Indian J Med Res 134, July 2011, pp 101-106

Larvicidal activity of medicinal plant extracts against *Anopheles subpictus* & *Culex tritaeniorhynchus*

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Received December 17, 2009

***Background & objectives*: Mosquitoes transmit serious human diseases, causing millions of deaths every year and the development of resistance to chemical insecticides resulting in rebounding vectorial capacity. Plants may be alternative sources of mosquito control agents. The present study assessed the role of larvicidal activities of hexane, chloroform, ethyl acetate, acetone, and methanol dried leaf and bark extracts of *Annona squamosa* L., *Chrysanthemum indicum* L., and *Tridax procumbens* L. against the fourth instar larvae of malaria vector, *Anopheles subpictus* Grassi and Japanese encephalitis vector, *Culex tritaeniorhynchus* Giles (Diptera: Culicidae).**

***Methods*: Larvicidal activities of three medicinal plant extracts were studied in the range of 4.69 to 1000 mg/l in the laboratory bioassays against early 4th instar larvae of *An. subpictus* and *Cx. tritaeniorhynchus.* The mortality data were subjected to probit analysis to determine the lethal concentrations (LC_{50} and LC_{90}) to kill 50 and 90 per cent of the treated larvae of the respective species.**

***Results*: All plant extracts showed moderate effects after 24 h of exposure; however, the highest toxic effect of bark methanol extract of *A. squamosa*, leaf ethyl acetate extract of *C. indicum* and leaf acetone extract of *T. procumbens* against the larvae of *An. subpictus* (LC_{50} = 93.80, 39.98 and 51.57 mg/l) and bark methanol extract of *A. squamosa,* leaf methanol extract of *C. indicum* and leaf ethyl acetate extract of *T. procumbens* against the larvae of *Cx. tritaeniorhynchus* (LC_{50}=104.94, 42.29 and 69.16 mg/l) respectively.**

***Interpretation & conclusions*: Our data suggest that the bark ethyl acetate and methanol extract of *A. squamosa,* leaf ethyl acetate and methanol extract of *C. indicum*, acetone and ethyl acetate extract of *T. procumbens* have the potential to be used as an ecofriendly approach for the control of the *An. subpictus*, and *Cx. tritaeniorhynchus*.**

Key words *Anopheles subpictus* - *Culex tritaeniorhynchus* - medicinal plant extracts - larvicide

Mosquitoes are the major vector for the transmission of malaria, dengue fever, yellow fever, filariasis, schistosomiasis and Japanese encephalitis (JE)[1]. In India, malaria is one of the most important causes of direct or indirect infant, child and adult mortality with approximately two to three million new cases arising every year. *Anopheles subpictus* Grassi is distributed throughout India, Afghanistan, Borneo,

China, Malaysia, Philippines, Sri Lanka, Java and Indonesia. It is a dominant species in Haryana and Uttaranchal states[2]. Though it is a non-vector species, same infected specimens with malarial parasite have been reported from India, Indonesia and Java[3]. *An. culicifacies* is the main vector of malaria, and *An. subpictus* is a significant secondary vector in Sri Lanka[4]. *An. subpictus* is recognized as the secondary vector of malaria in South East Asia, with a large number of cases being reported from India. 2,400 million (about 40%) of the world's population[5,6]. India contributes 77 per cent of the total malaria in Southeast Asia[7]. *Cx. tritaeniorhynchus* is a primary vector of Japanese encephalitis (JE) virus, with a distribution throughout Southeast Asia and South Asia. Keiser *et al*[8] have reported that global annual incidence and mortality estimates for JE are 30,000 to 50,000 and 10,000 respectively.

