Original Article

Screening of Methanolic Plant Extracts against Larvae of *Aedes aegypti* and *Anopheles stephensi* in Mysore

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Abstract

Background: Mosquitoes transmit serious human diseases, causing millions of death every year. Vector control is facing a threat due to the emergence of resistance to synthetic insecticides. Insecticides of botanical origin may serve as suitable alternative biocontrol techniques in the future. Nine different locally available medicinally important plants suspected to posse larvicidal property were screened against fourth instar larvae of *Aedes aegypti* and *Anopheles stephensi* to a series of concentrations of the methanolic extracts.

Methods: Susceptibility tests on *Ae. aegypti* and *An. stephensi* were conducted using standard WHO methods. The larvae of two mosquito species were exposed to methanolic extracts and mortality counts were made after 24 hours of exposure as per WHO method. Larvae of *Ae. aegypti* were more susceptible than that of *An. stephensi*.

Results: Among the nine plant species tested, *Annona reticulata* leaf extract was more effective against *Ae. aegypti* larvae with LC_{50} and LC_{90} values of 95.24 and 262.64 ppm respectively and against *An. stephensi* larvae 262.71 and 636.94 ppm respectively. The least efficacy was in *Cosmos bipinnatus* with LC_{50} and LC_{90} values of 442.6 and 1225.93 ppm against *Ae. aegypti* and LC_{50} and LC_{90} values of 840.69 and 1334.01 ppm of *Thespesia populnea* against *An. stephensi*.

Conclusion: The crude methanolic extract of the *An. reticulata* with good larvicidal efficacy could be considered for further characterization to control mosquito vectors instead of chemical insecticides. High efficacy found in *An. reticulata* extract will be considered for further studies to isolate the bioactive compound.

Keywords: *Ae. aegypti*, *An. stephensi*, *Annona reticulata*, Larvicide, *Thespesia populnea*

Introduction

Many new and re-emerging diseases are transmitted by Arthropod vectors (Brogdon and Mc Alister 1998). "Vector borne diseases account for around 17% of the estimated global burden of all infectious diseases" (WHO 2006). Over 350 species of mosquitoes are vectors of pathogens that cause diseases in humans and domesticated animals. More than fourteen mosquito genera are known to harbour arboviruses (Mattingly 1973). "These diseases contribute significantly to disease burden, death, poverty and social debility in tropical countries" (Yang et al. 2004). The proliferation of the diseases is not only due to the higher number of breeding places in urban agglomeration, but also due to increasing resistance of mosquitoes to current commercial insecticides such as organo-chlorides, organophosphates, carbamates, pyrethroids and also to biological insecticides (Goettel et al. 1992, Das and Amalraj 1997, Yadav et al. 1997).

Thus, mosquitoes are responsible for the transmission of more diseases than any other group of arthropods and play an important role as etiologic agents of devastating malaria, filariasis, dengue, yellow fever, Japanese encephalitis, chikungunya and other viral diseases. In addition, they also cause irritation

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to human by causing allergic responses that include local skin reactions as well as systemic reactions such as angioedema and urticaria (Peng et al. 1999). *Aedes aegypti*, a vector of yellow fever, dengue and chikungunya is widely distributed in the tropical and subtropical zones. About two-third of the world's population, live in areas infested with dengue vectors, mainly *Ae. aegypti* (Hahn et al. 2001). *Anopheles stephensi* is the primary vector of malaria in India and other west Asian countries. Every year, an estimated 300–500 million new infections and 600,000 cases based on world malaria report 2013 (WHO 2013) deaths result from malaria worldwide.

In the past decade, chikungunya - a virus transmitted by *Ae.* spp. mosquitoes - has re-emerged in Africa, southern and southeastern Asia, and the Indian Ocean Islands as the cause of large outbreaks of human disease **(**Burt et al. 2012**)**. Malaria is a protozoan infection of erythrocytes caused in human beings by five species of the genus *Plasmodium* (*P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*). In most cases, malaria is transmitted via the bite of an infected female anopheline mosquito, but congenital malaria and acquisition through infected blood transfusion are well described (Falade et al. 2007). "More than 40 per cent of the world's population-approximately 3 billion people are exposed to malaria in 108 endemic countries "(WHO 2009). About one million cases of malaria are reported in India every year.

