomedical Research* **8**(1): 31-33.

Redwane, A., Lazrek, H.B., Bouallam, S., Markouk, M., Amarouch, H. & Jana, M. (2002). Larvicidal activity of extracts from *Querus Lusitania* var *infectoria* galls (Oliv). *Journal of Ethnopharmacology* **79**: 261-263.

Sukumar, K., Parich, M.J. & Boobar, C.R. (1991). Botanical derivatives in mosquito control. A review. *Journal of American Mosquito Control Association* **7**: 210-287.

WHO. (1996). Report of the WHO informal consultation on the evaluation and testing of insecticides. *CTD/WHOPES/IC/96* **1**: 69.