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Larvicidal activities of the leaf extracts and essential oil of *Premna latifolia* Roxb. (Verbenaceae) against *Aedes albopictus* Skuse (Diptera: Culicidae)

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ABSTRACT

In the context of rapid emergence of resistance among vector mosquitoes to synthetic insecticides, use of natural products of plant origin has gained considerable importance as potential agents for vector control. The current investigation attempts to evaluate the mosquito larvicidal efficacies of the crude aqueous, chloroform and methanol extracts and essential oil from the leaves of *Premna latifolia* Roxb. (Verbenaceae) against the fourth instar larvae of *Aedes albopictus* Skuse (Diptera: Culicidae). The chemical composition of essential oil also was analyzed using gas chromatography–mass spectrometry. The extracts as well as the essential oil showed good larvicidal effects; however, the highest larval mortality was found in essential oil followed by chloroform extract. Their LC_{50} values in 24 h were 38.9 ppm (LC_{90} = 86.9 ppm) and 76.1 ppm (LC_{90} = 272.1 ppm) respectively. The principal chemical constituents found in the essential oil were β-cadinene (33.62%) and phytol (27.28%), followed by α-selinene (9.42%). The study demonstrates that crude extracts and essential oil of *P. latifolia* leaves have good potential to be used as biodegradable agents for the control of the mosquito vector *A. albopictus* and could be a potent source for the production of biodegradable natural larvicides.

INTRODUCTION

Despite the advances made in medical sciences and the advent of new drugs, the diseases transmitted by mosquitoes, including malaria, filariasis, dengue and viral encephalitis, are still the most important human diseases, with an estimated two billion people in the world living in areas where these diseases are endemic (WHO, 1999). The Asian tiger mosquito, *Aedes albopictus*, is currently the most invasive mosquito species in the world (Benedict *et al*., 2007). This species is an aggressive daytime biter that can efficiently transmit several arboviruses including Dengue, Yellow Fever, Encephalitis, Chikungunya and West Nile, all having considerable medical and veterinary importance (Mitchell, 1995).

Dengue is an emerging infectious disease that is estimated to affect 50-100 million individuals each year in tropical and subtropical areas and *A. albopictus* stands just second to *Aedes aegypti* L. as a vector of dengue (Halstead, 2007). In India, *A. albopictus* is often been cited as the major dengue vector, particularly in urban settlements and occasionally in rural backgrounds also.

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Chikungunya virus is yet another mosquito transmitted virus that has received much global attention recently, due to the series of large-scale epidemics it caused between 2004-2007 in Africa, Indian Ocean basin and South-East Asia. The transmission of chikungunya virus also is partially been attributed to *A. albopictus* (Tsetsarkin *et al*., 2011). Though *A. albopictus* is native to the tropical and subtropical areas of South East Asia, in the past couple of decades it has become a very serious pest throughout the world by increasing air travel and transport of goods across countries. The expansion of the area of activity of the Asian tiger mosquito, along with its resistance to insecticides is creating new opportunities for dengue and chikungunya viruses to invade and circulate in new areas and has resulted in more dengue and chikungunya outbreaks in several parts of the world. Controlling the vectors concerned deserves special consideration in these two diseases since they belong to those classes of communicable diseases against which effective vaccines have not been evolved so far. Repeated use of chemical insecticides for vector control can lead to the development of resistance in mosquito vectors and also leads to many undesirable side effects on non-target organisms including human beings. Plant extracts and essential oils might serve as natural insecticides, since they comprise a rich source of bioactive compounds that are environment safe, more target

specific, biodegradable and hence are widely accepted as an important alternative strategy of vector control (Cheng *et al*., 2008).

