Innovations

Larvicidal activity of different solvent extracts of Nerium oleander (Apocynaceae) red coloured flowers against Aedes aegypti, Anopheles stephensi and Culex quinquefasciatus (Diptera: Culicidae)

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Abstract

Larvicidal activity of various solvent extracts of Nerium oleander red colored flower was studied against third instar larvae of three important vector mosquitoes viz., Aedes aegypti, Anopheles stephensi and Culex quinquefasciatus. Preliminary phytochemical analysis was done to all the solvent extracts showed the presence of Tannins, saponins, flavonoids, quinones, terpenes, phenols and coumarines are present in the red colour flowers extracts. Among the solvent extracts acetone extract was most effective when compared to other extracts. Cx. quinquefasciatus was the most susceptible (LC_{50}=20.4 mg/L) and An. stephensi was the most tolerant (LC_{50}=34.0 mg/L). Also, the extract was subjected to GC-MS analysis and 16 major biochemicals were detected. The present study clearly indicated that bioactive molecules present in the acetone extract of red color flower of N. oleander are promising botanical pesticide against vector mosquitoes.

Keywords: *1. Botanical pesticide, 2. Nerium oleander, 3. Different solvent extracts,4. Vector mosquitoes, 5.GC-MS.*

Highlights

- *Nerium oleander* flower extract was prepared with various solvents like Hexane, Petroleum ether, Dichloromethane, Chloroform, Ethyl acetate, Acetone, Methanol and Distilled water using Soxhlet apparatus.
- Acetone extract was effective against the third instar larvae of three important vector mosquitoes viz., *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus.*
- Acetone extract was fractionated using silica gel column chromatography and impure compounds were isolated. Then the compounds were subjected to GC-MS analysis and 16 major biochemicals were detected.

Introduction

Mosquitoes transmit serious human diseases, causing millions of deaths every year and the development of resistance to chemical insecticides resulting in rebounding vectorial capacity. The efficacy of plant extracts, plant compounds, seed oils and plant essential oils in vector mosquito management has been well documented by many investigators (Shaalan, 2015). Pavela *et al.,* (2019) has reviewed mosquito larvicidal plants and reported that 29 plants out of 400 plant species possessed outstanding larvicidal activity with LC_{50} values below 10 ppm against major vector mosquitoes including *Anopheles, Aedes* and *Culex.*

Nerium oleander is a common ornamental plant in India. It possesses many medicinal properties. Various research have been reported on the mosquito larvicidal activity of different parts of *N. oleander*. Kumar *et al*. (2012) have studied the mosquitocidal properties of *N. oleander* leaf extracts against *Culex tritaeniorhynchus* and *Culex gelidus.* In the present study, eight different solvent extracts of red colored flowers of *N. oleander* were tested for their larvicidal activity against three important vector mosquitoes namely *Ae. aegypti, An. stephensi* and *Cx. quinquefasciatus*.

Materials and Methods

Collection of *Nerium oleander* flowers and preparation of extracts: Fresh red color flowers of *N. oleander* were collected from Chennai, Tamil Nadu, India and plant identity was authenticated. The flowers were washed with dechlorinated tap water to remove dusts and shade-dried at room temperature. Dried flowers were powdered using an electric blender. One kilogram of the powder was sequentially extracted with three litres each of hexane, petroleum ether, dichloromethane, chloroform, ethyl acetate, acetone, methanol and distilled water using a Soxhlet apparatus (Vogel 1978). The different solvent extracts were filtered separately through a Buchner funnel with Whatman number 1 filter paper. The solvent in the extract was evaporated using a rotary vacuum evaporator to obtain the crude extract of the flower. The extracts were stored separately in glass vials at dry, dark and low temperature conditions.

Larvicidal bioassay: The larvae of *Ae. aegypti, An. stephensi* and *Cx. quinquefasciatus* were acquired from Entomology Research Institute, Loyola College, Chennai, India. The larvae were free of prior exposure to all types of insecticides and were maintained in an isolated insectary with a mean room temperature of 27±1°C; relative humidity of 70%-80% and photoperiod of 10±1 h. Larvicidal experiments were carried out following the guidelines of World Health Organization with some modifications (2005). Larvicidal activity of crude extracts was tested at five different concentrations viz., 62.5, 125, 250, 500 and 1000 mg/L. Larvicidal activity of isolated compound was tested at five different concentrations viz., 12.5, 25, 50, 100 and 200 mg/L. The required test concentrations and quantity of test solution was prepared by serially diluting one per cent stock solution of the crude extract or compound.