Many approaches have been developed to control mosquito menace. One such approach to prevent mosquito borne disease is by employing larvicide. The current mosquito control approach is based on synthetic insecticides. Even though their effectiveness, they created many problems like insecticide resistance (Liu et al. 2005), pollution and toxic side effects on human beings (Lixin et al. 2006). This has necessitated the need for research and development of environmentally safe, biodegradable, indigenous method for vector control. Botanicals offer great promise as source of phyto-chemicals with proven potential as insecticides which can play an important role in the control of mosquitoes and in the interruption of disease transmission at individual as well at community level (Mittal and Subbarao 2003). Six plant families with several representative species, Asteracae, Cladophoraceae, Labiatae, Meliaceae, Oocystacae and Rutaceae appear to have the greatest potential for providing future mosquito control agents. Insecticides of plant origin do not cause toxicity to human and domestic animals and are easily biodegradable. In the present study leaf extract of the plant Passifloraceae, Annonaceae, Asteraceae, Lauraceae, Malvaceae, Lamiaceae was studied for its insecticidal activity against malaria and dengue vectors. The application of botanical derivatives against mosquito has been reviewed in detailed by Sukumar et al. (1991).

In this regard, of late the researchers have shown more interest on plant derivatives as botanicals offer great promise as sources of molecules for the control of both agricultural pests and medically important insect species. Now a days, the increased use of phytochemicals for the control of these insects may be attributed to the fact that population throughout the world are aware of the danger inherent in conventional insecticides, particularly the detrimental effect on the environment (Pitasawat et al. 1998). It is in this regard, plant derived products have received increased attention from scientists and more than 2000 plant species are already known to have insecticidal properties (Balandrin et al. 1985, Sukumar et al. 1991). Natural insecticides such as pyrethrum, rotenone and nicotine among others have been extensively used until recently for insect control (Balandrin et al. 1985).

To search derivatives with insecticidal property from local plants, the present in-

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vestigation has been made in the Vector Biology Research Lab, Department of Studies in Zoology at University of Mysore.

Materials and Methods

Aedes aegypti and *An. stephensi* larvae available at the mosquito colony maintained in Vector Biology Research Lab, Department of Studies in Zoology, University of Mysore by following standard rearing techniques. The larvae were reared in large enamel or plastic trays (30x24x5 cm) containing dechlorinated water and fed using finely powdered dog biscuits and dry yeast in the ratio of 2: 1.

Plant material and extraction

Nine plant species as listed in Table 1 were collected from in and around Mysore, Karnataka, from March 2012 to Jan 2013 and the leaves were shade dried, powdered manually and subjected to methanol solvent extraction in a Soxhlet apparatus until exhaustion, to obtain non-polar bioactive constituents. The pooled extract was evaporated in a rotory vacuum evaporator at 40 ºC to dryness and stored at 4 ºC in an air tight bottle for further analysis. This was later dissolved in acetone and employed to prepare different concentrations.

Larval bioassay

Bioassays on mosquito larvae were performed on late third or early fourth instars, according to standard guidelines of WHO (2005). The required quantity of plant extract of different concentrations was prepared in acetone as solvent. One ml of each of the concentration was mixed thoroughly with 249 ml of dechlorinated water in 500 ml glass beakers. Larvae were exposed in an ascending series of five concentrations according to log dose (Table 2). Parallel control tests were also maintained by adding 1ml of the solvent to 249 ml of dechlorinated water. Twenty five early fourth instar larvae were transferred to each of the beakers. A minimum of three replicates were kept for each concentration along with the control. Observation for the dead or moribund larvae was carried out after 24 h duration at 25±2 ºC and 75±5 % of relative humidity (RH).

Data analysis

Larval mortality counts were adjusted for the mortality in control, if any, by employing Abbott's formula (Abbott 1975) to give an estimate of the plant extract attributable mortality. The corrected mortality data were subjected to regression analysis of probit mortality on log dosage (Finney 1971). The significant difference in LC_{50} is based on the non-overlapping of 95 % Fiducial limits.

