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NOTES

Larvicidal activity and chemical compositions of *Juniperus phoenicea* L. leave extract against *Culex pipiens* L.

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Mosquitoes are of great concern in many countries. *Culex pipiens* L. (Diptera:Culicidae) is the vector of the West Nile virus and Usutu Viruses. Intense use of insecticidal agents has resulted in increased mosquitocidal resistance, and thereby search for new effective mosquitocidal agents. In this context, we have explored the larvicidal potential of Phoenician juniper [*Juniperus phoenicea* L. (Fam. Cupressaceae)] against *Cx. pipiens* and studied the chemical composition of the leaf extract. Fresh leaves of *J. phoenicea* were macerated in 70% methanol. The LC_{50} at 24, 48 and 72 h post-treatment with methanol extract were 7.14, 3.97 and 3.17 µg/mL, respectively, against *Cx. pipiens larvae*. In treated larvae, the cells lost integrity, nucleus and cell membrane damage, basal lamina detached, and lost nuclear and cytoplasmic materials. From GC–MS analysis, 19 compounds were detected. Some of the phytochemicals detected in *J. phoenicea* extract were 9,12-octadecadienoic acid (Z,Z)-(50.2.%), podocarpa-8,11,13-trien-3-one, 14-isopropyl-13-methoxy-(13.2%), -Amino-7,10-dimethyldibenzo [b,f][1,4]oxazepin-11(10H)-one (8.4%), and n-hexadecanoic acid (5.6%). *J. phoenicea* offered promising larvicidal activity against *Cx. pipiens*.

Keywords: Cupressaceae, Mosquitocidal resistance, 9,12-Octadecadienoic, Phoenician juniper, Saint Louis encephalitis virus, West Nile virus, Usutu virus

Culex pipiens (Linnaeus, 1758), the principal vector of the West Nile virus and Usutu viruses (USUV) are major health concerns in several nations. WNV is commonly found in Africa, Europe, West Asia, Australia, Canada, and Venezuela. The USA, Russia, Greece, Romania and the Middle East faced the most severe outbreaks. (WHO, 2023). Similarly, USUV was detected in Switzerland, the Czech Republic, Serbia, Italy, Hungary, Greece, France, Spain, Croatia, Belgium, Austria, Germany, Tunisia, Burkina Faso, Uganda, Nigeria, Ivory Coast and Senegal[1]. *Cx. pipiens* also transmit several other arboviruses, such as the Sindbis virus, Japanese encephalitis virus, and Rift Valley fever virus, and act as vectors for filarial worms and plasmodia that cause avian malaria[2-4]. Vector control using insecticidal agents is one of the standard methods to control disease transmission. Acetofenate, propoxur and dichlorvos are commonly used insecticidal agents. The intense use of these insecticides by household communities, private companies, and the government has resulted in resistance in *Culex populations*[5]. Resistance to these insecticidal agents has been recorded in many countries[6-8].

The mosquitocidal resistance is increasing, and the success rate of new insecticidal agents in biocontrol programs is low[9,10]. Therefore, this necessitates searching for new mosquitocidal agents. Botanical mosquitocides have a long history and are a safer alternative to controlling various insect pests. Secondary metabolites extracted from plants usually have low toxicity, degrade rapidly (within 3-4 days), such as Neem insecticides[11], and act faster than biological control[12]. *Juniperus phoenicea* L. (Cupressaceae) is a small tree with a wide geographic distribution and can be found from Portugal to Saudi Arabia[13]. This wide geographical range allows genetic variability[14]. Decoctions from the leaves are used to treat diuretic diarrhea, rheumatism, bronco-pulmonary illness, and diabetes[15]. A hypoglycemic agent is made from the berries and leaves of this plant[16]. Here, we evaluated the larvacidal efficacy and chemical constituents of *J. phoenicea* leaf extract against *Cx. pipiens* larvae.

