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Ovicidal, larvicidal and pupicidal activity of *Nelumbo nucifera* Gaertn against the filarial vector *Culex quinquefasciatus* Say (Diptera: Culicidae)

Annie Rubens[1*] and P. Philip Samuel[2]

[1]Department of Advanced Zoology and Biotechnology, Women's Christian College, Chennai 600006, Tamil Nadu, India; [2]ICMR-Vector Control Research Centre Field station, Department of Health Research, Madurai 625002, Tamil Nadu, India. Email: rubensannie93@gmail.com

ABSTRACT: In the current investigation, different solvent leaf extracts of *Nelumbo nucifera* were tested for their mosquitocidal potential against the filarial vector *Culex quinquefasciatus*. Further, bioactive compounds of the crude leaf extracts of *N. nucifera* were identified using GC-MS analysis. The benzene leaf extracts of *N. nucifera* showed significant egg, larval and pupal mortality at concentrations of 62.5, 125, 250 and 500 ppm, respectively. Mortality of ovicidal, larvicidal and pupicidal effect at 500 ppm at 48 h period were 89, 38 and 67 per cent with LC50 values 4.247, 6.694 and 4.975 and LC90 values 5.881, 9.628, 6.565 ppm, respectively. GC-MS profile of the leaf showed seven peaks and the major components identified as nuciferine, steporphine and mecambroline (29.40%). The study confirms *N. nucifera* has significant mosquitocidal effects.

KEYWORDS: *Nelumbo nucifera,* mosquitocidal potential, nuciferine, steporphine, mecambroline

INTRODUCTION

Among all insect vectors of human diseases, mosquitoes stand out as the most medically important fauna causing significant public health problem. Pathogens and parasites are the causative agents of vector-borne diseases (VBDs) and serve as major cause of death especially in the tropical and subtropical countries (Kovendan *et al*., 2012; Singh *et al*., 2014). Mosquitoes act as vectors for maladies such as filariasis, malaria, dengue, chikungunya, yellow fever, West Nile, and Zika viral disease (WHO, 2014, 2016). The control of mosquitoes has become a major public concern all over the world (Islam *et al*., 2011).

Spread of mosquito-borne diseases and deaths can be controlled by preventing the mosquito larvae from maturing into adults (Al-Doghariri and Elhag, 2003). Control of VBDs using broad-spectrum insecticides caused undesirable side effects (Youdeowei and Service, 1983). India has a vast resource of plants which contain phytochemicals which are virtually an untapped reservoir of pesticides that can be used directly or as templates for synthetic pesticides due to their eco-friendly nature (Sekar, 2010; Okwute, 2012). *Nelumbo nucifera* Gaertn.(Nymphaeaceae) known as Indian lotus or sacred lotus is a hydrophyte that produces individual leaves and flowers directly from the root

* Author for correspondence

system (Shen Miller *et al*., 2002). The whole plant has various medical properties such as diuretic, astringent and palliative and finds therapeutic use in the treatment of diarrhea, tissue irritation and homeostasis. *N.nucifera* leaves are used for treatment of hematemesis, epistaxis, hematuria, and metrorrhagia. Several bioactive compounds such as alkaloids, steroids, triterpenoids, flavonoids, glycosides and polyphenols were identified from different parts of the plant (Paudel and Panth, 2015).The current study was undertaken to examine the effects of five different solvent extracts of the leaves of *N. nucifera* against the egg, larvae and pupal stages of *Cx. quinquefasciatus*.

MATERIALS AND METHODS

Plant material and extraction procedures: Fresh and mature leaves of *N. nucife*ra were collected thoroughly washed, shade dried and pulverized using a blender. By using a Soxhlet apparatus (Vogel, 1978) about one kg of the pulverized leaves were serially extracted with three liters of the solvents (hexane, benzene, ethyl acetate, methanol and distilled water) to obtain the active compounds. The extracts were filtered and further dried using a rotary evaporator. Stock solution (1 %) was prepared by adding required quantity of acetone to each extract and stored at 4^0C until further bioassay (Annie *et al*., 2015).

