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Mosquitocidal potential of *Rhodomyrtus tomentosa* leaf extracts against Dengue and Zika virus vector, *Aedes aegypti*

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Abstract
Dengue is a mosquito-borne flavivirus found in tropical and sub-tropical regions of the world, mostly in urban and semi-urban areas. *Aedes aegypti* is primary vector and its day-biting mosquitoes spread disease, 40% of the world's population living in an area at risk for dengue. The present study explored the effects of *Rhodomyrtus tomentosa* acetone and methanol leaf extract of *R. tomentosa* on larvicidal and pupicidal toxicity against dengue and zika virus vector, *Aedes aegypti*. The *R. tomentosa* dried plant materials (powder), 300 g of the plant materials was extracted with 1 L of organic solvents of acetone and methanol for using a Soxhlet apparatus boiling point range 60–80°C for 8 h at room temperature (27±2°C). The extracts were concentrated at reduced temperature on a rotary vacuum evaporator and stored at a temperature of 4 °C. The yields of the *R. tomentosa* acetone and methanol crude extracts were 12.86g and 14.11g, respectively. The larvicidal and pupicidal lethal toxicity was observed after 24 h of exposure; No mortality was observed in the control group. The LC_{50} and LC_{90} of *R. tomentosa* leaf acetone extract (RTAE) against the first to fourth instar larvae and pupae *A. aegypti* were; the LC_{50} value of first instar was 312.17, 352.65, 407.21, 471.60 and 523.91 ppm and 722.67, 739.19, 848.42, 944.81 and 1083.29 ppm, respectively. The LC_{50} and LC_{90} values of *R. tomentosa* leaf methanolic extract (RTME) against the first to fourth instar larvae and pupae of *A. aegypti* were; the LC_{50} value of LC_{90} values were; 263.82 ppm (I), 299.43 ppm (II), 331.64 ppm (III), 386.16 ppm (IV) and 419.56 ppm (pupae), respectively. The LC_{90} value of 1st instar was 609.83 ppm, 2nd instar was 677.22 ppm, 3rd instar was 746.13 ppm, 4th instar was 858.83 ppm, and pupa was 908.72 ppm (pupae), respectively. Overall, highlighted the larvicidal and pupicidal evaluation of acetone and methanolic leaf extract of *Rhodomyrtus tomentosa* against Dengue and Zika virus vector, *Aedes aegypti.* This is a new eco-friendly approach for the control of dengue and zika virus vector, *Ae. aegypti* mosquito as target species. The natural products of biopesticides are eco-friendly for the dengue vector control management programs.

Keywords: larvicidal, pupicidal, eco-friendly, biopesticides and vector borne diseases

Introduction

Dengue is a mosquito-borne flavivirus found in tropical and sub-tropical regions of the world, mostly in urban and semi-urban regions. Dengue virus is transmitted by female mosquitoes mainly of the species *Aedes aegypti* and transmits chikungunya, yellow fever and Zika infection [1]. It is the fastest spreading vector-borne viral disease and is now endemic in over 100 countries, resulting in 40% of the world's population living in an area at risk for dengue. Dengue fever/dengue hemorrhagic fever (DHF) continues to be of major public health importance in countries of the Western Pacific and Southeast Asia. It is caused by one of four distinct serotypes (DEN-1, DEN-2, DEN-3 and DEN-4) [1].

The chemical based insecticides are play a very important role in controlling mosquito vector in their breeding sites, but this kind of control methods showed a negative impact in areas of beneficial and nontarget organisms and still mosquito vectors resistance to synthetic insecticides. In view of the recently increased interest in developing plant origin insecticides as an alternative to synthetic insecticides [2, 3]. The environmental safety and low cost insecticide is considered to be of paramount importance and should not cause mortality on non-target organism in order to be acceptable [4].

Chemical control is an effective strategy used extensively in daily life and is presently at the forefront among mosquito-controlling agents. However, the environmental threat that these chemicals pose, effects on non-target organisms, and the resistance of mosquitoes to insecticides have all increased during the last five decades [2, 5]. Biopesticides provide an alternative to synthetic pesticides because of their generally low environmental pollution, low toxicity to humans, and other advantages [6]. Many herbal products have been used as natural insecticides before the discovery of synthetic organic insecticides [7].

