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Nerium oleander Leaf Extract Causes Midgut Damage and Interferes with the Survival of *Culex pipiens* L. Larvae

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ABSTRACT

Background: Diseases vectored by mosquitoes are one of the major causes of diseases and death worldwide. Insecticide resistance of *Cx. pipiens* increased the suffering of the people. Plants are a promising source of secondary metabolites that could provide valuable insights in the search for effective insecticidal agents and help address the issue of insecticidal resistance.

Methods: This study investigated the larvicidal potential of *N. oleander* methanol aqueous extract against *Cx. pipiens*.

Result: Post 24 h, 48 h and 72 h of exposure, the LC_{50} value was 4.94 mg/mL and the LC_{90} value was 8.01 mg/mL. After 72 h of exposure, the values were 2.93 mg/mL and 4.79 mg/mL, respectively. Larvae treated with *N. oleander* leaf extract displayed degraded microvilli, degenerating peritrophic membrane and degenerating nuclei with blebbing compared to the control. The GC-MS analysis revealed the presence of eleven phytoconstituents in the leaf extract. 1-Methyl-1H-Tetrazol-5-yl) thioacetic acid (80.67%), 2-Furancarboxaldehyde, 5^{-1} (hydroxymethyl)- (8.10%) and 9,12-Octadecadienoic acid (Z, Z)- (4.33%) were the main secondary metabolites found in the leaf extract.

Key words: Antioxidant activity, Biological control, *Cx. Pipiens*, Larvicide, Midgut cells, *N. oleander*.

INTRODUCTION

One of the leading causes of illness and death globally is diseases spread by mosquitoes (Akram *et al*., 2011). Dengue fever, Malaria, Japanese encephalitis and Filariasis are diseases vectored by the genera *Anopheles*, *Culex* and *Aedes* of mosquitoes (Borah *et al*., 2010). *Cx. pipiens* is of great concern in many countries. *Cx. pipiens* transmit several viruses, such as West Nile virus, Rift Valley fever virus, Japanese encephalitis virus, Usutu Viruses and Sindbis virus (ECDC, 2022) (Huijben *et al*., 2007; Martinet *et al*., 2019; Otranto *et al*., 2013).

One of the used procedures for mosquito control is synthetic insecticides. However, these insecticides adversely affect the environment by contaminating the soil, water and air (Shivakumar *et al*., 2013). To these insecticides, mosquitoes also developed resistance that amplified mosquito management. *Cx. pipiens* has become resistant to many synthetic insecticides such as temephos, malathion, fenthion and fenitrothion (El-Akhal *et al*., 2015). Finding alternatives to pesticides in the form of natural plant biocides is urgently needed.

Plants are a promising source of natural therapeutic secondary metabolites. Apocynaceae family is one of the largest plant groups, with around 400 genera and 4,555 species. Members of this family are rich in secondary metabolites that possess several biological activities and are traditionally used for treating several ailments (Bhadane *et al*., 2018).

Nerium oleander L. (Apocynaceae) is a traditional medicinal plant that belongs to the Apocynaceae family. In traditional medicine, the roots are used to treat headaches and colds (El-Seedi *et al*., 2013). Decoctions of the leaves are used for skin diseases and against paralysis and pain in extremities (Kuete, 2014). Besides these, the bark, stem, leaves, flowers and roots of *N. oleander* possess antibacterial (Hussain and Gorsi, 2004), anticancer (Rashan *et al*., 2011), antidiabetic (Dey *et al*., 2015) and larvicidal (El-Akhal *et al*., 2015) activities.

The present study aimed to assess the larvicidal potential of *N. oleander* extract against the 4th instars of *Cx. pipiens*. The study further evaluated the impact of the active extract on the midgut region of the *Cx. pipiens*. Chemical characterisation of the extract causing larval toxicity was carried out using GC-MS analysis.

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MATERIALS AND METHODS

Plant collection and extraction

The *Nerium oleander* L. used in our work was collected in February near Oud Saf-Saf in Salah Bouchaour Skikda, Algeria. The leaves were collected, air dried in the dark at 28°C and powdered using an electric grinder. The powdered material (100 grams) was macerated for 24 h in 70% methanol and filtered using Wattman filter paper. The filtrates were evaporated using a rotary evaporator under a vacuum at 65°C and the yield was calculated.

Quantitative study

The total phenolic and flavonoid contents were evaluated following the Folin-Ciocalteu method described by (Wong *et al*., 2006) and the aluminium trichloride (AlCl3) method cited by (Djeridane *et al*., 2006) respectively. The results are expressed in micrograms of quercetin equivalent per milligram of extract (µg EQ/mg E) for flavonoids. For phenols contents, the results are expressed as gallic acid equivalents per milligram of extract (µgEAG/mg E).