Results

Table 2 provides the mortality rate of mosquito larvae against different concentrations of methanol extracts. Table 3 and 4 provide the efficacy of methanol extracts tested against the dengue vector *Ae. aegypti* and malarial vector *An. stephensi* larvae. Fig. 1 and 2 show the log dose- probit mortality responses of all plant extracts. Though the plants exhibited larvicidal activity against the two mosquito species, out of the nine plants screened using methanol as solvent, the *Annona reticulata* (Annonaceae) was found to possess better larvicidal activity against *Ae. aegypti* followed by *An. stephensi*. The high percentage of mortality with low concentration was recorded of in the species against *Ae. aegypti* has been with LC_{50} and LC_{90} values being 95.24 and 262.64 ppm respectively (Table 3). Likewise LC_{50} and LC_{90} values against *An. stephensi* are 262.71 and 636.94 ppm respectively (Table 4). The 95% Fiducial Limits (FL) of *An. reticulata* for LC_{50} is 46.83–139.69 and 95% FL for LC_{90} is 172.23–931.30 and the slope is 2.90±0.55

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against *Ae. aegypti*. Similarly, the 95% FL for LC_{50} is 197.21–137.54 and for LC_{90} it is 457.41-1434.98 with the slope being 3.33±0.49 against *An. stephensi*. Among these two mosquito species, susceptibility of *Ae. aegypti* was found to be significantly more than that of *An. stephensi*. Further, the larvicidal efficacy of *An. reticulata* was found to be significant by more compared to other plants (P< 0.05). The figure depicts the log dose-probit mortality responses and slope regression lines of tested plants.