Mosquito culture: *Cx. quinquefasciatus* immature stages were collected from local water bodies and colonized in the laboratory to obtain the F1 generations. The larvae were maintained in enamel trays at 27 ± 2°C and 70 to 80 per cent relative humidity and fed with powdered dog biscuit and yeast in the ratio 3:1 (Annie *et al*., 2015).

Ovicidal bioassay: The leaf extracts were screened for their ovicidal efficacy using conventional WHO susceptibility test method (WHO, 2005). The egg rafts laid by F1 generation of the laboratory colonized mosquitoes were collected. By serial dilution of 1 per cent stock solution, test concentrations (62.5, 125, 250 and 500 ppm) of each of the crude extract was prepared. In all, 20 fresh eggs were separated from the egg raft with the aid of a dissection microscope and brush and were introduced into 250mL plastic containers containing 200 ml of test concentration and water. For each concentration, five replicates per trial were performed along with appropriate controls. Addition of acetone to distilled water served as treated control, while distilled water alone served as an untreated control. After 48 h the hatchability was calculated (Veni *et al*., 2017).

Larvicidal and Pupicidal Bioassay: The solvent extracts were screened for their efficacy against larval and pupal stages of *Cx. quinquefasciatus* using WHO standard susceptibility test method (WHO, 2005).The required test concentrations (62.5, 125, 250 and 500 ppm) was prepared by serial dilution of 1 per cent stock solution for each crude extract. For the present study 20 IV instar larvae and pupae were collected from laboratory colonized F1 generations mosquitoes and introduced separately into 250ml plastic containers containing 200ml of test concentration and water. For each concentration, five replicates per trial were performed along with treated and untreated controls.

Gas chromatography mass spectrum (GC-MS) Analysis: Among the solvents evaluated, benzene extract of the leaf of *N. nucifera* showed maximum mosquitocidal activity. GC-MS was performed to identify the phytochemicals responsible for the mosquitocidal activity (Abirami and Rajendran, 2012). The GC–MS analysis was performed using Clarus 500 Perkin – Elmer Gas Chromatograph coupled to Perkin Elmer Turbomass 5.1 spectrometer. The starting temperature of the oven was 110^oC, and maintained for 2 min at this temperature. After the time period of 2 min the oven temperature was raised up to 280^oC, at the rate 5^oC/min, and subsequently maintained at this temperature for 9 min. Temperature at the injection port was maintained at 250^oC and Helium was used as a carrier gas with a flow rate as one ml/min. Ionization voltage was 70eV. The samples were injected in split mode as 10:1. The individual phytochemicals in the crude extracts were separated by the gas chromatography column and the separated compounds enter the Mass Spectrum (MS) and get ionized. The MS ionizing spectrum

was recorded and compared to the MS spectrum of known compounds within the NIST library. Compound name, retention time, area per cent, molecular formula and molecular weight of the components of the subjected extracts were determined.

Data analysis: Mortality for all the three bioassays was calculated (Veni *et al*., 2017) and Abbott's formula was used when control mortality varied between 5 to 20 per cent (Abbott *et al*., 1925). Data from all replicates pertaining to ovicidal, larvicidal and pupicidal bioassay were pooled for statistical analysis. SPSS software by probit analysis (SPSS, 2009) was used for calculating LC50 and LC90 values. Difference in mortality between concentrations was determined using ANOVA and results with P<0.05 level were considered to be statistically significant.

RESULTS AND DISCUSSION

Benzene extracts of the leaves of *N. nucifera* exhibited significant mosquitocidal activity against *Cx. quinquefasciatus*. Ovicidal, larvicidal and pupicidal activity at 500ppm after 48 h period was 89, 38 and 67 per cent, with LC50 and LC90 values of 4.247, 6.694, 4.975 and 5.881, 9.628, 6.565 ppm respectively. A direct correlation was observed between the ovicidal, larvicidal and pupicidal activity and the extract concentration (62.5 to 500 ppm), the rate of mortality was directly proportional to the concentration indicating that the extracts exhibited a dose-dependent mortality effect on all the stages. No mortality was noticed in the controls (Table 1 and 2).