Antioxidant activity

Antioxidant activity was assessed spectrophotometrically using a plate reader by monitoring the disappearance of 2, 2′-Diphenyl-1-picrylhydrazyl (DPPH) at 517 nm as previously described (Sánchez-Moreno, 2002). The reaction mixtures consisted of 400 µL of extract and 1600 µL of 0.04% $DPPH^{\cdot}$ dissolved in 70% methanol. The samples were incubated for 30 min at 25°C. The samples were measured in triplicate. Methanol (70%) was used as a control and the experiments were carried out in triplicate. The DPPH activity was calculated as a percentage of inhibition using Eq.

The anti-free radical activity is given by the following formula (Yen and Duh, 1994):

$$\text{Antioxidant activity (\%)} = \frac{\text{Abs negative control - Abs sample}}{\text{Abs negative control}} \times 100$$

IC_{50} determination

IC_{50} are calculated graphically by the formula of regression of inhibition percentages against different concentrations of the tested extract.

Collection and identification of larvae

The larvae of *Cx. pipiens* was collected from waste water sites in the Frères Souissi in Azzaba Skikda. The samples were then transported to the laboratory for identification. Morphological identification of the larval species was identified following (Brunhes *et al*., 2000).

Larval susceptibility testing

The tests were carried out using the standard protocol of WHO developed in 2005 (WHO, 2005). Solutions at different concentrations (0.5 mg/ml; 1 mg/ml; 2 mg/ml; 4 mg/ml and 8 mg/ml) were prepared for each extract. Each concentration was placed in a plastic vessel containing water (50 mL) to which 10 larvae of stages 3rd and 4th instars were added. Control was also maintained following the same procedure without adding the extract. Five independent duplicates for the treated and control groups were carried out. We counted the number of live and dead larvae 24, 48 and 72 hours post-exposure. The percentage of mortality compared to the control was used to express the susceptibility testing results. If the mortality rate in the control group is more than 5%, Abbott's formula must be used to adjust the mortality rate in the extract-exposed larvae (Abbott, 1925).

Histology analysis

The process was carried out according to Abutaha *et al*., 2022. In brief, post-exposure to plant extract, the fixed (2.5% glutaraldehyde solution) midguts of treated and control groups were washed with distilled water, dehydrated in ascending ethanol series (70, 80, 90, 95 and 100%) and embedded in resin, sectioned (4 µm thin) and stained with haematoxylin and eosin. The stained midguts were examined under an Olympus BX60 microscope (Olympus, USA) and photographed with a digital camera.

Gas chromatography-mass spectrometry (GC-MS)

Chemical contents of *N. oleander* leaf extract were performed using a GC-MS (Agilent Technologies, USA). HP-88 capillary (100 m, ID: 250 µm, film thickness: 0.20 µm) standard column was employed for the analysis. Two microliters were the injected volume. Initially, the column temperature started at 50°C and was increased to 250°C at 5°C/min. The carrier gas used was Helium. The flow rate was 1.0 ml/min. The phytochemicals have been identified by comparing the mass spectrum patterns of the phytochemicals to the reference mass spectra found in the National Institute of Standards and Technology (NIST) Mass Spectra Database.

Statistical analysis

All the assays were performed in triplicate and the means and standard deviation were calculated. Larval mortality was assessed using one-way ANOVA followed by Tukey's test. SPSS (SPSS version 15, USA) was used to perform the analyses. A p-value of less than 0.05 indicated statistical significance.

RESULTS AND DISCUSSION

The application of natural products remains the method that has many advantages for the health of the living being and its environment compared to the products of chemical synthesis that globally contaminate the biosphere (Ben ayed, 2007). Integrating pest management relies on a combination of practical and affordable technologies to decrease mosquito populations while having the fewest negative impacts on people and the environment. Botanical extracts are a good candidate for integrated pest management strategies because of their significant efficacy, availability and low cost (Al-Mehmadi and Al-Khalaf, 2010).

Plants are sources of bioactive compounds and can be used as alternatives to conventional insecticides in mosquito control programs. According to (Jacobson, 1989), more than 2,000 plant species with insecticidal properties have been identified. Our results show that the larvae have different percentages of mortality responses based on the concentrations and the duration of exposure to the extract. *Aedes aegypti* and *Cx. Pipiens* are larvicidal targets for *N. oleander's* bark, stem, leaves, flowers and roots (Aouinty *et al*., 2006; El-Akhal *et al*., 2015; El-Sayed and El-Bassiony, 2016). The extract of *N. oleander* also exhibited ovicidal properties (Kumar *et al*., 2017) and adulticidal activity against *Anopheles stephensi* (Roni *et al*., 2013).