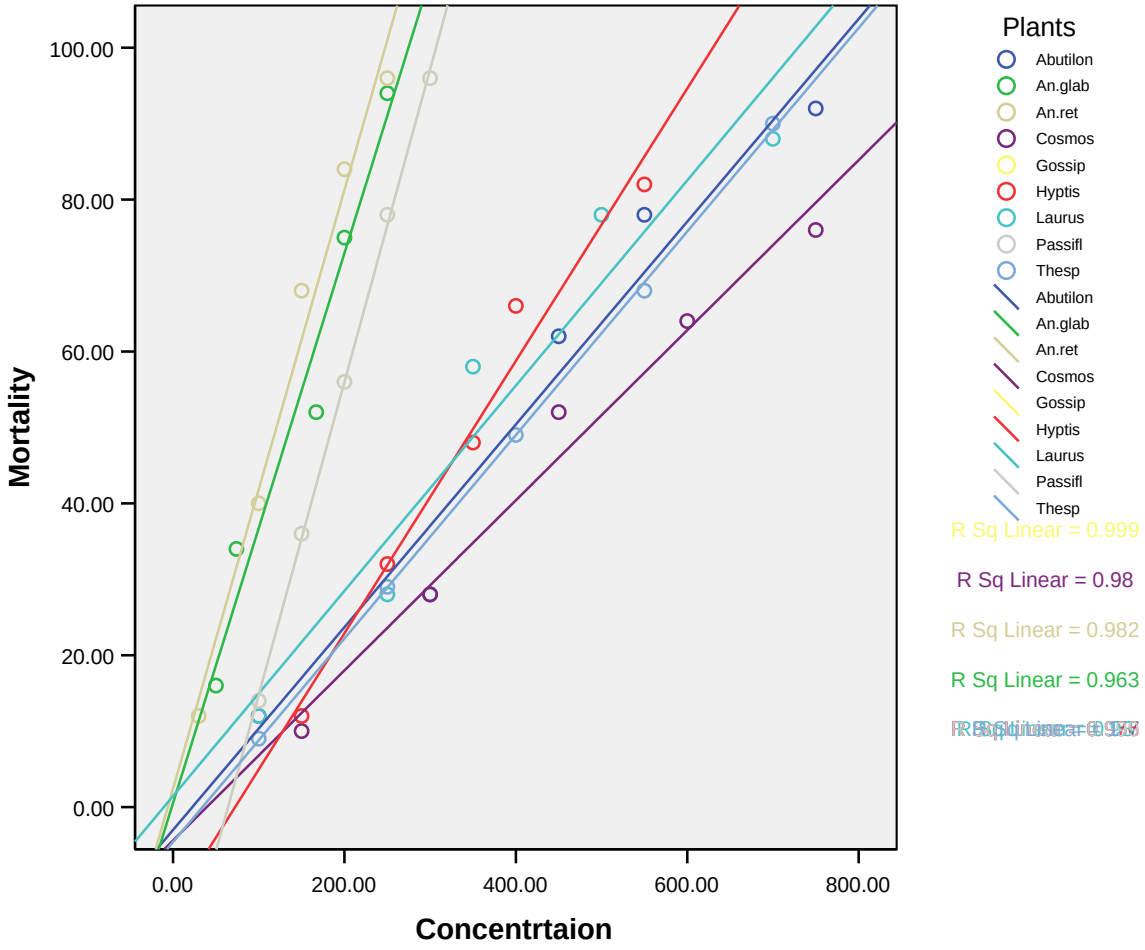

Fig. 1. Regression graph showing mortality of methanolic extracts of different plant species on *Aedes aegypti*

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Table 1. List of plant screened against *Aedes aegypti* and *Anopheles stephensi*

No	Plants name	Family	Medicinal/ toxic property
1	***Passiflora foetida***	Passifloraceae	Pitta, inflammation,insomnia, depression and anxiety disorders
2	***Annona glabra***	Annonaceae	Anticancer effects, substantial antimicrobial, antifungal and moderate insecticidal, sporicidal and cytotoxic
3	***Cosmos bipinnatus***	Asteraceae	Jaundice, intermittent fever and splenomegaly
4	***Laurus nobilis***	Lauraceae	rheumatism and dermatitis
5	***Abutilon indicum***	Malvaceae	Febrifuge, anthelmintic, antiemetic, anti-inflammatory, and in urinary and uterine discharges, piles, and lumbago
6	***Gossypium herbaceum***	Annonaceae	Febrifuge, anthelmintic, antiemetic, anti-inflammatory.
7	***Annona reticulata***	Annonaceae	Febrifuge, anthelmintic, antiemetic, anti-inflammatory, and in urinary and uterine discharges, piles, and lumbago
8	***Hyptis suoveolens***	Lamiaceae	Gastrointestinal infections, cramps, and pain, skin infections
9	***Thespesia populnea***	Malvaceae	Antifertility, antibacterial, antiinflammatory, antioxidant, purgative and hepatoprotective activity

Table 2. Concentration and Mortality of Nine plant species against *Aedes aegypti* and *Anopheles stephensi*

No	Plant Name	*Aedes aegypti*		*Anopheles stephensi*	
		Concentration(ppm)	Mortality (%)	Concentration(ppm)	Mortality (%)
1	***Passiflora foetida***	100	14	200	12
		150	36	350	30
		200	56	500	54
		250	78	650	74
		300	96	800	90
2	***Annona glabra***	100	16	50	10
		150	34	200	28
		250	60	400	48
		400	74	550	70
		600	90	700	92
3	***Cosmos bipinnatus***	150	10	50	12
		300	28	200	30
		450	52	350	52
		600	64	500	78
		750	76	750	90
4	***Laurus nobilis***	100	12	100	14
		250	28	250	36
		350	58	400	52
		500	78	750	74
		700	88	900	92

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Table 2. Continued…

5	***Abutilon indicum***	100	12	150	16
		300	28	300	36
		450	62	450	50
		550	78	700	76
		750	92	750	90
6	***Gossypium herbaceum***	150	10	100	08
		200	34	250	30
		250	62	400	48
		300	76	550	74
		350	90	800	88
7	***Annona reticulata***	30	12	100	12
		100	40	200	30
		150	68	300	52
		200	84	400	70
		250	96	500	90
8	***Hyptis suoveolens***	150	12	150	08
		250	32	300	32
		350	48	450	50
		400	66	600	76
		550	82	750	94
9	***Thespesia populnea***	100	10	500	10
		250	28	700	30
		400	48	900	52
		550	68	1100	78
		700	90	1300	92

Table 3. Efficacy of methanolic extracts of nine plant species against *Aedes aegypti*