Annona squamosa, commonly known as custard apple is a native of West Indies and is cultivated throughout India, mainly for its edible fruit. Das *et al*[9] reported that the ethanol leaf extract of *A. squamosa* was found to have the most promising larvicidal activity against *Cx. quinquefasciatus* larvae. The larvicidal and mosquitocidal activities of ethanolic water mixture extract of *A. squamosa* and *Centella asiaticai* were effective against larvae of *An. stephensi*[10]. The leaves methanolic extract and the seeds petroleum ether extract of *A. squamosa* showed larvicidal activity when tested against *An. stephensi*, *Cx. quinquefasciatus* and *Aedes aegypti*[11]. *Chrysanthemum indicum* is a traditional herb used to treat various disorders, hypertension symptoms and several infectious diseases in Korean and Chinese medicine[12]. Beninger *et al*[13] reported that the leaves of *C. morifolium* extracted sequentially with hexane, ethyl acetate, and methanol were found to reduce the growth of *Trichoplusia ni* larvae. *Tridax procumbens* is commonly used in Indian traditional medicine as anticoagulant, antifungal and insect repellent, in bronchial catarrh, diarrhoea and dysentery[14]. The entire plant is used for the treatment of malaria, leishmaniasis, vaginitis, dysentery and gastrointestinal disorders[15].

Though larvicides play a vital role in controlling mosquitoes in their breeding sites, these also show a negative impact in areas of beneficial and non-target organisms. In view of an increasing interest in developing plant origin insecticides as an alternative to chemical insecticide, this study was undertaken to assess the larvicidal potential of the extracts from the medicinal plants against two medically important species of malaria vector, *An. subpictus* and JE vector, *Cx. tritaeniorhynchus*.

Material & Methods

Collection of plant materials: The bark of *Annona squamosa* L. (Annonaceae), leaf of *Chrysanthemum indicum* Linné (Compositae), *Tridax procumbens* Linn. (Asteraceae) were collected from Chitheri Hills, Dharmapuri District, Tamil Nadu, India in March 2009 and the taxonomic identification was made by Dr. C. Hema, Department of Botany, Arignar Anna Govt. Arts College for Women, Walajapet, Vellore, India. The voucher specimen was numbered and kept in the research laboratory of Unit of Bioactive Natural Products, Post Graduate & Research Department of Zoology, C. Abdul Hakeem College, Melvisharam, Vellore District, Tamil Nadu, India for further reference.

Preparation of plant extracts: The leaf and bark were dried for 7-10 days in the shade at the environmental temperatures (27-37° C day time). The dried bark (600 g) and leaf (700 g) were powdered mechanically using commercial electrical stainless steel blender and extracted with hexane (2,200 ml, Fine Chemicals, Mumbai), chloroform (1,000 ml, Fine Chemicals, Mumbai), ethyl acetate (2,500 ml, Qualigens, Fine Chemicals, Mumbai, India), acetone (1,000 ml, Qualigens), and methanol (2,800 ml, Qualigens), in a soxhlet apparatus (boiling point range 60–80°C) for 6 h. The extracts were filtered through a Buchner funnel with Whatman number 1 filter paper. The extract was concentrated under reduced pressure 22 - 26 mm Hg at 45°C and the residue obtained was stored at 4°C. The residues were then made in to a 1 per cent stock solution with acetone (stock solution). From the stock solution, 1000-4.69 mg/l, dilutions were prepared with dechlorinated tap water. Polysorbate 80 (Qualigens) was used as an emulsifier at the concentration of 0.05 per cent in the final test solution[16,17].

Mosquito culture: *An. subpictus* and *Cx. tritaeniorhynchus* larvae were collected from rice field and stagnant water areas of Melvisharam and identified in Zonal Entomological Research Centre, Vellore, Tamil Nadu, to start the colony and larvae were kept in plastic and enamel trays containing tap water. They were maintained and all the experiments were carried out at 27 ± 2°C and 75–85 per cent relative humidity under 14:10 h light and dark cycles. Larvae were fed a diet of Brewer's yeast, dog biscuits and algae collected from ponds in a ratio of 3:1:1, respectively as per the method of Kamaraj *et al*[17].