Phytochemical studies on *N. oleander* have allowed the isolation of a large number of secondary metabolites such as cardenolides, triterpenes, pregnanes, flavonoids, coumarins and steroidal derivatives (Hanson, 1985). The observed larvicidal effect may be attributed to the presence of polyphenols and flavonoids in the leaf extract of *N. oleander*, as reported by Aouinty *et al*. (2006). In the present study the polyphenol content is calculated from the calibration curve of gallic acid (Y= 0.048x + 0.164, R^2= 0.960) and quercetin (Y= 0.0345x + 0.232, R^2= 0.9095). The total content of phenols and flavonoids were 147.32±5.22 µg EAG/mg and 8.31±0.39 µg EQ/mg respectvily.

The findings indicated that the mortality rate of *Cx. pipiens* larvae was significantly influenced by both the concentration of *N. oleander* extract and the duration of treatment. The half-lethal concentrations of this biocide were LC_{50}: 2.93 mg/mL, LC_{90}: 4.79 mg/mL and LC_{95}: 5.03 mg/mL generated after 72h of exposure to the biocide. The analysis of variance (ANOVA) shows a significant difference ($p<0.001$) between the various *N. oleander* concentrations used after 24, 48 and 72 hours. The results show that the crude extract of *N. oleander* has good larvicidal activity. The multiple comparisons of mortality after 24, 48 and 72 h by the Tukey test show a difference between the concentrations. After 24 hours of treatment, the results showed correlated increase in the mortality with the increase of the dose, *i.e*. a value of 6.22% for the 0.5 mg/mL dose against 67.33% for the 8 mg/mL dose (Table 1).

The midgut cells were severely affected in the third instar of *Cx. pipiens* post-treatment with *N. oleander* extract compared to the control (Fig 1). The control midgut cells appeared normal, with an intact epithelial layer (EL), normal peritrophic matrix (Pm) and well-distinguished gut lumen (GL). Midgut cells treated with *N. oleander* extract displayed most cellular morphological changes in the gut lumen, such as degraded microvilli (DMV), degenerating peritrophic membrane (DPM), degenerating nuclei (DN) with blebbing (BC) as compared to the control (Fig 1). This damage to the digestive cells in the midgut of *Cx. pipiens* larvae, caused the impairing digestion and absorption, endangering survival and interfering with larval mosquito development. The regenerative cells are essential in renewing the intestinal epithelium, a necessary step in metamorphosis (Nishiura *et al*., 2003). The disruption of regenerative cell division in treated larvae hindered the midgut's metamorphosis and organ remodelling during pupation. Since enteroendocrine cells have been shown to release monoamines, peptides and other compounds that regulate the production of digestive enzymes, injury to these cells disturbs homeostasis (Brown *et al*., 1985). Botanical-derived insecticides have shown harmful effects on the mosquito's midgut, similar to the *N.* oleander leaf extract. For example, cell hypertrophy, vacuolisation, cell lysis, as well as microvilli damage and death were caused by the Magonia pubescens bark (Nishiura *et al*., 2003), *Derris urucu* root (Gusmão *et al*., 2002), Annona squamosa (Costa *et al*., 2014) and Melia azedarach (Al-Mehmadi and Al-Khalaf, 2010) extracts in the midgut of mosquito larvae. Similar to this, isolated chemicals like pellitorine led to epithelial cell degeneration, injury to the gastric caeca and osmoregulation failure (Perumalsamy *et al*., 2013).