The genus *Premna* is a widely distributed, medicinally important member of the family Verbenaceae, many species of which are extensively used in traditional medicine for the treatment of various disorders. A good volume of phytochemical work has been done on *Premna* species and several new terpenoid compounds have been isolated (Rao *et al*., 1985; Suresh *et al*., 2011). *Premna latifolia* Roxb. is a small deciduous tree, widely distributed both in tropical and sub-tropical areas. Leaves are aromatic when crushed and diuretic when given internally and are applied externally in dropsy (Anonymous, 1969). Milk of the bark is applied to boils and the juice is given to cattle in colic (Kirtikar and Basu, 1980). Many plants in the Verbenaceae family have been found to be effective against a variety of mosquito vectors (Karunamoorthi *et al*., 2008; Costa *et al*., 2010). The present investigation was designed to examine the larvicidal efficacy of different solvent extracts and essential oil obtained from the leaves of *P. latifolia* against the fourth instar larvae of *A. albopictus* and also to identify the active constituents of the essential oil.

MATERIALS AND METHODS

Collection of Plant Material

Fresh, mature and green twigs of *P. latifolia* were collected during June-July, 2012, from the plants growing within Calicut University campus (geographical co-ordinates 11.25 °N and 75.78°E), Kerala, India. The taxonomic identity of the plant was established by Dr A. K. Pradeep, Assistant Professor, Department of Botany, University of Calicut. The voucher specimen (CALI 123729) was deposited at the Herbarium (CALI) of Department of Botany, University of Calicut.

Extraction of Essential Oil

After collection, the leaves were initially rinsed with distilled water and further shade dried and mechanically ground by an electric blender. 50 g of the powder was weighed and was subjected to hydrodistillation using a modified Clevenger apparatus for 6 h. The yield of essential oil obtained was averaged over three experiments and calculated according to dry weight of the plant materials. Essential oil was stored in airtight glass vials in the refrigerator at 4°C until prior to further chemical analysis and larvicidal bioassays.

Gas Chromatography-Mass Spectrometry (GC/MS) Analysis

Analysis of essential oil was performed by gas chromatography coupled with mass spectrometry (GC/MS) on a Hewlett Packard (HP) 6890 GC interfaced with a Hewlett Packard 5973 Mass Selective Detector (MSD) system operating at 70 eV and 250°C, equipped with a splitless injector. The GC column used was a cross-linked 5 % phenyl methyl siloxane column HP-5(DB5), with 320 µm × 3 m and 0.25 µm film thicknesses. Helium was used as the carrier gas at a flow rate of 1.4 mL/min. The temperature program for the HP-5 column was set initially at 60°C for 1 min and then heated at the rate of 3°C /min to 246°C. Runtime was 61 min. The presence of various oil components was analyzed and the identification of individual components was done using NIST MS Search. The relative quantity of each compound in essential oil was determined based on the percentage peak area integrated by the analysis program.

Preparation of Plant Extracts

10 g of the dried powder was weighed and extracted with 100 ml of 100% chloroform, methanol and distilled water, separately in a Soxhlet apparatus for 6 h. The extracts were filtered through Whatman No.1 filter paper. After complete evaporation of the solvent, the concentrated extracts were stored in closed amber coloured glass bottles and kept under refrigeration till use.

Mosquito Larvicidal Bioassays

All bioassays were performed essentially following the standard protocols of WHO (1981). The experiment was carried out using laboratory reared larvae obtained from the mosquito colonies that were maintained at the Cell and Molecular Biology Division, Department of Botany, University of Calicut, India. The larval colonies were grown in open plastic trays and dishes containing tap water and fed a diet of decaying leaves and yeast granules. The early fourth instar larvae only were used for the study. For experimental treatment, 20 mg of each of the extracts was dissolved in 2 mL of Dimethyl sulphoxide (DMSO) and shaken gently to form a homogenous stock solution. In the same way, the essential oil obtained was also used to prepare a 1 % stock solution in acetone. Graded series of test solutions of different concentrations (25, 50, 100, 200, 300, 400 and 500 ppm) were prepared from the stock solutions by serial dilution with distilled water. Three control tests were set up in parallel for comparison at a time; one consisted of distilled water and acetone, another of distilled water and DMSO and the third, distilled water alone. The fourth instar larvae (25 each) were tested for seven different concentrations as well as controls. Five replicates were made per concentration. Mortality and survival of the larvae were recorded after 24 h of exposure and the larvae were starved during this period. The larvae were considered dead, if they were not responsive to a gentle prodding with a fine needle. The percentage mortality of the larvae was reported from the average of five replicates. Toxicity and effect were reported as LC_{50} and LC_{90}, representing the concentrations in ppm with 50% and 90% larval mortality rate in 24 h, respectively.