Ten early third instar larvae of each species were introduced into plastic cups (250ml) containing 100mL of test solution prepared with tap water. Untreated control (tap water only) was maintained separately. Larval mortality was observed exactly 24 h after treatment. Moribund larvae were scored to be dead when they showed no signs of movement when gently probed by a needle on their respiratory siphon. The per cent larval mortality was calculated and larval mortality was corrected when larvae in the control set showed mortality between 5-20% using Abbot's formula (Sureshkumar *et al.,* 2009). Five replicates for each concentration and control were maintained.

Corrected larval mortality = 1-*n* in T after treatment / *n* in C after treatment x 100

Where, *n* = number of larvae; T= treatment and C= control

Preliminary Phytochemical analysis of extracts: The methods proposed by Harborne (1973), were followed in preliminary phytochemical analysis.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis : GC-MS analysis was performed with a SHIMADZU-QP2010 with helium as a carrier gas with a linear velocity flow on a Resteck-624 ms column (30.0m x 250µm). Column flow rate was 1.491 ml/min. The oven was programmed to rise to 45°C (4 min) isotherm, and then to 175 leads to 240°C at a rate of 10°C/min and 25°C/min respectively. Injector and detector temperatures were 140°C. The identification of the constituents was performed by computer library search and retention indices.

Statistical analysis: Mean values and standard deviation for mean of larvicidal activity were calculated. One way Analysis of Variance (ANOVA) was used to find out the significance of the treatment and mean values were separated using Tukey's multiple range test using statistical software package (Panagoda, 2017). Probit analysis was carried out to determine median lethal concentration (LC_{50}) and LC_{90} of extracts. Chi-square analysis was performed and the differences were considered statistically significant at $P \leq 0.05$.

Results and Discussion

Larvicidal activity of red flower extracts of *N. oleander:* Hexane, ethyl acetate and acetone extract of red flower recorded larvicidal activity at all concentrations. Acetone and ethyl acetate extracts of red flower showed the higher larvicidal activities. Ethyl acetate extract of red flower recorded 12, 24, 36, 68 and 100% larvicidal activity in *Ae. aegypti* at 62.5, 125, 250, 500 and 1000 mg/L concentrations, respectively. Acetone extract of red flower recorded 10, 18, 34, 62 and 100% larvicidal activity in *Ae. aegypti* at 62.5, 125, 250, 500 and 1000 mg/L concentrations, respectively (Table 1). Among the different red flower extracts, acetone extract recorded the highest larvicidal activity against *An. stephensi*. The acetone extract showed 24, 40, 62, 86 and 100% larvicidal activity in *An. stephensi* at 62.5, 125, 250, 500 and 1000 mg/L concentrations, respectively (Table 2). In *Cx. quinquefasciatus* larvae, both acetone and ethyl acetate extracts recorded 100 percent larvicidal activity at 1000 mg/L concentration (Table 3). Dichloromethane extract did not cause larval mortality at all concentrations. Many plant species in Apocynaceae family were reported for their potential mosquito larvicidal effects. The ethanolic extract of *N. oleander* was tested for its larvicidal property against *Cx. pipiens*. Median lethal concentration (LC_{50}) and LC_{90} of ethanolic extract were calculated as 57.57 and 166.35 mg/mL, respectively.

Previously some investigators have studied the leaf extract of *N. oleander* against mosquito larvae.The leaf extract at 1:1 ratio of petroleum ether and ethyl acetate, showed good larvicidal activity against *Cx. quinquefasciatus* and *An. Stephensi*[31]. Lokesh *et al*.[32] tested the larvicidal effect of aqueous extract of *N. oleander* against *An. stephensi, Ae. aegypti* and *Cx. quinquefasciatus*. The treatment killed 91% *Ae. aegypti* and 43% *Cx. quinquefasciatus* at 3% concentration in 24 h. However, the effect of 3% concentration in their study is very low compared to the efficacy of acetone and ethyl acetate extracts of flowers of *N. oleander* in the present study.