Larvicidal bioassay: During preliminary screening with the laboratory trial, the larvae of *An. subpictus* and *Cx. tritaeniorhynchus* were collected from the insect rearing cage and identified in Zonal Entomological Research Centre, Vellore. From the stock solution, 1000 mg/l extract was prepared with dechlorinated tap water. The larvicidal activity was assessed by the procedure of WHO[18] with some modification and as per the method of Rahuman *et al*[19]. For the bioassay test, larvae were taken in five batches of 20 in 249 ml of water and 1.0 ml of the desired plant extract concentration. The control was set up with acetone and polysorbate 80. The numbers of dead larvae were counted after 24 h of exposure and the percentage of mortality was reported from the average of five replicates. The experimental media in which 100 per cent mortality of larvae occurs alone were selected for dose response bioassay. Hundred per cent mortality was observed in bark methanol extract of *A. squamosa* at concentration of (500, 250, 125, 62.5, 31.25, 15.63 and 7 mg/l), leaf methanol (300, 150, 75, 37.5, 18.75, 9.38 and 4.69 mg/l) and ethyl acetate (500, 250, 125, 62.5, 31.25, 15.63 and 7 mg/l) extract of *C. indicum*, leaf acetone (800, 400, 200, 100, 50, 25 and 12.5 mg/l) and ethyl acetate (600, 300, 150, 75, 37.5, 18.8 and 9.4 mg/l) extract of *T. procumbens* tested against *An. subpictus and Cx. tritaeniorhynchus* (Table I).

Table I. Larvicidal activity of different concentrations against *An. subpictus* and *Cx. tritaeniorhynchus* (mg/l)

Plant species / Parts used	Concentration (mg/l) and % mortality	
	An. subpictus	*Cx. tritaeniorhynchus*
A. squamosa/	Methanol	Methanol
Bark	500 - 100 ± 0.00	500 - 100 ± 0.00
	250 - 82.6 ± 2.46	250 - 88.4 ± 1.64
	125 - 63.0 ± 1.84	125 - 52.6 ± 4.63
	62.5 - 48.2 ± 4.62	62.5 - 34.2 ± 2.84
	31.25 - 16.4 ± 2.04	31.25- 20.6 ± 1.67
	15.63 – 92.0± 3.28	15.63- 12.4 ± 2.45
	7.82 - 4.6 ± 4.60	7.82 - 6.8 ± 1.87
C. indicum/	Methanol	Ethyl acetate
Leaf	300 - 100 ± 0.00	500 - 100 ± 0.00
	150 - 86.4 ± 4.02	250 - 87.0 ± 2.84
	75 - 68.2 ± 2.34	125 - 64.8 ± 1.64
	37.5 - 52.6 ± 2.60	62.5 - 48.2 ± 2.41
	18.75- 36.4 ± 1.86	31.3 - 34.0 ± 2.83
	9.38 - 18.2 ± 4.20	15.65- 14.8 ± 4.85
	4.69 - 8.6 ± 2.04	7.82 - 8.4 ± 1.24
T. procumbens/	Acetone	Ethyl acetate
Leaf	800 - 100 ± 0.00	600 - 100 ± 0.00
	400 - 84.2 ± 1.46	300 - 80.2 ± 1.80
	200 - 70.6 ± 4.08	150 - 56.4 ± 2.19
	100 - 56.4 ± 2.42	75 - 40.2 ± 4.32
	50 - 42.8 ± 4.62	37.5 - 26.8 ± 2.63
	25 - 28.6 ± 1.64	18.8 - 12.4 ± 4.86
	12.5 - 12.6 ± 2.20	9.4 - 6.4 ± 2.82

Values are mean ± SD of five replicates

Dose-response bioassay: From the stock solution, different concentrations ranging from 4.69-1000 mg/l were prepared. Based on the preliminary screening results 100 per cent mortality of larvae extracts alone were tested at different concentrations. Crude different solvents of leaf, and bark extracts prepared from *A. squamosa*, *C. indicum* and *T. procumbens* was subjected to dose response bioassay against *An. subpictus*, and *Cx. tritaeniorhynchus* respectively. The numbers of dead parasite and mosquito larvae were counted after 24 h of exposure, and the percentage mortality was reported from the average of five replicates. However,

Table II. Preliminary screenings of different solvent extracts against fourth instar larvae of *An. subpictus* and *Cx. tritaeniorhynchus* at1000 mg/l