Rhodomyrtus tomentosa (Aiton) Hassk. (Family Myrtaceae) is an ornamental, evergreen shrub grows up to four meters tall. This plant species is native to southern and south-eastern Asia [8]. It has been often used in traditional medicine to treat colic diarrhoea [9], dysentery, abscesses, haemorrhage, and gynecopathy[10]. In addition, it is used to formulate skin-whitening, antiaging, and skinbeautifying agents[11]. The extract from this plant possess strong inhibitory activity against gram-positive bacteria [12, 13], antimicrobial properties [14, 15, 16, 17]. The *R. tomentosa* plant part has been employed in traditional medicine to treat colic diarrhoea, haemorrhage, dysentery, abscesses, and gynaecopathy [18, 19, 20, 21]. No data regarding its mosquitocidal properties are available. Hence, the present investigation was aimed to explore the mosquitocidal potential of the extracts (Ethanol and Methanol) from *Rhodomyrtus tomentosa* leaves against dengue and zika virus vector, *Aedes aegypti* under the laboratory condition.

Material and Methods

Collection of eggs Maintenance of larvae *Aedes aegypti*

The eggs of *Aedes aegypti* were collected from Vector Control Research Centre (VCRC-ICMR, Pondicherry), India without expose to any insecticide and in and around Coimbatore district, India at different breeding habitats with the help of 'O' type brush. These eggs were brought to the laboratory and were transferred to 18 X 13 X 4 cm size enamel trays containing 500 ml of water and kept for larval hatching. The mosquito larval culture was maintained in our laboratory at 27±2°C, 75-85% RH, under14L: 10D photoperiod cycles. The mosquito larvae were fed with dog biscuits and yeast at 3:1 ratio. The feeding was continued till the larvae were transformed into pupae.

Maintenance of pupae and adult *Aedes aegypti*

The pupae were collected from the culture trays and were transferred to plastic containers (12 X 12 cm) containing 500 ml of water. The plastic jars were kept in 90 X 90 X 90 cm sized mosquito cage for adult emergence. The freshly emerged adults were maintained 27±2°C, 75-85% RH, under 14L: 10D photoperiod cycles. The adults were fed with 10% sugar solution for a period of three days before they were provided an animal for blood feeding.

Blood feeding of adult Mosquitoes

The female mosquitoes were allowed to feed on the blood of a rabbit (exposed on the dorsal side) for two days. The males were provided with 10% glucose solution on cotton wicks. The cotton was always kept moist with the solution and changed every day. An egg trap (cup) lined with filter paper containing water was placed at a corner of the cage egg collection.

Collection of plant materials

The tribal medicinal plant, *Rhodomyrtus tomentosa* were collected from Nilgiri hills, Western Ghats, Tamil Nadu, India. The plants were identified (Taxonomy) at BSI (Botanical Survey of India, Coimbatore, Tamil Nadu, India.) and the specimens were deposited at Zoology Department, Annamalai University, Annamalai Nagar, Chidambaram, Tamil Nadu, India. This plant is a small tree growing up to 5 to 10 feet. It is an edible plant of higher altitude. It's used by the native tribal for stomach pain.

Taxonomic position of *Rhodomyrtus tomentosa*

Kingdom - *Plantae* - Plants
Subkingdom - *Tracheobionta* - Vascular plants
Superdivision - *Spermatophyta* - Seed plants
Division - *Magnoliophyta* - Flowering plants
Class - *Magnoliophyta* - Dicotyledons
Subclass - Rosidae
Order - myrtales
Family - Myrtaceae-myrtce family
Genus - *Rhodomyrtus* (DC) RCHB-*rhodomyrtus*
Species - *tomentosa* (Aiton) Hasaaic rose myrtus

Preparation of plant extracts

The *Rhodomyrtus tomentosa* plant was collected in and around Mettuppalayam, Coimbatore, India. *R. tomentosa* plant was washed with tap water and shade dried at room temperature (27±2°C). An electrical blender powdered the dried plant materials (leaves). From the powder, 300 g of the plant materials was extracted with 1 L of organic solvents of Acetone and Methanol for using a Soxhlet apparatus[22] boiling point range 60–80°C for 8 h. The extracts were filtered through a Buchner funnel with Whatman number 1 filter paper. The crude plant extracts were evaporated to dryness in rotary vacuum evaporator. One gram of the plant residue was dissolved in 100 ml of acetone (stock solution) and considered as 1% stock solution. From this stock solution, different concentrations were prepared ranging from 120 to 600 ppm, respectively.