No	Plant name	LC_{50} (ppm)	95% FL	LC_{90} (ppm)	95% FL	Slope±SE	Heterogeneity (df)	Regression equation
1	***Annona reticulata****	95.24	46.83-139.69	262.64	172.23-931.30	2.90±0.55	5.20(3)	Y=2.90X±0.75
2	***Annona glabra***	114.47	54.99-191.18	320.82	191.84-3738.15	2.86±0.64	6.98(3)	Y=2.86X±0.89
3	***Passiflora foetida***	172.31	143.20-200.36	300.99	96.24-242.06	5.29±0.70	2.73(3)	Y=5.29X±6.83
4	***Gossypium herbaceum***	216.23	194.57-239.21	620	524.18-778.30	2.79±0.24	1(3)	Y=2.79X±1.53
5	***Laurus nobilis***	302.66	207.85-409.02	813.94	555.81-2193.14	2.98±0.47	3.35(3)	Y=2.98X±2.39
6	***Hyptis suaveolens***	327.18	303.55-352.76	720.37	624.61-880.33	3.73±0.35	1(3)	Y=3.73X±4.40
7	***Abutilon indicum***	332.98	123.63-546.59	903.76	549.46-692.12	2.95±0.71	7.40(3)	Y=2.95X±2.45
8	***Thespesia populnea***	353.07	236.81-498.69	969.48	635.18-3376.91	2.92±0.50	3.79(3)	Y=2.92X±2.44
9	***Cosmos bipinnatus***	442.60	402.11-488.40	1225.93	1013.27-2710	2.89±0.28	1(3)	Y=2.89X±2.66

Note: LC_{50} - median lethal concentration, FL - fiducial limits; LC_{90} - lethal concentration, df - degree of freedom.
*Difference in LC_{50} from the extracts of other eight plants is significant based on non-overlapping 95% fiducial limits ($P< 0.05$).

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Table 4. Efficacy of methanolic extracts of nine plant species against *Anopheles stephensi*

No	plant name	LC_{50} (ppm)	95% FL	LC_{90} (ppm)	95% FL	Slope±SE	Heterogeneity (df)	Regression equation
1	***Annona reticulata****	262.71	197.21-337.54	636.94	457.41-1434.98	3.33±0.49	2.89(3)	Y=3.33X±3.06
2	***Cosmos bipinnatus***	258.39	105.36-471.41	1048.00	544.56-1326.18	2.10±0.45	5.74(3)	Y=2.10X±0.68
3	***Gossypium herbaceum***	298.80	62.13-768.79	1221.72	564.60-1130.12	2.09±0.55	8.40(3)	Y=2.09X±0.18
4	***Laurus nobilis***	336.57	222.28-475.98	1197.75	757.60-3516.32	2.32±0.33	2.82(3)	Y=2.32X±0.87
5	***Abutilon indicum***	372.81	231.41-532.21	1000.95	655.62-4054.35	2.98±0.56	4.51(3)	Y=2.98X±2.68
6	***Hyptis suaveolens***	391.66	361.46-422.54	843.60	748.86-986.10	3.84±0.31	1(3)	Y=3.84X±4.97
7	***Passiflora foetida***	441.01	409.18-473.62	922.27	820.34-1077.59	3.99±0.33	1(3)	Y=3.99X±5.57
8	***Annona glabra***	448.78	338.06-570.24	900.18	675.62-1951.54	4.23±0.69	3.94(3)	Y=4.23X±6.24
9	***Thespesia populnea***	840.69	801.16-880.57	1344.01	1249.14-1478.27	6.28±0.50	1(3)	Y=6.28X±13.39

Note: LC_{50} median lethal concentration, FL, fiducial limits, LC_{90} lethal concentration, df, degree of freedom.
*Difference in LC_{50} from the extracts of other eight plants is significant based on non-overlapping 95 % fiducial limits (P< 0.05).

