



Larvicidal Activity of Algerian Cypress (*Cupressus sempervirens* L.) Against *Culex pipiens* with Chemical Profiling and Molecular Docking Insights

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Abstract: Increasing resistance to synthetic insecticides complicates control of *Culex pipiens* L., a key vector, making plant-derived essential oils a promising eco-friendly alternative. This study aims to investigate the chemical composition, larvicidal activity, and potential molecular mechanisms of *Cupressus sempervirens* essential oil from Algeria against fourth-instar *Culex pipiens* larvae. The extraction yield was $0.87 \pm 0.21\%$. Gas Chromatography–Mass Spectrometry profiling identified 46 compounds, dominated by monoterpene hydrocarbons, including α -pinene (21.37%) and 3-carene (18.36%). Larvicidal assays demonstrated potent activity, with LC_{50} and LC_{90} values of 6.59 ppm (95% CI: 4.65–8.29) and 23.40 ppm (95% CI: 17.74–37.76), respectively, showing a dose dependent mortality. Molecular docking analysis suggested favourable binding affinities of α -pinene and 3-carene toward acetylcholinesterase and glutathione S-transferase, with ligand accommodation within the active sites through predominantly hydrophobic interactions. These results suggest possible interference with neurotransmission and detoxification pathways. These findings indicate that *Cupressus sempervirens* essential oil is a promising natural larvicidal agent and support its potential application in environmentally friendly mosquito management strategies.

Key words: acetylcholinesterase, 3-carene, *Cupressus sempervirens*, glutathione S-transferase, *in silico*, α -pinene

Introduction

Vector-borne diseases are human illnesses caused by parasites, viruses, and bacteria transmitted by vectors, primarily hematophagous insects such as mosquitoes. These diseases cause more than 700 000 deaths an-

nually worldwide, representing a major global public health burden (WHO 2020), particularly in Mediterranean and North African regions, where *Culex pipiens* L. (*C. pipiens* L.) is among the most widespread and epidemiologically significant mosquito species. This mosquito plays a central role in the transmission

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of numerous pathogens of medical and veterinary importance, including West Nile virus, Rift Valley fever virus, and various filarial parasites (Fesce & Ferraguti 2025). In recent decades, mosquito control programs have relied extensively on synthetic insecticides, including organophosphates (e.g., temephos, malathion), pyrethroids (e.g., permethrin, deltamethrin), carbamates (e.g., propoxur, bendiocarb), and organochlorines (e.g., DDT) (Khater & Geden 2019). However, despite their initial effectiveness in reducing mosquito populations and disease transmission, the intensive and prolonged use of these chemicals has led to the widespread development of insecticide resistance, environmental persistence and bioaccumulation of toxic residues, and adverse effects on non-target organisms, thereby raising serious ecological and public health concerns related to water and food contamination (Pavela & Benelli 2016, Baz et al. 2025). These limitations have stimulated increasing interest in alternative and sustainable vector control strategies, particularly those based on natural products such as plant-derived essential oils (EOs). These extracts are complex mixtures of bioactive secondary metabolites, mainly mono- and sesquiterpenes, known to exhibit broad-spectrum insecticidal and larvicidal activities through multiple and complementary modes of action, including neurotoxicity, enzyme inhibition, and disruption of cellular membranes (Regnault-Roger et al. 2012). Their chemical complexity and multi-target effects reduce the likelihood of resistance development, making them promising candidates for environmentally sustainable mosquito management (Isman, 2020). Moreover, EOs are generally recognised as environmentally friendly, rapidly biodegradable, minimally toxic to mammals, and have demonstrated repellent and toxic effects against various mosquito species (Huong et al. 2020). *Cupressus sempervirens* L. (*C. sempervirens*) is an evergreen conifer tree belonging to the Cupressaceae family widely distributed in Algeria and throughout the Mediterranean region, where it is native (Chaudhary et al. 2012). It is morphologically characterised by a monoecious conifer with a slender trunk, columnar to pyramidal crown, and densely branched shoots. Its gray-reddish bark and dark green, scale-like leaves cover branchlets completely. Rounded woody cones (2–3 cm) with 8–12 scales release seeds at maturity (Amara & Boughérara 2017). Commonly known as Mediterranean or Algerian cypress, this species has been traditionally used for its antimicrobial, antiviral, antioxidant, anticancer, anticoagulant, anti-inflammatory, wound-healing, and insect-repellent properties (Al-Snafi 2016). Despite its wide distribution in Algeria and throughout the Mediterranean basin, mo-