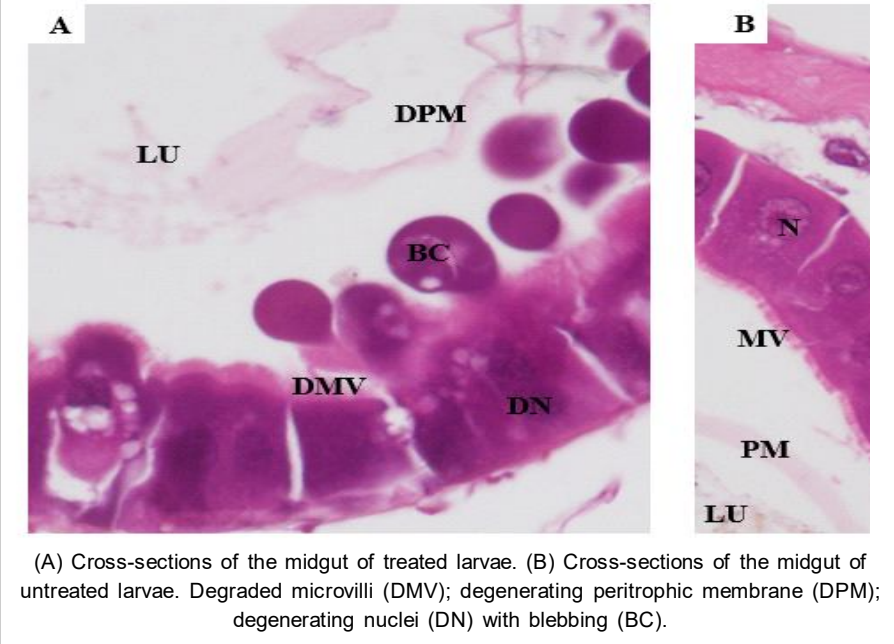
Eleven potential phytochemicals were found in *N. oleander* leaf extract using GC-MS profiling (Table 2). The major compound identified with a high concentration in leaf extract was (1-Methyl-1h-tetrazol-5-yl)thio]acetic acid (80.67%), 2-Furancarboxaldehyde, 5-(hydroxymethyl)- (8.10%) and 9, 12-Octadecadienoic acid (Z, Z)- (4.33%) (Fig 2). Chemical compounds from *N. oleander* leaf extract were mainly fatty acids, fatty acid methyl esters, polyphenols and

Table 1: Variations in mortality of *Culex pipiens* larvae treated with *Nerium oleander* as a function of concentration and time.

Concentration	Time			df	F
	24	48	72		
0.5	6.22±2.55aC	10.94±0.46aD	11.39±0.28aE	2	3.625
1	10.94±0.46cBC.	21.88±0.96bD	31.99±0.82aD	2	192.274
2	18.89±2.28cB	37.78±3.28bC	53.33±1.36aC	2	50.231
4	19.44±2.53cB	54.45±3.24bB	65.41±1.35aB	2	92.236
8	67.33±3.36bA	89.5±4.50aA	89.56±5.48aA	2	7.996
LC_{50}	4.94	3.08	2.93		
LC_{90}	8.01	4.84	4.79		
LC_{95}	8.39	5.06	5.03		
F	101.804	111.457	131.437		
df	4	4	4		

flavonoids. All these compounds were included in various patented pesticide formulations. (Reid *et al*., 2015 and Anderson *et al*., 2003) designed formulation containing various adjuvants within the pesticide composition. Oleic acid, tetradecanoic acid, palmitic acid, octadecenoic acid (Z)-methyl ester, hexadecanoic acid methyl ester, 9- (fatty acid methyl ester) and catechin (flavanols) were also detected in their formulation.

The bioactive molecule *n*-Hexadecanoic acid has been reported to have multiple biological properties in *Vitex negundo* (Kumar *et al*., 2019); (Enerijiofi *et al*., 2021). The furfural, a bioactive compound reported earlier in several species like Boswellia dalzielii, with diversified medicinal uses (Jung *et al*., 2007); (Kohoude *et al*., 2017). The compound 9,12-Octadecadienoic acid (Z, Z) was known to possess anticarcinogenic, antioxidant, anti-inflammatory (Arora and Meena, 2017) and larvicidal properties (Rahuman *et al*., 2008). The other bioactive compounds identified were 2-Furancarboxaldehyde, 5-(hydroxymethyl) and butanoic acid, 3-hydroxy-, methyl ester reported earlier in *Callistemon*



(A) Cross-sections of the midgut of treated larvae. (B) Cross-sections of the midgut of untreated larvae. Degraded microvilli (DMV); degenerating peritrophic membrane (DPM); degenerating nuclei (DN) with blebbing (BC).

Fig 1: Photomicrograph of the midguts of *Culex pipiens* 4th instars treated with extract of *Nerium oleander* at 24 h post-treatment.