In the present study, the acetone and ethyl acetate extracts gave higher larvicidal activities against all the three species of mosquitoes. Many earlier studies have reported that ethyl acetate and acetone extracts presented higher larval mortality against mosquitoes.

Preliminary phytochemical analysis: The results of preliminary phytochemical analysis are given in Table 4. Acetone and petroleum ether extracts had more phytochemical groups. Tannins, flavonoids, quinones, phenols and coumarins were present in all solvent extracts. Tannins, saponins, flavonoids, quinones, terpenes, phenols and coumarines are present in the red colour flowers of acetone and ethyl acetate extracts. These phytochemicals were already reported as toxins to mosquito larvae.

GC-MS analysis: There were 16 different compounds with different percent area were detected by GC-MS analysis at different retention times. The GC-MS analysis showed that the isolated compound was not pure; it was a mixture of three major compounds. 1-Naphthalenepropanol,.alpha.-ethyldecahydro-5-(hydroxymethyl)-.alpha.,5,8a-trimethyl-2-methylene (Torulosol, dihydro-) was the major component (Area = 16.639%) followed by 1-Hexyl-1-nitrocyclohexane (13.557%) and 4-(2,2-Dimethyl-6-methylenecyclohexyl) butanal (12.882%). The molecular formula of Torulosol, dihydro- is $C_{20}H_{36}O_2$. The molecular formula of 1-Hexyl-1-nitrocyclohexane is $C_{12}H_{23}O_2N$ and 4-(2,2-Dimethyl-6-methylenecyclohexyl) butanal has the molecular formula of $C_{13}H_{22}O$. Some more compounds namely 1-Methylene-2b-hydroxymethyl-3,3-dimethyl-4b-(3-methylbut-2-enyl)-cyclohexane (9.545%), 1-Tetradecyne (8.491%), 4-Pentadecyne, 15-chloro (6.011%) and 2-Methyl-6-methylene-octa-1,7-dien-3-ol (6.552%) were also found to be present at considerable amounts (Table 5; Fig.1). Till date, there is no insecticidal activity reported for these compounds. Derwich *et al*. (2010) isolated essential oil from *N. oleander* flowers. The oil presented good antibacterial activity and the GC-MS analysis showed that the oil had 34 compounds. Nériine (22.56%), digitoxigénine (11.25%), Amorphane (8.11%), 1.8-cineole (6.58%), α-pinene (5.54%), calarene (5.12%) and Limonene (5.01%) were reported as major compounds.

Conclusions

The present study clearly showed that red colour flowers of *N. oleander* possessed mosquito larvicidal molecules. The extracts of red flowers of *N. oleander* can be used as a botanical pesticide against vector mosquitoes. Further studies are necessary to isolate and characterize pure active compounds from the red flowers and to utilize it for the eradication of vector mosquitoes without polluting the environment.

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Table 1 Larvicidal activity (%) of *Nerium oleander* red flower extracts against *Aedes aegypti* (Mean±SD) (n=6)

Extract	Concentration (mg/L)				
	62.5	125	250	500	1000
Hexane	4.0 ±1.9^{b}	10.0 ±2.2^{c}	18.0 ±3.3^{b}	26.0 ±3.4^{c}	54.0 ±4.8^{b}
Petroleum Ether	0^{c}	0^{e}	0^{d}	6.0 ±1.9^{e}	24.0 ±3.4^{d}
Dichloromethane	0^{c}	0^{e}	0^{d}	0^{f}	0^{e}
Chloroform	0^{c}	0^{e}	0^{d}	6.0 ± 2.4^{e}	23.8 ±2.7^{d}
Ethyl acetate	10.0 ±2.8^{a}	18.0 ±3.8^{a}	34.0 ±2.6^{a}	62.0 ±4.6^{a}	100.0 ± 0^{a}
Acetone	12.0 ±2.6^{a}	24.0 ±2.9^{b}	36.0 ±4.3^{a}	68.0 ±4.8^{b}	100.0 ± 0^{a}
Methanol	0^{c}	8.0 ±2.8^{c}	14.0 ±2.6^{b}	22.0 ±3.2^{cd}	44.0 ±3.8^{c}
Aqueous	0^{c}	2.0 ±0.4^{d}	6.0 ±0.8^{c}	20.0 ±2.9^{d}	0^{e}