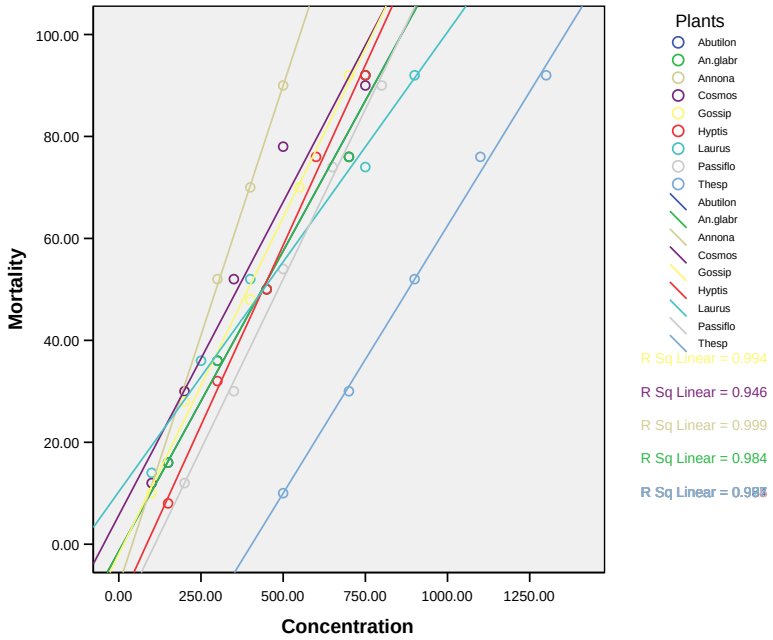

Fig. 2. Regression graph showing mortality of methanolic extracts of different plant species on *Anopheles Stephensi*

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Discussion

The results of the larvicidal bioassay employing different plant extracts against two different mosquito species (Table 3, 4) indicate significant larvicidal activity ($P< 0.05$) with methanol extract. The biological activity of these plant extract may be due to various compounds such as, phenolics, terpenoids, flavonoids and alkaloids (Gohil et al. 2010). Such compounds may jointly or independently contribute to produce toxic activity against the mosquito species.

Environmental safety of an insecticide is of paramount importance while employing against pests and vectors. An insecticide need not cause high mortality on target organisms in order to be acceptable (Kabaru and Gichia 2001). Resistance to insecticides dates back to the beginning of application of chemicals, since DDT was initially introduced for mosquito control in 1946 and just in one year, the first case of DDT resistance occurred in *Ae. tritaeniorhynchus* and *Ae. sollicitans* (Hemingway and Ranson 2000). More than 500 species of arthropods are reported to be resistant to various insecticides (Shelton et al. 2007). In this regard, phytochemicals may serve as suitable alternatives to synthetic insecticides in future, as they are relatively safe, inexpensive and are readily available throughout the world. According to Bowers et al. (1995), the screening of locally available medicinal plants for mosquito control will be cost effective, reduce dependence on expensive imported products and stimulate local efforts to enhance public health. It is in this regard, the present study adds to our knowledge on the efficacy of the locally available medicinal plants.

An earlier report points that, ethanolic extract of *Annona squamosa* leaf has the most promising larvicidal activity against *Cx. quinquefasciatus* larvae (Das et al. 2007). The larvicidal and growth regulating activities of *Annona squamosa* and *Syzygium cumini* two related species have been reported against *An. stephensi* and other mosquitoes (Saxena et al. 1993 and Kaushik and Saini 2008). The significant activity demonstrated by extracts of *A. squamosa* and *A. senegalensis* suggest that the two plants may have strong killing effects against insects particularly mosquitoes, hence giving a promising source of larvicidal agents (Magadula et al. 2009). Previously, a collection of *A. squamosa* plant materials from Brazil indicated larvicidal effect against *Ae. albopictus* and *Culex quinquefasciatus* (Das et al. 2007) and against *An. stephensi* (Saxena et al. 1993). However, no reports on the larvicidal efficacy are available on *An. reticulata*. By comparing our results with the earlier studies, it is evident that the methanolic extracts of *An. reticulata* leaf have promising larvicidal activity against two mosquito species. It shows the LC_{50} value of 95.24 against *Ae. aegypti* and 262.71 against *An. stephensi* (Tables 3, 4).