Materials and Methods

Collection and extraction

Juniperus phoenicea fresh leaves were collected in February-March from Djerma Batna city, Algeria, and identified by Sakhraoui N, Professor of Botany, Natural and Life Sciences Department of University 20th August 1955 Skikda, Algeria. The voucher sample (ALJP22026) was deposited in the same department. The leaves were washed using distilled

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water, dried at 25ºC in a well-ventilated area, and ground using a commercial grinder. A hundred grams of plant powder was macerated in 70% methanol (300 mL) and filtered, and the filtrate was rotary evaporated (Heidolph, Germany), and the yield was calculated.

Test mosquitoes

Larvae of *Cx. pipiens* were collected from Natural and Life Sciences Department, University of 20th August 1955 Skikda, Algeria. They were housed in 35, 20 and 15 cm plastic trays and fed fish flakes (Aquafin max feeding) at 30°C. With a sieve, the pupae were manually separated into a 500 mL beaker of tap water. Adult mosquitoes were fed glucose using tissue paper soaked in a 10% glucose solution and occasionally blood-fed using trapped mice. The laid eggs were used for control and bioassay tests.

Larvicidal bioassay

The larvicidal bioassay was conducted based on WHO guidelines (WHO, 2005). Different concentrations of *J. phoenicea* extract (0.5, 1, 2, 4 and 8 mg/mL) were prepared. The prepared concentrations were used to investigate the larvicidal potential against *Cx. pipiens* (3rd instars). Fifty millilitres of dechlorinated tap water were placed in plastic boxes, to which 10 larvae were added. Control (0.01% methanol) and treated larvae were maintained under similar laboratory conditions for colony maintenance without providing fish flake. Five replicates of each concentration were used in the experiment. The dead larvae were counted after 24, 48 and 72 h of treatment. A larva was considered dead if it did not move when touched with a fine wooden rod. The observed mortality of the larvae is calculated using the formula:

$$\text{Observed mortality \%} = \frac{\text{Total number of dead mosquitoes}}{\text{Total sample size}} \times 100$$

Observed mortalities were corrected by the Abbott (1925) formula:

$$\text{Corrected mortality \%} = \frac{(\text{\% observed mortality} - \text{\% control mortality})}{(100 - \text{\% control mortality}} \times 100$$

which allows natural mortality to be eliminated.

Histologic analysis

Cx. pipiens third-instar larvae were subjected to treatment with the 24 h LC_{50} value (7.14 µg/mL) of *J. phoenicea* extract. At 24 h post-treatment, larvae treated and untreated with the extract were immediately fixed in formaldehyde (10%). Afterwards, sections with a thickness of 5 µm were cut from the embedded larval preparations. The sections were deparaffinized and rehydrated with ethanol in phosphate-buffered saline (PBS) solutions. The sections were stained with hematoxylin and eosin using a Leica Biosystems autostainer (Wetzlar, Germany). A microscope was used to view and record images (Olympus BX53, Japan).

GC–MS analysis

GC–MS analysis was carried out using a Perkin Elmer GC Calarus 600T combined with a Clarus 600T single quadrupole mass spectrometer attached to an autosampler and gas chromatography interfaced to a mass spectrometer (GC–MS) instrument. The following parameters were used: helium (99.999%) was used as the carrier gas at a constant flow of 1.491 mL/min and injection volume of 1.0 mL, injector temperature was 140°C, and ion source temperature was 200°C. The capillary column was 624 ms (30 m 0.32 mm 1.8 m) operating in an electronic mode at 70 eV. The oven was set to a temperature of 45°C. At 70 eV, mass spectra were recorded.

Statistical analyses

Each assay's means and standard deviation were computed after being carried out in triplicate. Tukey's test was used after one-way ANOVA to determine the larval mortality rate. The analyses were done using SPSS (SPSS version 15, USA). Statistical significance was indicated by a *P*-value less than 0.05.

Result and Discussion

After the extraction process, 100 g of *J. phoenicea* extract yielded 12 g of extract. The results show a remarkable mortality rate based on the concentration and duration of larvae treated with *J. phoenicea* extract. The death rate increased when the dose was increased; for the 0.5 dose, mortality was not noted until 72 h after treatment. After 24 h, mortality reached 34.33% for the 8 mg/mL dose against 21.33% for the 4 mg/mL doses. After 48 h of treatment, the 8 mg/mL dose gave 68.66% mortality. Finally, 100% mortality was reported with the 8 mg/mL dose after 72 h of exposure. The analysis of variance (ANOVA) displayed a highly significant difference ($P \leq 0.001$) between the different concentrations of *J. phoenicea* post-24, 48 and 72 h (Table 1) of exposure. Different concentrations had different larvicidal effects, as evidenced by the numerous comparisons of mortality at 24, 48 and 72 h.