(Values carrying different alphabets in a column are significantly different at p=0.05 by Tukey's multiple range test)

Table 2 Larvicidal activity (%) of *Nerium oleander* red flower extracts against *Anopheles stephensi* (Mean±SD) (n=6)

Extract	Concentration (mg/L)				
	62.5	125	250	500	1000
Hexane	0^{d}	10.0 ±2.4^{c}	14.0 ±2.2^{de}	22.0 ±3.7^{de}	42.0 ±4.8^{d}
Petroleum Ether	6.0 ±0.8^{c}	10.0 ±1.8^{c}	18.0 ±2.8^{cd}	34.0 ±3.8^{b}	46.0 ±3.8^{d}
Dichloromethane	0^{d}	0^{e}	0^{f}	0^{f}	0^{e}
Chloroform	0^{d}	0^{e}	8.0 ±1.7^{e}	18.0 ±2.2^{e}	44.0 ±2.9^{d}
Ethyl acetate	10.0 ±2.0^{b}	14.0 ±2.9^{b}	28.0 ±3.5^{b}	34.0 ±3.8^{b}	68.0 ±5.4^{b}
Acetone	24.0 ±3.6^{a}	40.0 ±4.7^{a}	62.0 ±5.3^{a}	86.0 ±6.3^{a}	100.0 ± 0^{a}
Methanol	4.0 ±0.8^{c}	12.0 ±2.8^{bc}	22.0 ±2.7^{c}	28.0 ±3.0^{c}	54.0 ±5.1^{c}
Aqueous	0^{d}	4.0 ±0.5^{d}	12.0 ±2.2^{e}	26.0 ±2.8^{cd}	44.0 ±3.4^{d}

(Values carrying different alphabets in a column are significantly different at p=0.05 by Tukey's multiple range test)

Table 3 Larvicidal activity (%) of *Nerium oleander* red flower extracts against *Culex quinquefasciatus* (Mean±SD) (n=6)

Extract	Concentration (mg/L)				
	62.5	125	250	500	1000
Hexane	12.0 ±2.6^{cd}	10.0 ±2.3^{d}	22.0 ±3.9^{c}	36.0 ±4.6^{c}	64.0 ±4.5^{c}
Petroleum Ether	10.0 ±2.2^{d}	12.0 ±3.8^{cd}	22.0 ±2.6^{c}	26.0 ±4.0^{d}	48.0 ±3.3^{e}
Dichloromethane	0^{f}	0^{f}	0^{e}	0^{e}	0 ^{g}
Chloroform	0^{f}	4.0 ±0.6^{e}	16.0 ±2.7^{d}	38.0 ±3.9^{c}	76.0 ±4.6^{b}
Ethyl acetate	22.0 ±3.4^{a}	38.0 ±4.2^{a}	54.0 ±5.7^{a}	80.0 ±5.3^{a}	100.0 ± 0^{a}
Acetone	14.0 ±2.8^{b}	26.0 ±3.6^{b}	48.0 ±4.8^{b}	74.0 ±5.7^{b}	100.0 ± 0^{a}
Methanol	10.0 ±1.6^{d}	14.0 ±2.3^{c}	22.0 ±2.9^{c}	38.0 ±3.8^{c}	42.0 ±3.8^{f}
Aqueous	6.0 ±0.5^{e}	10.0 ±2.1^{d}	20.0 ±2.1^{c}	30.0 ±2.7^{d}	56.0 ±4.2^{d}

(Values carrying different alphabets in a column are significantly different at p=0.05 by Tukey's multiple range test)

Table 4 Preliminary Phytochemical analysis of pink, white and red flower extracts of *Nerium oleander* Tannins, flavonoids, quinones, phenols and coumarins