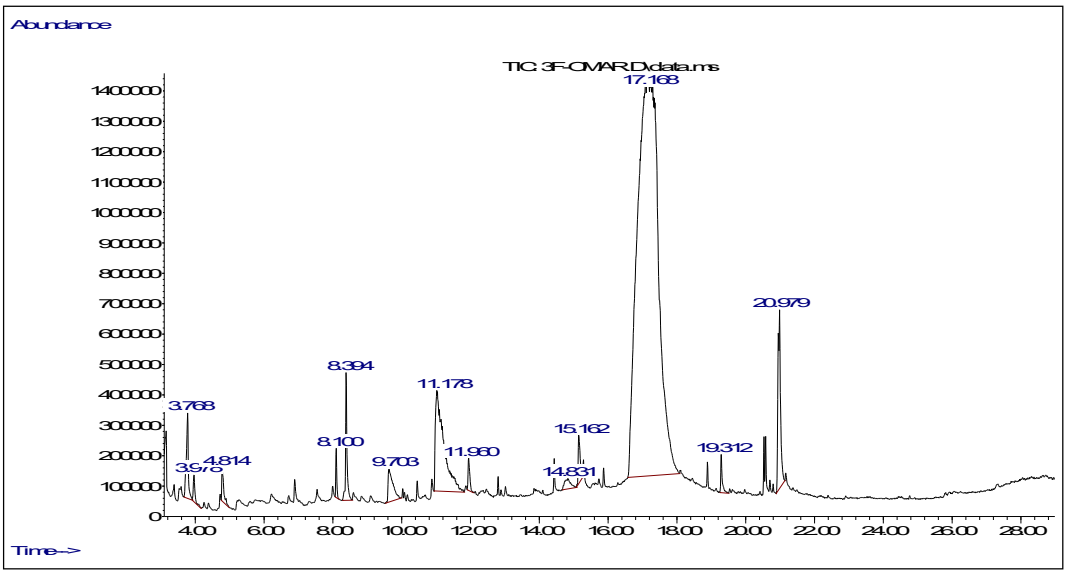


Fig 2: GC-MS of methanol extract of *Nerium oleander*.

Table 2: Components of methanol extract of *Nerium oleander* as determined by GC-MS.

Chemical formula	Formula	Molecular weight	Rt (min)	% of total
Furfural	$C_5H_4O_2$	96.021	4.817	0.615065
Butanoic acid, 3-hydroxy-, methyl ester	$C_5H_{10}O_3$	118.063	8.098	0.454458
Thiophene, 2-butyltetrahydro-	$C_8H_{16}S$	144.097	8.391	1.723451
1,3-Dioxolane, 2,4,5-trimethyl-	$C_6H_{12}O_2$	116.084	9.706	1.521338
2-Furancarboxaldehyde, 5 (hydroxymethyl)-	$C_6H_6O_3$	126.032	11.181	8.107072
4-Hydroxy-3-methylacetophenone	$C_9H_{10}O_2$	150.068	11.956	0.523222
Dodecanoic acid	$C_{12}H_{24}O_2$	200.178	14.83	0.591202
2,3,5,6-Tetrafluoroanisole	$C_7H_4F_4O$	180.02	15.161	0.903206
Acetic acid, 2-[(1-methyl-1H-1,2,3,4-tetrazol-5-yl)thio]-	$C_4H_6N_4O_2S$	174.021	17.166	80.67582
n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.24	19.312	0.546813
9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	280.24	20.976	4.338356

lanceolatus and *Rheum ribes*, known to possess antioxidant, anticancer and anti-inflammatory properties (Achakzai *et al*., 2019; Ahmad *et al*., 2018).

The results of the antioxidant characteristics of the *N. oleander* crude extract demonstrate that this plant has a promising antioxidant activity on DPPH radicals with an LC_{50} of 29.05±0.68 µg/mL; however, this antioxidant capacity is still less than that of vitamin C (IC_{50}= 5.06 0.15 µg/ml). The antioxidant activity of the extract could be related to the composition, mainly phenolic and flavonoid compounds.

CONCLUSION

The larvicidal activity of the hydroethanolic extract of *N. oleander* against *Cx. pipiens* had LC_{50} and LC_{90} values of 4.94 mg/ml and 8,01 mg/mL, respectively. These consequences are linked to the gut's structural damage and disorganisation. The larvicidal activity of the extract could be attributed to the Acetic acid, 2-[(1-methyl-1H-1,2,3,4-tetrazol-5-yl)thio], the major compounds detected. Additional research is required to determine the extract's active component/s.

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Authors contribution

S. Benzazia and F. Boulkenafet designed the study, F.A. Al-Mekhlaf and N. Abutaha conducted data analyses and wote the manuscript. F.A. Al-Mekhlaf performed light microscopy experiments. MT and L. Mellahi helped in writing the manuscript and conducted data analyses.

Ethical approval

The conducted research is not related to either human or animal use.

Data availability statement

All the data is available within the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

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