Karunamoorthi and Ilango (2010) have reported that theLC_{50} and LC_{90} values of methanol leaf extracts of *Croton macrostachyus* (*C. macrostachyus*) were 89.25 and 224.98 ppm, respectively against late third instar larvae of malaria vector, *An. arabiensis* (*An. arabiensis*). The crude leaf extract of *Azadirachta indica* with different solvents, viz. benzene, chloroform, ethyl acetate and methanol were tested for larvicidal activity against *An. stephensi*. The LC_{50} values were 19.25, 27.26, 23.26 and 15.03, respectively. Kamaraj et al. (2008a) have reported that the peel methanol extract *C. sinensis*, leaf and flower ethyl acetate extracts of *Ocimum canum* against larvae of *An. stephensi* (LC_{50} =95.74, 101.53, 28.96, LC_{90}=303.20, 492.43 and 168.05 ppm) respectively. The highest larval mortality was found in methanol extract of *O. canum* against the larvae of *Ae. aegypti* (LC_{50}=99.42, 94.43 and 81.56 ppm)

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and against *Cx. quinquefasciatus* (LC_{50}= 44.54, 73.40 and 38.30 ppm), respectively (Kamaraj et al. 2008b). *An. stephensi* and *Ae. aegypti*. Chowdhury et al. (2009) have reported that the chloroform and methanol extracts of mature leaves of *Solanum villosum* showed the LC_{50} value for all instars between 24.20 and 33.73 mg/l after 24 h and between 23.47 and 30.63 mg/l after 48 h of exposure period against *An. subpictus*. Govindarajan (2010) evaluated larvicidal activity of crude extract of *Sida acuta* against three important mosquitoes with LC_{50} values ranging between 38 and 48 mg/l. The crude extract had strong repellent action against the three species of mosquitoes as it provided 100 per cent protection against *An. stephensi* for 180 min followed by *Ae. aegypti* (150 min) and *Cx. quinquefasciatus* (120 min).

Dua et al. (2009) showed that the LC_{50} values of the neem oil were 1.6 and 1.7 ppm while LC_{90} values were 3.4 and 3.7 ppm against *An. stephensi* and *Ae. aegypti* respectively. Shivakumar and Kataria (2011) have reported that *An.* showed a high susceptibility to very low concentrations of *Azadirachta indica* (1–5 ppm), whereas in the case of *Ae. aegypti*, the diagnostic concentration is slightly higher at 10ppm. The larvicidal action on *An. stephensi*, at a concentration of 3ppm resulted in 100% mortality within 72 h and a concentration of 15 ppm produces 90% mortality within 24 h of the treatment. In the present investigation out of the nine plants screened using methanol as solvent, *Annona reticulata* (Annonaceae) was found to possess better larvicidal activity against early fourth instar larvae of *Ae. aegypti* followed by *An. stephensi* with LC_{50} and LC_{90} values being 95.24 and 262.64 ppm respectively (Table 3). Likewise LC_{50} and LC_{90} values against *An. stephensi* are 262.71 and 636.94 ppm respectively (Table 4).

Annona muricata was an active larvicide with a 48-hour LC_{50} value of 67.4 µg/mL. (Jacobson 1958). He has further reported similar results for the seed extracts of another *Annona* species, namely *A. cherimola*, *A. glabra* and *A. squamosa*, which had lethal effects on larvae of *Ae.* sp. Its potential as a larvicidal plant was further supported by the results of a recent study by Satoto (1993), who has found that *A. squamosa* seed was one of the most effective larvicides against both *Culex tritaeniorhynchus* and *Ae. aegypti*.

Earlier studies further indicated that crude extracts of leaves of *Annona reticulata* when evaluated for in vitro anthelmintic activity on Indian adult earth worms *Eisinia fetida*, a dose dependent inhibition of spontaneous motility (paralysis) of the worms was noticed (Sonal et al. 2011). There are no reports available on the toxicity of *Annona reticulata* against mosquito larvae. The present studies add that, apart from all medicinal property viz, antimicrobial, antitumoric, anthelmintic, antibacterial, cytotoxic etc, mosquito larvicidal property also present in this plant. Therefore, the methanolic extracts of this plant inhibiting the development of larval growth, indicates hopes for further characterization of the active compound in our lab.

Conclusion

The results from this study indicate that phyto-products isolated from the plants are more advisable to use for control of mosquito borne diseases. These plants are remarkably economical and ecofriendly with more larvicidal properties. Among the plants screened *An. reticulata* showed high larvicidal efficacy against two mosquito species. Hence, *An. reticulata* will be selected for further chemical isolation of the active ingredient in future studies. It could be considered as a potent resource for controlling mosquito larvae. Such practice would not

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only reduce the disadvantages of insecticides on the environment but also promote sustainable utilization of locally available bioresource by rural communities.

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