Since, the benzene extract of the leaves of *N. nucifera* was found to be more effective, it was further subjected to Gas Chromatography-Mass Spectral analysis to identify the phytoconstituents which might be responsible for their significant mosquitocidal activity. Comparison of the mass spectra of the constituents of the extract when matched with NIST library revealed the presence of seven phytocompounds with their corresponding peaks at different retention time (Fig 1). The Retention Time, peak area (%), molecular formula and molecular weight of the benzene leaf extract of *N. nucifera* were identified (Table 3). The compounds hexadecanoic acid (9.02%), hexadecatrienoic acid, linoleyl alcohol (8.74%), nornuciferine (3.15%), roemerine (4.86), 10 nondecanol (7.44%), oxirane, tetradecyl, tetradecanal (12.00%) were recorded. The major constituents were nuciferine, steporphine, mecambroline (29.40%).

Vector borne disease remains a major cause of mortality worldwide. In recent years, the significance of biological activity of phytocompounds in mosquito erradication programmes has gained importance because of their biodegradable nature. Insecticide-resistant

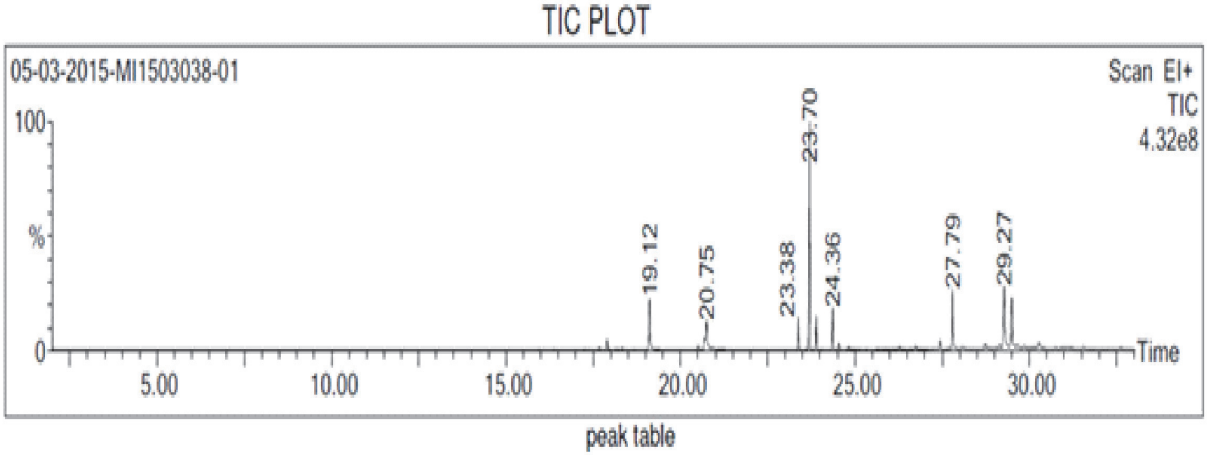

Fig. 1 Chromatogram of benzene extract of *N. nucifera*

Table 1.Ovicidal, larvicidal and pupicidal activity of *N. nucifera* crude leaf extract against *C. quinquefasciatus*