Larvicidal and pupicidal bioassay (WHO 2005)

The larvicidal bioassay was assessed by using slandered WHO Protocols[23] for experimental treatment, one ml of desired concentration obtained from stock solution of different products of *Rhodomyrtus tomentosa* extracts. Laboratory colonies of mosquito larvae/pupae were used for the larvicidal/pupicidal activity. Twenty-five numbers of I to IV instar larvae and puape were introduce into 500 ml glass beaker containing 249 ml of de-chlorinated water and 1ml of desired concentrations of biopesticide. Larval food was given for the test larvae. At each tested concentration 2 to 5 trials were made and each trial consisted of three replicates. Experiments were conducted at 27±1°C, 85% RH with photoperiod of 12L:12D. Symptoms of the treated larvae were observed and recorded immediately and at timed intervals and no foods offer to the larvae, mortality and survival was registered after 24 hours of the exposure period. dead larvae were identified when they failed to move after probing with a needle in the siphon or cervical region, moribund larvae were those incapable of rishing to the surface (within a reasonable period of time) or showing the characteristic diving reaction when the water was disturbed. The larvae showed discoloration, unnatural positions, tremors, unco-ordianation or rigor were also counted.

In case of experiment for determining pupicidal activity, the mouth of each bowl containing pupae was covered with muslin cloth to prevent the escape of any emerged adult mosquitoes. Mortality in larvae and pupae was recorded at 24 hrs. The larvae/pupae exposed to de-chlorinated water without biopesticide served as control. The control mortalities were corrected by using Abbott's formula [24].

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

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

Statistical analysis

All data were analyzed using the SPSS Statistical Software Package version 16.0. Means were separated using Duncan's multiple range test. Furthermore, mosquitoes mortality data from laboratory assays were analyzed by probit analysis, calculating LC_{50} and LC_{90} following the method by [25]. Mosquito larval density data from field assays were analyzed using a two-way ANOVA with two factors (i.e. the mosquitocidal treatment and the elapsed time from treatment).

Results and Discussion

Vector control programme is one of the most important and potent weapons in the procedure of managing vector populations to reduce/interrupt the transmission of vector borne disease [26]. Mosquitoes in the immature stage (larval and pupal) are attractive targets for pesticides because mosquitoes breed in water, which makes it easy to deal with them in this habitat. Biopesticides, especially those derived from natural resources (plants and seaweeds) are more promising in this aspect. Aromatic plants and their essential oils are very important sources of many compounds that are used in different respects [2]. In laboratory assays, the larvicidal and pupicidal toxicity of two solvent crude extracts of *Rhodomyrtus tomentosa* was evaluated for the potential larvicidal and pupicidal activity of plants used for this purpose. The crude acetone and methanol extracts of the leave of the plant *Rhodomyrtus tomentosa* were studied for use as eco-friendly insecticides instead. Table 1 show that the larvicidal and pupicidal toxicity of *R. tomentosa* leaf acetone extract against the Dengue and Zika virus vector, *Ae. aegypti* (first to fourth instar larvae and pupae) after the treatment at different concentrations viz., 120 ppm, 240 ppm, 360 ppm, 480 ppm and 600 ppm respectively. 27.4% mortality was noted at first instar larvae by the treatment of acetone extract of *R. tomentosa* (RTAE) at 120 ppm, whereas it has been increased to 81.2% at 600 ppm; similar trend has been noted for all the instars and pupae of *Ae. aegypti*. The LC_{50} and LC_{90} values were represented as follows; LC_{50} value of first instar was 312.17 ppm, second instar was 352.65 ppm, third instar was 407.21 ppm, fourth instar was 471.60 ppm, and pupa was 523.91 ppm, respectively. The LC_{90} value of first instar was 722.67 ppm, second instar was 739.19 ppm, third instar was 848.42 ppm, fourth instar was 944.81 ppm, and pupa was 1083.29 ppm, respectively. No mortality was observed in the control. Among different larval stages, the 1st instars (Younger) larvae were more susceptible than the other instars (Older) larvae. The regression equation and chi-square values were significant at $P<0.05$ level (Table 1).