Statistical Analyses

The percentages of larval mortality at different concentrations of the essential oil and extracts were calculated and the data were subjected to probit analysis (Finney, 1971) to calculate the LC_{50}, LC_{90}, their fiducial limits at 95% confidence limits, regression equations and chi square values. The one way factorial ANOVA of the mortality data was conducted employing Duncan Mean Range Test (DMRT) to analyze for significant differences among the tested materials' activity against the larvae.

All statistical analyses were performed by using the computer software SPSS 21.0 for Windows. Results with $P < 0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

The results of the present study have proved that different solvent extracts and essential oil of *P. latifolia* are highly effective against the fourth instar larvae of *A. albopictus* (Table 1 and Figure 1).

Table 1. Regression parameters of probit analysis for mortality of the fourth instar larvae of *A. albopictus* to different solvent leaf extracts and essential oil of *P. latifolia*.

Oil/Solvent Extract	Regression equation	χ^2	LC_{50} (Fiducial limits) ppm	LC_{90} (Fiducial limits) ppm
Essential oil	3.673X-5.840	27.636	38.9 (35.186-42.643)	86.9 (76.791-101.672)
Chloroform extract	2.316X-4.357	45.831	76.1 (65.918-86.742)	272.1 (230.133-333.570)
Methanolic extract	1.929X-4.181	63.528	147.0 (124.370-173.050)	678.7 (521.334-967.768)
Aqueous extract	1.845X-4.347	48.104	226.9 (195.914-265.549)	1122.9 (840.368-1663.676)

LC_{50} lethal concentration that kills 50% of the exposed larvae, LC_{90} lethal concentration that kills 90% of the exposed larvae, χ^2 chi-square, X concentration in parts per million.

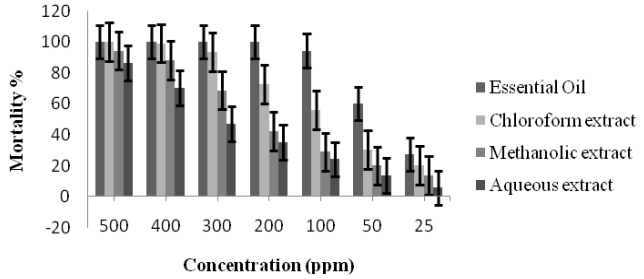

Fig 1. Mosquito larvicidal effects of leaf extracts and essential oil from *P. latifolia* against fourth instar larvae of *A. albopictus* in 24 h.

The highest larval mortality was shown by the essential oil. The LC_{50} and LC_{90} values of the leaf essential oil were 38.9 and 86.9 ppm, respectively. Among the three crude extracts, chloroform extract was found to exhibit relatively high larvicidal activity (LC_{50} = 76.1 ppm; LC_{90} = 272.1 ppm). This was followed by the methanolic extract with LC_{50} and LC_{90} of 147.0 and 678.7 ppm, respectively. The aqueous extract also showed moderate larvicidal activity with LC_{50} and LC_{90} of 226.9 and 1122.9 ppm.

The standard errors with means of larval mortalities were shown in Table 2. Larval mortality rates were found to be dosages dependent since significant differences were found to exist in mortality as a function of concentration (Table 2). No trace of mortality was observed in the control treatments.