Stages	Solvents	Concentration (ppm)			
		62.5	125	250	500
Ovicidal	Hexane	0.8±1.78^{a} (4.00)	2.2±3.49^{ab} (11.00)	4.2±1.30^{ab} (21.00)	6.0±4.0^{b} (30.00)
	Benzene	4.4±2.30^{b} (22.00)	9.4±1.34^{c} (47.00)	14.2±2.68^{d} (71.00)	17.8±1.48^{e} (89.00)
	Ethyl Acetate	1.6±1.34^{a} (8.00)	5.4±2.60^{b} (27.00)	6.8±2.16^{b} (34.00)	10.2±1.78^{c} (51.00)
	Methanol	1.2±0.83^{ab} (6.00)	2.4±1.51^{bc} (12.00)	2.6±1.34^{bc} (13.00)	3.4±1.34^{c} (17.00)
	Aqueous	2.8±1.92^{ab} (14.00)	4.0±1.87^{bc} (20.00)	6.8±1.30^{cd} (34.00)	8.6±2.60^{d} (43.00)
Larvicidal	Hexane	0.2±0.44^{a} (1.00)	0.8±0.83^{a} (4.00)	1.6±1.81^{ab} (8.00)	3.4±1.51^{b} (17.00)
	Benzene	2.0±1.41^{ab} (10.00)	2.6±1.81^{ab} (13.00)	4.0±1.41^{b} (20.00)	7.6±2.07^{c} (38.00)
	Ethyl Acetate	1.0±1.22^{ab} (5.00)	2.2±0.83^{bc} (11.00)	3.2±1.30^{c} (16.00)	4.0±1.58^{c} (20.00)
	Methanol	0.6±1.34^{a} (3.00)	1.0±1.41^{a} (5.00)	1.6±1.67^{a} (8.00)	2.0±1.0^{a} (10.00)
	Aqueous	0.2±0.44^{a} (1.00)	0.4±0.54^{a} (2.00)	0.6±0.54^{a} (3.00)	1.0±1.0^{a} (5.00)
Pupicidal	Hexane	2.2±0.44^{ab} (11.00)	6.0±4.24^{bc} (30.00)	9.8±5.16^{c} (49.00)	10.6±3.28^{c} (53.00)
	Benzene	5.0±4.58^{ab} (25.00)	6.6±3.28^{b} (33.00)	9.8±4.49^{bc} (49.00)	13.4±1.34^{c} (67.00)
	Ethyl Acetate	0.2±0.44^{a} (1.00)	0.6±0.89^{ab} (3.00)	1.0±1.0^{ab} (5.00)	2.2±1.78^{b} (11.00)
	Methanol	0.2±0.44^{a} (1.00)	0.4±0.54^{a} (2.00)	1.2±0.44^{b} (6.00)	1.4±0.54^{b} (7.00)
	Aqueous	6.0±2.23^{b} (30.00)	7.8±4.76^{b} (39.00)	9.8±3.42^{b} (49.00)	10.4±2.07^{b} (52.00)

Mean values of five replicates per trials ±standard deviation. Values in parenthesis denote % mosquitocidal mortality. Different superscript alphabets specify statistical significant difference in mosquitocidal mortality between concentrations at P<0.05 level by one way ANOVA followed by Tukey's test.

population of *Cx. quinquefasciatus* has been reported worldwide and as a result of which plant based products may be considered as alternate biopesticide in Integrated Vector Management Programme (Bowers *et al*., 1995; Tennyson *et al*., 2012).

Outcome of the present investigation can be compared with previous reports on the ovicidal activity against *Cx. quinquefasciatus*, where benzene leaf extract of *Ervatamia coronaria* was more effective in exerting ovicidal and larvicidal activity at 300, 250 and 200 ppm against the egg/

Table 2.Probit analysis of ovicidal, larvicidal and pupicidal activity of *N. nucifera* crude leaf extract *against C. quinquefasciatus*