Table 2 provides the results of larval and pupal mortality of *Ae. aegypti* (1st to 4th instar larvae and pupae) after the treatment of methanolic leaf extract of *R. tomentosa* (RTME) at different concentrations (120 to 600 ppm). 32.4% mortality was noted at first instar larvae by the treatment of *R. tomentosa* at 120 ppm, whereas it has been increased to 92% at 600 ppm; 20.2% pupal mortality was noted at 120 ppm and 65.4% pupal mortality at 600 ppm of *R. tomentosa* leaf methanolic extract treatment. Similar trend has been noted for all the instars of *Ae. aegypti* at different concentration of *R. tomentosa* treatment. The LC_{50} and LC_{90} values were represented as follows; LC_{50} value of 1st instar was 263.82 ppm, 2nd instar was 299.43 ppm, 3rd instar was 331.64 ppm, 4th instar was 386.16 ppm and pupa was 419.56 ppm, respectively. The LC_{90} value of 1st instar was 609.83 ppm, 2nd instar was 677.22 ppm, 3rd instar was 746.13 ppm, 4th instar was 858.83ppm, and pupa was 908.72 ppm, respectively. The regression and Chi-square value are also given in Table 2.

Table 1: Larvicidal and pupicidal toxicity of *R. tomentosa* leaf acetone extract against the Dengue and Zika virus vector *Ae. aegypti*.

Target	Mortality (%) ± SD					Regression equation	LC_{50} (LC_{90})	95% confidence Limit LC_{50} (LC_{90})		x^2 (d.f.=4)
	120 ppm	240 ppm	360 ppm	480 ppm	600 ppm			LFL	UFL	
I	27.4±2.19^a	41.2±3.19^b	55.4±2.88^c	70.8±1.64^d	81.2±1.09^a	y=0.975+0.003x	312.17 (722.67)	270.86 (646.03)	349.28 (839.77)	0.052 n.s
II	22.6±1.14^a	33.8±2.48^b	51.8±1.30^c	67.2±1.78^d	78.8±1.78^{ab}	y=1.169+0.003x	352.65 (739.19)	316.46 (664.59)	388.28 (850.75)	0.217 n.s
III	18.6±1.34^a	30.8±2.16^b	47.4±2.30^c	61.4±1.67^d	67.8±3.19^c	y=1.183+0.003x	407.21 (848.42)	367.60 (748.69)	451.53 (1007.34)	1.454 n.s
IV	15.4±1.18^a	27.2±1.30^b	39.4±2.19^c	53.6±1.51^d	60.8±1.09^d	y=1.277+0.003x	471.60 (944.81)	426.96 (821.58)	529.93 (1150.43)	0.913 n.s
Pupa	13.2±2.68^a	25.4±1.67^b	35.6±1.34^c	45.4±1.81^d	56.4±2.96^{de}	y=1.331+0.003x	523.91 (1083.29)	471.54 (881.29)	600.44 (1285.85)	0.718 n.s

The larval mortalities are expressed as Mean±SD of five replicates. Nil mortality was observed in the control. Within a column means followed by the same letter(s) are not significantly different at 5% level by Duncan's multiple range test. *LFL* - Lower Fiducidal Limit; *UFL* - Upper Fiducidal Limit. x^2, Chi-square value. *Significant at P< 0.05 level.

Table 2: Larvicidal and pupicidal toxicity of R. tomentosa leaf methanolic extract against the Dengue and Zika virus vector Ae. aegypti.

Target	Mortality (%) ± SD					Regression equation	LC_{50} (LC_{90})	95% confidence Limit LC_{50} (LC_{90})		x^2 (*d.f.*=4)
	120 ppm	240 ppm	360 ppm	480 ppm	600 ppm			LFL	UFL	
I	32.4±1.67[a]	46.2±1.14[b]	59.4±1.94[c]	77.8±1.92[d]	92.0±2.30[a]	y=0.977+0.004x	263.82 (609.83)	224.69 (554.31)	296.98 (689.36)	2.038 n.s
II	29.6±1.48[a]	39.8±1.92[b]	56.2±1.64[c]	73.2±1.30[d]	85.8±2.38[ab]	y=0.912 +0.003*x*	299.43 (677.22)	260.42 (610.87)	334.06 (775.23)	0.774 n.s
III	25.6±1.81[a]	37.8±1.09[b]	54.8±1.14[c]	68.4±2.30[d]	78.8±1.81[c]	*y*=1.025+0.003*x*	331.64 (746.13)	291.59 (665.93)	369.13 (869.29)	0.185 n.s
IV	22.4±1.92[a]	34.2±0.89[b]	49.4±1.09[c]	61.6±1.48[d]	69.8±1.64[d]	*y*=1.047+0.003*x*	386.16 (858.83)	343.71 (751.51)	431.61 (1034.95)	0.595 n.s
Pupa	20.2±1.51[a]	31.4±1.22[b]	46.6±1.51[c]	58.4±1.30[d]	65.4±1.14[de]	*y*=1.099+0.003*x*	419.56 (908.72)	375.92 (789.49)	470.77(1108.79)	0.987 n.s

The larval mortalities are expressed as Mean±SD of five replicates. Nil mortality was observed in the control. Within a column means followed by the same letter(s) are not significantly different at 5% level by Duncan's multiple range test. *LFL* - Lower Fiducidal Limit; *UFL* - Upper Fiducidal Limit. x^2, Chi-square value. *Significant at $P< 0.05$ level.