The gas chromatogram of *P. latifolia* leaf essential oil is shown in Figure 2 and the chemical composition of essential oil is presented in Table 3. The GC-MS data revealed that the oil of *P. latifolia* is rich in non-polar compounds namely terpenes. The yield of leaf essential oil from the hydrodistillation was found to be 0.07%. The essential oil consisted mainly of sesquiterpenoids along with a diterpene alcohol, phytol. The essential oil composition of the present study differs significantly from an earlier report (Kumar *et al*., 2011). The variations in the essential oil composition could be due to different chemotypes for the same species or from environmental, developmental or other differences (Sefidkon *et al*., 2004).

Application of insecticides of plant origin has been gaining more importance as it could possibly bring down the cost of production without leaving any harmful residues in the ecosystem. Vieira *et al*. (2001) stated that terpenoids are an important source of insecticide substances, which can defend plants against a variety of insect pests. According to Paluch *et al*. (2009), one of the important functional roles of the diverse sesquiterpenes in plants is to provide protection against pest arthropods. Plant essential oils containing relatively high proportions of sesquiterpene hydrocarbons have been shown to have excellent larvicidal properties in the past also (Aguiar *et al*., 2010; Lima *et al*., 2011; Kiran and Devi, 2007). Sesquiterpenes isolated from the roots of *Inula helinium* have been found to be highly potent against the third and fourth instar larvae of *A. albopictus* (Konishi *et al*., 2008). These findings point to the fact that the potential larvicidal property of *P.latifolia* could possibly be attributed to the presence of high proportion of various sesquiterpenes. Further investigations on the identification of the active compound(s) responsible for the activity and their mode of action should be evaluated in detailed field trials. This might eventually lead to the development of an effective commercial mosquitocidal formulation of plant origin. Use of plant derived preparations can contribute a lot to overcome the limitations of the conventional larvicides and so is recommendable as an auxiliary control measure for mosquito larvae. Before any attempts of a large scale application, however, the mechanism of its action in the target species as well as the possible effects on non-target organisms should be well documented.

Table 2. Mortality effect induced by different concentrations of leaf extracts and essential oil from *P. latifolia* against fourth instar larvae of *A. albopictus* in 24 h treatment.

Treated substance (Oil/solvent extract)	Larval Mortality *						
	500ppm	400ppm	300ppm	200ppm	100ppm	50ppm	25ppm
Essential oil	25.0±0^a	25.0±0^a	25.0±0^a	25.0±0^a	23.6±0.98^a	15.0±1.64^a	6.8±0.73^a
Chloroform extract	25.0±0^a	24.8±0.2^a	23.4±0.68^b	18.2±0.86^b	14.0±1.4^b	7.6±0.75^b	5.0±0.45^b
Methanolic extract	23.6±0.75^a	22.0±0.7^b	17.2±0.37^c	10.6±0.87^c	7.2±1.24^c	5.0±0.55^{bc}	3.4±0.5^b
Aqueous extract	21.6±0.87^b	17.6±1.08^c	11.8±0.58^d	8.8±0.58^c	6.6±0.75^c	3.4±0.93^c	1.4±0.4^c

*Each value represents the mean ± SE of five replicates of larvae dead at each concentration of the treated substance.
Controls—nil mortality
Means in a column followed by the same superscript letters are not significantly different (P<0.05, one-way ANOVA, DMR test).