Botanical name/ Family (Herbarium numbers) Vernacular names	Parts used	Species	% mortality *± SD				
			Ace	Chl	Eac	Hex	Met
Annona squamosa L. /Annonaceae	Bark	*An. subpictus*	75 ± 2.058	73 ± 2.408	75 ± 2.345	78 ± 1.941	100 ± 0.000
(ZD/AS/045-08) Pangiee/Sita		*Cx. tritaeniorhynchus*	78 ± 2.570	77 ± 2.701	71 ± 2.529	82 ± 2.350	100 ± 0.000
Chrysanthemum indicum Linné / Asteraceae	Leaf	*An. subpictus*	70 ± 2.121	74 ± 3.701	73 ± 2.581	61 ± 1.788	100 ± 0.000
Saamandi		*Cx. tritaeniorhynchus*	78 ± 2.325	84 ± 2.359	100 ± 0.000	68 ± 1.140	84 ± 1.483
Tridax procumbens L. / Asteraceae	Leaf	*An. subpictus*	100 ± 0.000	79 ± 1.699	83 ± 1.682	84 ± 1.698	80 ± 1.695
Thata poodu		*Cx. tritaeniorhynchus*	75 ± 1.765	86 ± 1.709	100 ± 0.000	71 ± 1.924	82 ± 1.140

Control - Nil mortality; *Mean value of five replicates; Ace, acetone; Chl, chloroform; Eac, ethyl acetate; Hex, hexane; Met, methanol

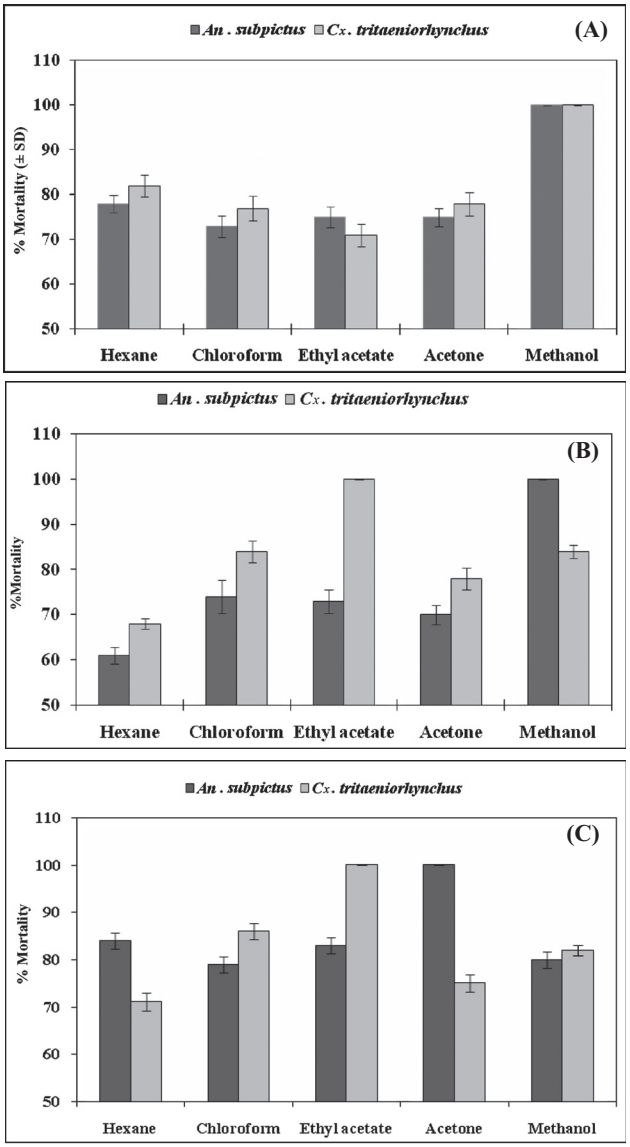

Fig. Graph showing the larvicidal activity of **(A)** *Annona squamosa* bark **(B)** *Chrysanthemum indicum* leaf and **(C)** *Tridax procumbens* leaf crude extracts against the fourth instar larvae of *An. subpictus* and *Cx. tritaeniorhynchus* at 1000 mg/l. Values are mean ± SD of 5 replicates.

at the end of 24 h, the selected test samples turned out to be equal in their toxic potential.

Statistical analysis: The average larval mortality data were subjected to probit analysis for calculating $LC_{50,}$ LC_{90}, and other statistics at 95 per cent fiducial limits of upper confidence limit and lower confidence limit, and Chi-square values were calculated by using the software developed by Reddy *et al*[20]. $P<0.05$ was considered to be statistically significant.