Botanical products have become more prominent in assessing current and future pest control alternatives [27]. Subramaniam *et al.*, [28] have reported that the toxicity of larval mortality in the all the instars (I-IV) of *Ae. aegypti* after the treatment of petroleum ether extract of *A. vera* noted at different concentrations. The LC_{50} and LC_{90} values were represented as follows; LC_{50} value of I instar was 162.74 ppm, II instar was 201.43 ppm, III instar was 253.30 ppm, and IV instar was 300.05 ppm, respectively. The LC_{90} value of I instar was 442.98 ppm, II instar was 518.86 ppm, III instar was 563.18 ppm and IV instar was 612.96 ppm, respectively.

The methanol leaf extracts of *C. colocynthis* and *C. maxima* showed potential larvicidal effectiveness against filarial vector, *C. quinquefasciatus*; LC_{50} values were 117.73 and 171.64 ppm, respectively [29]. Kovendan *et al.* [30] have reported the methanolic leaf extract of *Jatropha curcas* against *Culex quinquefasciatus* and *Leucas aspera* leaf methanolic extract against *Anopheles stephensi* respectively [31]. Subramaniam and Murugan [32] have reported that the Larval and pupal mortality of methanolic extract of *M. fragrans* seeds against *A. stephensi*. The LC_{50} LC_{90} values were represented as follows: LC_{50} value of I instar was 113.26 ppm, II instar was 126.94 ppm, III instar was 155.95 ppm, and IV instar was 173.53 ppm. The LC_{90} value of I instar was 279.26 ppm, II instar was 332.19 ppm, III instar was 398.21 ppm and IV instar was 442.29 ppm. The LC_{50} value of pupae was 197.93 ppm, and the LC_{90} value of pupae was 493.34 ppm respectively. Similarly, Many medicinal plant extracts have been reported to show insecticidal activities against agricultural, medical and veterinary importance insect pests [33, 34, 35, 36]. Yang *et al.* [37] have reported that the toxicities of methanol extracts from 28 medicinal plant species to early 4th instar larvae of *Aedes aegypti*, *Ochlerotatus togoi {Aedes togoi)*, and *Culex pipiens pallens* at a concentration of 100 ppm, >90% mortality of *Cinnamomum cassia* bark, *Illicium verum* fruit, *Piper nigrum* fruit, *Zanthoxylum piperitum* fruit, and *Kaempferia galanga* rhizome. *P. nigrum* fruit extract gave 100% mortality of larvae of *Ae. aegypti* and *O. togoi* at 5 ppm and 96% mortality of larvae of C. *pipiens pallens* at 2.5 ppm. Z. *piperitum* fruit extract gave 85, 100, and 48% mortality in larvae of *Ae. aegypti*, *O. togoi*, and *Cx. pipiens pallens* at 10 ppm, respectively. For instance, Kamaraj et al.[38] have reported that the LC_{50} value of petroleum ether extracts of *Jatropha curcas* (*J. curcas*), *Pedilanthus tithymaloides*, *Phyllanthus amarus*, *Euphorbia hirta* and *Euphorbia tirucalli* against larvae of *Ae. aegypti* 8.79, 55.26, 90.92, 272.36, and 4.25 ppm, respectively, Karunamoorthi and Ilango[39] have reported that the LC_{50} and LC_{90} values of methanol leaf extracts of *Croton macrostachyus* were 89.25 and 224.98 ppm, respectively against late third instar larvae of malaria vector, *Anopheles arabiensis*.

Conclusion

In conclusion, the larvicidal and pupicidal evaluation of acetone and methanolic leaf extract of *Rhodomyrtus tomentosa* against Dengue and Zika virus vector, *Aedes aegypti* were evaluated. These plant extracts are showed that they have excellent effective mosquito control at immature stages of mosquito vector and this work shows potential results. The natural products of biopesticides are eco-friendly for the dengue vector control management programs. Besides, further investigation regarding the effect on non-target organism is extremely important and crucial in the near future.

Conflicts of interest

The authors declare no conflicts of interest.

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