Table 1 — Variations in mortality of *Culex pipiens* 3rd instar treated with *Juniperus phoenicea* as a function of concentration and time

Conc. (mg/mL)	Time (h)			df	F
	24	48	72		
0.5	0.00±0.00aD	0.00±0.00aE	2.00±2.00aE	2	1.00
1	0.00±0.00bD	8.00±2.00aD	12.67±1.85aD	2	16.62
2	8.00±4.47cC	31.33±0.82bC	43.55±0.89aC	2	179.47
4	21.33±1.22cB	53.33±1.36bB	74.66±1.90aB	2	374.86
8	34.33±3.20cA	68.66±2.86bA	95.55±2.72aA	2	160.22
LC_{50}	7.14	3.97	3.17		
LC_{90}	11.59	6.16	4.78		
LC_{95}	12.14	6.43	4.98		
F	174.96	289.40	411.27		
Df	4	4	4		

[Identical capital letters in columns and identical small letters in rows denote differences that are insignificant at $P \leq 0.001$]

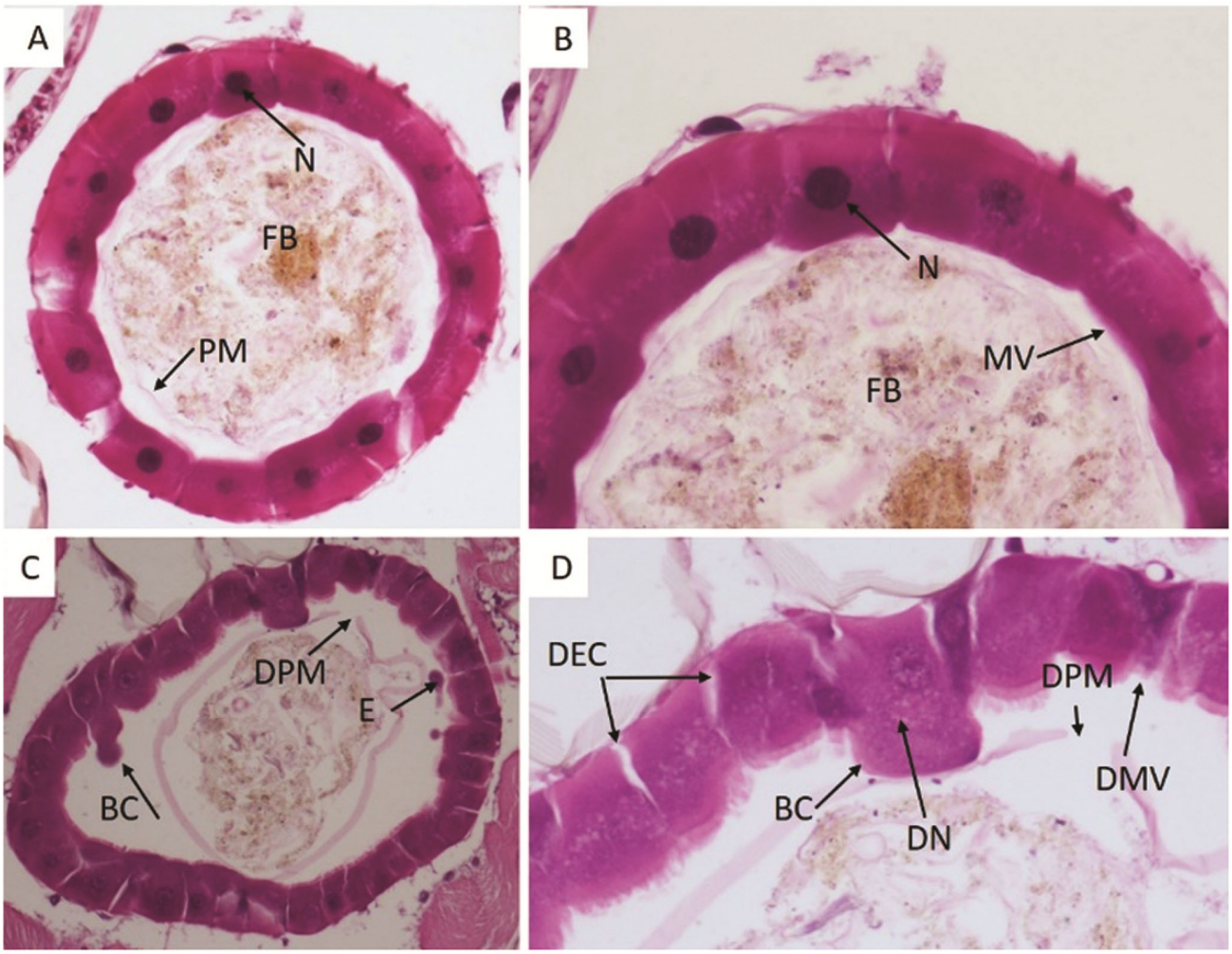

Fig. 1 — Photomicrograph of midgut histology of *Cx pipiens* larvae 3rd instar. (A & B) Control; and (C & D) Treated (with *Juniperus phoenicea* extract). [In the extract-treated group, the larvae showed peritrophic membrane degradation, displacement of the epithelial layer, irregular blebbing, and sloughing. blebbing (Bc), Degraded microvilli (DMV), degenerating epithelial cells (DEC), degenerating nuclei (DN), degenerating peritrophic membrane (DPM), extrusion (E), food bolus (FB), and nuclei (N). Magnification: A & C 200X, B & D 400X]

Histopathological study

In the control group, the cytoplasm in gut epithelial cells (cuboidal/flattened cells) was clear with a rounded nucleus (Fig. 1 A & B). However, in treated larvae, the midgut cells were swollen, and vesicles of different sizes were detected (Fig. 1 C & D). The *J. phoenicea* extract-treated larvae had deteriorated microvilli, blebbing and degenerating nuclei,