Stages	Solvents	LC_{50}(ppm)	95% Confidence Limit		LC_{90}(ppm)	95% Confidence Limit	
			LL(ppm)	UL(ppm)		LL(ppm)	UL(ppm)
Ovicidal	Hexane	6.916	6.119	8.711	9.677	8.123	13.504
	Benzene	4.247	4.078	4.420	5.881	5.617	6.213
	Ethyl Acetate	5.732	5.428	6.130	8.168	7.139	8.514
	Methanol	8.640	7.460	11.050	12.774	10.542	17.463
	Aqueous	6.081	5.688	6.628	8.945	8.113	10.203
Larvicidal	Hexane	7.999	7.111	9.892	10.745	9.134	14.299
	Benzene	6.694	6.188	7.465	9.628	8.599	11.290
	Ethyl Acetate	7.968	7.064	9.652	11.537	9.805	14.878
	Methanol	9.933	7.823	18.360	14.223	10.531	29.361
	Aqueous	11.515	8.696	26.653	15.937	11.342	41.005
Pupicidal	Hexane	5.386	4.977	5.964	7.760	6.948	9.168
	Benzene	4.975	4.563	5.521	7.349	6.565	8.711
	Ethyl Acetate	9.059	7.676	12.820	12.291	9.898	18.959
	Methanol	9.988	8.106	16.328	13.716	10.540	24.629
	Aqueous	5.211	4.710	5.953	8.165	7.110	10.133

LC50: Lethal concentration that causes 50% mortality of the exposed larvae; LC90: Lethal concentration that causes 90% mortality of the exposed larvae.

Table 3. Phytochemicals isolated from benzene extract of *N. nucifera*

S. No.	Retention time (RT)(mins)	Peak area %	Name of the compound	Molecular formula	Molecular weight
1	19.12	9.02	Hexadecanoic acid	$C_{16}H_{32}O_2$	256
2	20.75	8.74	Hexadecatrienoic acid	$C_{16}H_{26}O$	234
			Linoleyl alcohol	$C_{18}H_{34}O$	266
3	23.38	3.15	Nornuciferine	$C_{18}H_{19}No_2$	281
4	23.70	29.40	Nuciferine	$C_{19}H_{21}No_2$	295
			Steporphine	$C_{18}H_{17}No_3$	295
			Mecambroline	$C_{18}H_{17}No_3$	295
5	24.36	4.86	Roemerine	$C_{18}H_{17}No_2$	279
6	27.79	7.44	10 Nondecanol	$C_{19}H_{40}O$	284
7	29.27	12.00	Oxirane,tetradecyl	$C_{16}H_{32}O$	240
			Tetradecanal	$C_{14}H_{28}O$	212

egg rafts and larvae of *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus*, respectively (Govindarajan *et al*., 2011). Earlier studies on *N. nucifera* methanol and aqueous leaf extracts indicated higher larvicidal activity against *An. subpictus* and *Cx.* q*uinquefasciatus* (Santhosh kumar *et al*., 2011; Kamaraj *et al*., 2011). Ethyl acetate extracts of seed coats of *N. nucifera* had significant larvicide activity (Ray *et al*., 2014).

Citrullus vulgaris benzene leaf extracts yielded 100 per cent mortality at 250 ppm on the eggs of *An. stephensi* after 48h period (Mullai *et al*., 2008). Reports on *Cardiospermum halicacabum* methanol and benzene leaf extracts revealed complete mortality at 300 ppm against *Cx. quinquefasciatus* eggs (Govindarajan, 2011).

Pupicidal activity against *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus* was noted in petroleum benzene leaf extract of *Acanthospermum hispidum* (Vivekanandhan *et al*., 2018). While methanol leaf extract of *Annona reticulata* exhibited effective pupicidal activity of 98.90% at 200 ppm concentration against *Cx.* q*uinquefasciatus* (Selvakumar *et al*., 2015). Methanol leaf extracts of *Euphorbia hirta* exhibited pupicidal activity against *An. stephensi* was reported by Panneerselvam *et al*. (2013).

GCMS analysis of the benzene extracts of *N.nucifera* revealed the major compounds as neuciferine, steporphine and mecambroline which are aporphine alkaloids which probably have insecticidal properties. Paudel and Panth (2015) reported that the bioactive compounds present in *N. nucifera* are mainly alkaloids and flavonoids. GC-MS of the leaves of *N. nucifera* shows a rich source of a number of alkaloids which exhibit pesticidal properties (Kunitomo *et al*., 1964; Chen *et al*., 2013; Do *et al*., 2013). In the present study it is concluded that the leaf extract of *N. nucifera* has mosquitocidal property against all the stages of *Cx. quinquefasciatus*.

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