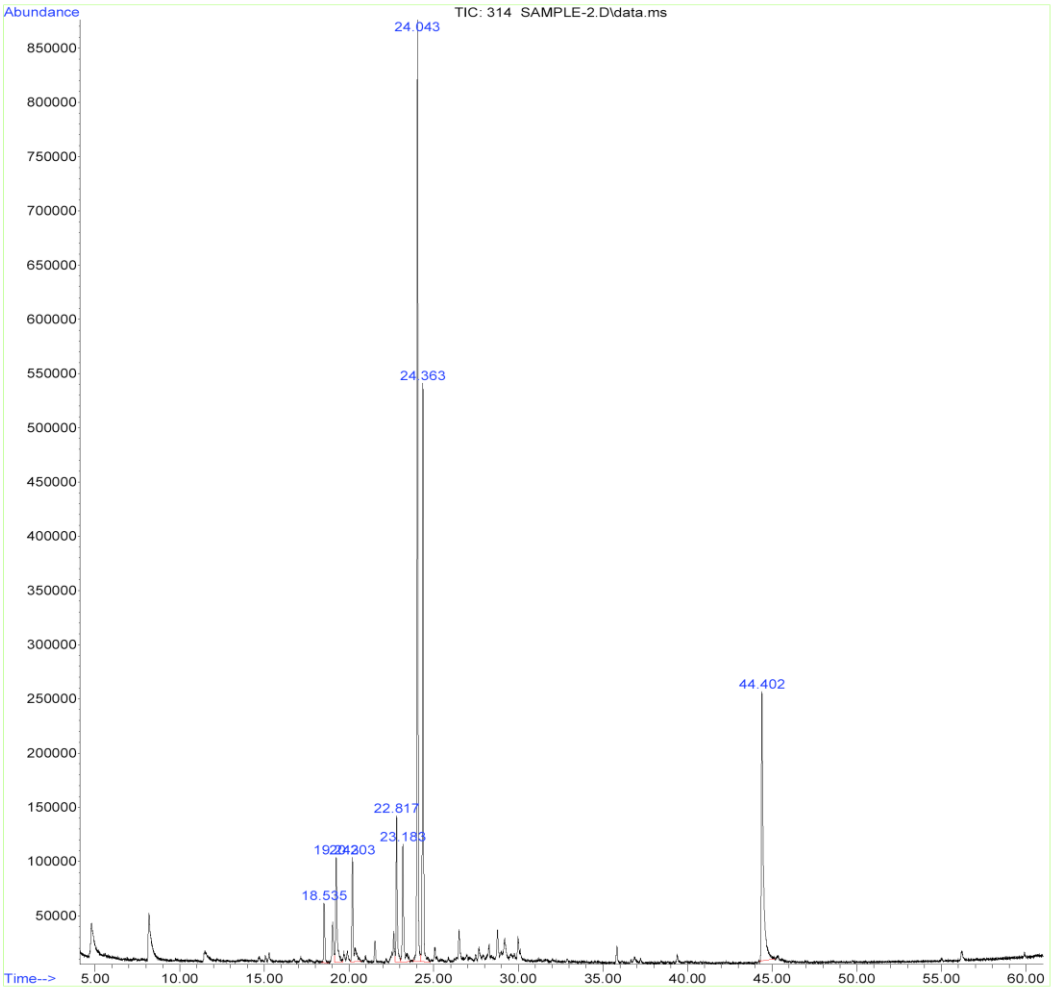

Fig 2. Gas chromatogram of *P. latifolia* leaf essential oil

Table 3. Chemical constituents of the leaf essential oil from *P.latifolia.*

Compounds	Retention time	Area percentage
Copaene	18.536	3.78
β-Elemene	19.242	8.28
β-Caryophyllene	20.198	8.43
α-Selinene	22.815	9.42
δ-Guaiene	23.179	9.18
β-Cadinene	24.363	37.26
Phytol	44.398	27.18

CONCLUSION

The results of the present investigation clearly indicate that *P. latifolia* can serve as a potent larvicide against *A. albopictus* larvae. The leaf essential oil and the crude extracts of *P. latifolia* with different solvents, *viz.* chloroform, methanol and water were tested for larvicidal activity against *A. albopictus* and are found to have significant larvicidal potential. Among them, the essential oil and the chloroform extract showed excellent inhibitory effects. To conclude, the leaves of *P. latifolia* contain effective biological compounds that could be targeted against specific mosquito larvae. The outcome of the study could be helpful in developing more selective, less toxic and easily degradable natural larvicides.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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