displacement of the epithelial layer, and partial loss of the peritrophic membrane.

GC-MS analysis

Preliminary investigation of *J. phoenicea* extract showed the presence of phytochemicals of diverse chemical nature. Some of the main phytochemicals identified in the extract are 9,12-octadecadienoic acid (Z,Z)- (50.2%), podocarpa-8,11,13-trien-3-one, 14-iso propyl-13-methoxy- (13.2%), -amino-7,10-dimethyl dibenzo[b,f][1,4]oxazepin-11(10H)-one (8.4%), n-hexa decanoic acid (5.6%) and Furo[2,3-b]quinoline, 4,6,7-trimethoxy (3.4%) (Table 2).

Untapped bioactive secondary metabolites from plants have intriguing phytochemicals that can replace synthetic insecticides. Natural products such as plants are rich in novel bioactive compounds, as drugs derived from plants have significantly impacted human health. The efficacy of phytochemicals on mosquito larvae has been demonstrated in several research. Phytochemicals are promising biodegradable larvicidal agents and have less harmful effects on non-target organisms[17]. The larvicidal potentials of 32 oils from various plants against the third instar of *Cx. pipiens* were reported by Baz *et al.*[18] All oils extracted showed larvicidal activity, and their LT_{50} values ranged from 9.67 to 37.64 h. Al-Solami[19] reported the efficacy of acetone extract of *Rhazya stricta*, *Ruta chalepensis*, *Lantana camara*, and *Amorpha fruticose* against *Cx. pipiens*. The results showed that the *L. camara* extract caused 98% mortality, followed by *R. stricta* (91%) compared to azadirachtin.