Results & Discussion

The activity of crude plant extracts is often attributed to the complex mixture of active compounds. In the preliminary screening, potential larvicidal activity of plants, different solvents crude extracts of three plants was noted (Table II, Fig. A, B and C). Five different solvents were tested against *An. subpictus* and *Cx. tritaniorhynchus* and 100 per cent larval mortality was observed in the bark methanol extract of *A. squamosa*, leaf ethyl acetate and methanol extract of *C. indicum* and leaf acetone and ethyl acetate extract of *T. procumbens* (Table II).

All plant extracts showed moderate toxic effect on *An. subpictus* and *Cx. tritaeniorhynchus* after 24 h of exposure at 1000 mg/l; however, the highest mortality was found in bark methanol extract of *A. squamosa*, leaf ethyl acetate extract of *C. indicum* and leaf acetone extract of *T. procumbens* against the larvae of *An. subpictus* (LC_{50}=93.80, 39.98, and 51.57 mg/l; LC_{90}=524.90, 145.70, and 226.56 mg/l); and bark methanol extract of *A. squamosa,* leaf methanol extract of *C. indicum* and leaf ethyl acetate of *T. procumbens* against the larvae of *Cx. tritaeniorhynchus* (LC_{50}=104.94, 42.29 and 69.16 mg/l; LC_{90}=443.79, 172.34, and 287.21 mg/l) respectively. The data obtained were analyzed using Chi-squared test, comparing experimental and control groups, with a significance level established at $P<0.05$. (Table III).

Table III. LC_{50}, LC_{90}, and other statistical analysis of different solvent plant extracts against, fourth instar larvae of *An. subpictus* and *Cx. tritaeniorhynchus*

Plant species	Parts used	Solvents	Species	LC_{50}±SE (mg/l)	UCL -LCL	LC_{90} ±SE (mg/l)	(UCL -LCL)
A. squamosa	Bark	Methanol	*An. subpictus*	93.80 ± 7.13	107.77 - 79.83	524.90 ± 69.90	661.92 - 387.31
			Cx. tritaeniorhynchus	104.94 ± 7.08	118.82 - 91.06	443.79 ± 50.94	543.63 - 343.96
C. indicum	Leaf	Methanol	*An. subpictus*	39.98 ± 2.57	44.89 - 35.07	145.70 ± 14.30	173.73 - 127.66
		Ethyl acetate	*Cx. tritaeniorhynchus*	42.29 ± 2.79	47.77 - 36.81	172.34 ± 18.21	208.03 - 136.65
T. procumbens	Leaf	Acetone	*An. subpictus*	51.57 ± 3.53	58.48 - 44.65	226.56 ± 26.17	227.86 - 175.25
		Ethyl acetate	*Cx. tritaeniorhynchus*	69.16 ± 4.63	78.22 - 60.10	287.21 ± 32.90	352.19 - 223.22

Control - Nil mortality; LC_{50} - Lethal concentration that kills 50 per cent of the exposed larvae; LC_{90}, Lethal concentration that kills 90 per cent of the exposed larvae; UCL, upper confidence limit; LCL, lower confidence Limit; $\chi 2$, Chi-square; df, degree of freedom; Chi-squared test, comparing experimental and control group, with a significance level established at $P<0.05$ and were chi-square value was significant

Crude extracts of leaves or bark of these plants have been tested earlier by several investigators[21-29] and larvicidal and antifeedent activities have been shown against *An. subpictus* and *Cx. tritaeniorhynchus,* and also *Leishmania maxicone*[30]. *C. fuscatum* and *C. myconis* flowers powders have been tested for the antifeeding and growth regulatory activity against *Spodoptera littoralis*[28]. The leaves essential oils of *T. procumbens* are more effective repellents activity at 6 per cent concentration against *An. stephens*[29] and leaves methanol extracts and purified compound (35)-16,17-didehydrofalcerinol (1) showed effective antileishmanial activity against promastigotes of *Leishmania mexicana*[30].

In conclusion, our findings showed that leaf and bark extract of *A. squamosa*, *C. indicum* and *T. procumbens* can be developed as ecofriendly larvicides. Also our results open the possibility for further investigations of the efficacy of larvicidal properties of natural product extracts.

Acknowledgment

The authors acknowledge C. Abdul Hakeem College Management, Dr S. Mohammed Yousuff, Principal, Dr K. Abdul Subhan, Associate Professor and Head, Zoology Department, and Dr Sait Sahul Hameed, Associate Professor in Zoology for their help and suggestions.

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