No	Phytochemical test	Solvents							
		A	DCM	PE	EA	CH	H	ME	AQ
1	Carbohydrates	+	+	+	+	+	+	-	+
2	Tannins	+	+	+	+	+	+	+	+
3	Saponins	+	+	+	+	+	+	+	-
4	Flavonoids	+	+	+	+	+	+	+	+
5	Alkaloid	+	-	+	-	-	+	-	-
6	Quinones	+	+	+	+	+	+	+	+
7	Glycosides	-	-	-	-	-	-	-	+
8	Cardiac glycosides	+	+	+	+	+	-	-	+
9	Terpenoids	+	+	+	+	-	+	+	+
10	Phenols	+	+	+	+	+	+	+	+
11	Coumarins	+	+	+	+	+	+	+	+
12	Steroids &Phytosteroids	+	+	+	-	+	+	-	+
13	Phlobatannins	-	-	-	+	-	-	+	-
14	Anthraquinones test	-	-	-	-	-	-	-	-

(+ indicates presence; - indicates absence) A: Acetone; DCM: Dichloromethane; PE: Petroleum Ether; EA: Ethyl Acetate; CH: Chloroform; H: Hexane; ME: Methanol; AQ: Aqueous

Table 5 GC-MS analysis of impure compound isolated by 1:1 ratio of acetone: methanol

Sl. No.	Matching Compound	Retention Time	Area (%)	Molecular Formula
1	1,5,9,11-TRIDECATETRAENE, 12-METHYL-, (E,E)-	15.188	2.126	$C_{14}H_{22}$
2	DIHYDRO-CIS-.ALPHA.-COPAENE-8-OL	15.213	2.633	$C_{15}H_{26}O$
3	1-METHYLENE-2B-HYDROXYMETHYL-3,3-DIMETHYL-4B-(3-METHYLBUT-2-ENYL)-CYCLOHEXANE	21.891	2.258	$C_{15}H_{26}O$
4	1-METHYLENE-2B-HYDROXYMETHYL-3,3-DIMETHYL-4B-(3-METHYLBUT-2-ENYL)-CYCLOHEXANE	24.557	9.545	$C_{15}H_{26}O$
5	1-HEXYL-1-NITROCYCLOHEXANE	27.038	13.557	$C_{12}H_{23}O_2N$
6	4-PENTADECYNE, 15-CHLORO	27.183	6.011	$C_{15}H_{27}Cl$
7	1-NAPHTHALENEPROPANOL, .ALPHA.-ETHYLDECAHYDRO-5-(HYDROXYMETHYL)-.ALPHA.,5,8A-TRIMETHYL-2-METHYLENE	27.413	16.639	$C_{20}H_{36}O_2$
8	1-TETRADECYNE	27.553	8.491	$C_{14}H_{26}$
9	4-PENTADECYNE, 15-CHLORO	27.693	2.763	$C_{15}H_{27}Cl$
10	4-(2,2-DIMETHYL-6-METHYLENECYCLOHEXYL)BUTANAL	27.829	12.882	$C_{13}H_{22}O$
11	1-HEXYL-1-NITROCYCLOHEXANE	28.039	5.758	$C_{12}H_{23}O_2N$
12	CHOLESTA-8,24-DIEN-3-OL, 4-METHYL-, (3.BETA.,4.ALPHA.)-	28.119	2.510	$C_{28}H_{46}O$
13	CHOLESTA-8,24-DIEN-3-OL, 4-METHYL-, (3.BETA.,4.ALPHA.)-	28.209	2.574	$C_{28}H_{46}O$
14	2-METHYL-6-METHYLENE-OCTA-1,7-DIEN-3-OL	28.464	6.552	$C_{10}H_{16}O$
15	1-OCTADECYNE	28.509	3.359	C18H34
16	9-OCTADECENOIC ACID (Z)-, PHENYLMETHYL ESTER	29.784	2.342	C25H40O2

from red colour flower acetone extract

Fig. 1 GC-MS analysis of impure compound isolated from acetone extract of red flowers of *Nerium oleander*

Qualitative Report

File: C:\TURBOMASS\2019.PRO\Data\3-(20ES-0125)-.raw
Acquired: 20-Feb-20 06:57:08 PM
Printed: 24-Feb-20 12:39 PM
Description:
GC/MS Method: GC: METHOD-1.mth MS: METHOD-1.EXP
Page 1 of 1
Sample ID: 3-(20ES-0125)
Vial Number: 29