The current study evaluated the histological changes that *J. phoenicea* extracts caused in the midgut of *Cx. pipiens* third instars. Some modifications include enlarged intercellular gaps, cytoplasmic vacuolization, fragmented nuclei and a distorted epithelial layer. Moreover, the separation of the peritrophic membrane and alteration of microvilli led to the destruction of midgut architecture. These histopathological changes caused by the *J. phoenicea* methanol extract on the *Cx. pipiens* midgut architecture were in agreement with several researchers using different plants[20-25]. Al-Mehmadi and Al-Khalaf[26] reported that treatment of Culex quinquefasciatus with Melia azedarach L. resulted in significant damage to the larvae's midgut epithelium, leading to cell vacuolization, microvilli damage, and cell death. Abutaha *et al.*[27] also reported morphological alternations in the larval midgut of *Cx. pipiens* when treated with *Cinnamomum burmannii* and *Syzygium aromaticum* combined extract. They observed different morphological alternations, such as microvilli degradation, peritrophic membrane damage, and loss of nuclei. From GC–MS analysis, 19 compounds were detected. Compounds, such as 9,12-octadecadienoic acid (Z,Z)- (50.2%) and n-hexadecanoic acid (5.6%) have been earlier reported for their larvicidal potential[28,29]. However, the other compounds detected were also reported from different plant extracts that showed larvicidal potential in

Table 2 — Compounds present in 70% methanol extract of *Juniperus phoenicea* leaves detected using GC-MS analysis

Compound	R.T. (min)	Area (%)	Biological activity
4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	9.76	2.19	Antioxidant, anti-inflammatory, and antimicrobial activities[31,32]
Phenol, 2,5-bis(1,1-dimethylethyl)-	14.46	0.96	Antifungal[6]
Azacyclohexane, 3-[1-pyrrolidyl]-	17.51	0.75	-
Pentadecanoic acid, 14-methyl-, methyl ester	18.52	1.78	Antimicrobial, antifungal[33]
n-Hexadecanoic acid	18.89	0.72	Larvicidal[27]
5-[3,4-Methylenedioxybenzyl]-2-thiohydantoin	19.32	5.64	-
9,12-Octadecadienoic acid, methyl ester	20.15	1.72	-
9,12-Octadecadienoic acid (Z,Z)-	20.53	1.77	-
10-Octadecenoic acid, methyl ester	20.59	2.38	-
9,12-Octadecadienoic acid (Z,Z)-	21.01	50.21	
Octadecanoic acid	21.19	0.98	Immunoregulatory and anti-inflammatory[34]
2-Amino-7,10-dimethyldibenzo[b,f][1,4]oxazepin-11(10H)-one	21.50	8.47	-
Methyl 9,12-heptadecadienoate	21.79	0.62	-
9,17-Octadecadienal, (Z)-	23.61	0.63	-
Furo[2,3-b]quinoline, 4,6,7-trimethoxy-	24.28	3.42	Anti-parasitic and antitumor activity[35]
1,3,12-Nonadecatriene	25.80	0.59	Antidiabetic and antilipidemic properties[36]
Podocarpa-8,11,13-trien-3-one, 14-isopropyl-13-methoxy-	25.91	13.16	-
3H-3a-Azacyclopenta[a]indene-2-carbonitrile, 3-oxo-1-(piperidin-1-yl)-4,5,6,7-tetrahydro-	26.83	3.21	-
beta-Sitosterol	27.311	0.83	Larvicide[37]

combination[30]. Thus, the larvicidal potential could be related to the individual compound or synergistic activity. Further investigation is needed to isolate these compounds to assess their larvicidal potential.

Conclusion

The research evaluated the larvicidal effectiveness and chemical composition of *Juniperus phoenicea* leaf extract against *Culex. pipiens* mosquito larvae. The study revealed a significant larvicidal effect, influenced by both concentration and exposure duration. Histopathological examinations demonstrated morphological changes in treated larvae, indicating a potential disruption of physiological processes. GC-MS analysis identified diverse phytochemicals in the *J. phoenicea* extract. Overall, the study suggests that *J. phoenicea* leaf extract has potent larvicidal properties against *Cx. pipiens*, possibly attributed to the identified phytochemicals. Further research is recommended to explore the extract's application as a natural larvicide for mosquito control, focusing on its safety and effectiveness in the broader ecological context.

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Conflict of Interest

Author declares no competing interests.

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