lecular-level investigations remain poorly explored. Although molecular docking studies on essential oil constituents and their interactions with insect target enzymes have previously been reported, integrated investigations combining the chemical characterisation, larvicidal activity, and exploratory docking analysis of Algerian *C. sempervirens* essential oil against *Culex pipiens* remain scarce.

Therefore, the present study aims to (i) characterise the chemical composition of *C. sempervirens* EO collected in Algeria using GC–MS analysis, (ii) evaluate its larvicidal activity against *C. pipiens* larvae, and (iii) investigate its potential molecular mechanisms of action through *in silico* docking analyses targeting acetylcholinesterase (AChE, PDB: 5X61) and glutathione S-transferase (GST, PDB: 18GS). This integrated approach contributes to the valorisation of Algerian cypress as a natural larvicidal resource and supports its potential application in environmentally sustainable mosquito control strategies.

Materials and Methods

Plant collection

The leaves of *C. sempervirens* were collected in March 2024 from the Afir region (36°46'03" N, 3°42'10" E), approximately 120 km east of Algiers. The species was selected for its availability and traditional bioinsecticidal use. Taxonomic identification was confirmed by a botanist at Boumerdes university.

Mosquito rearing

C. pipiens larvae used for bioassays were collected from untreated sites in Lake Reghaïa (36°46'17" N, 3°20'38" E), located 29 km east of Algiers. The larvae were transported to the laboratory in plastic containers filled with their native water and sorted according to their developmental stage. They were reared under controlled conditions at 27 ± 2 °C, $75 \pm 5\%$ relative humidity, and a 12:12 h light–dark photoperiod. The larvae were fed daily with a mixture of crushed biscuits and brewer's yeast (75:25, w/w) (Mir et al. 2022). Species identification was performed using the Culicidae identification software of Mediterranean Africa developed by IRD Montpellier (Brunhes et al. 2000) and confirmed with the identification key of Himmi et al. (1995).

Extraction and yield of the EO

EO was extracted from fresh leaves using the steam distillation method. The plant material was exposed to steam for 4 hours in a Clevenger-type apparatus. The obtained distillate was separated by decanta-

tion, and the EO was dried over anhydrous sodium sulfate (Na_2SO_4) to remove residual moisture. The oil was then stored in amber glass vials at 4 °C until further analysis.

The extraction yield was calculated based on 100 g of fresh plant material and expressed as a percentage (w/w) relative to the fresh weight according to the following formula:

Gas chromatography-Mass spectrometry analysis

The chemical composition of the EO was determined using gas chromatography–mass spectrometry (GC–MS). Analysis was performed on an Agilent 7890A gas chromatograph coupled to a 5975C mass selective detector (MSD), equipped with an HP-5MS capillary column (5% phenyl methyl siloxane; 30 m $\times$ 0.25 mm in internal diameter; film thickness 0.25 μm). Helium was used as the carrier gas at a constant flow rate of 1.5 mL/min. A 0.2 μL sample of the EO was injected in split mode (1:50), with the injector temperature maintained at 250 °C. The oven temperature was programmed from 60 °C (held for 8 min), then increased at a rate of 4 °C/min to 250 °C, and held at this final temperature for 25 min. The mass spectrometer was operated in electron impact (EI) mode at 70 eV, scanning in the m/z range of 40–550 amu. The identification of components was achieved by comparing their retention indices (RI), calculated relative to a homologous series of n-alkanes (C_6 – C_{28}), and by matching their mass spectra with those in the NIST MS.

Larvicidal bioassay

The larvicidal activity of *C. sempervirens* EO was evaluated against newly emerged fourth-instar larvae of *C. pipiens*, following the World Health Organization (WHO 2005) protocol with minor modifications. All bioassays were carried out under controlled laboratory conditions at 27 ± 2 °C and $75 \pm 5\%$ relative humidity. The light-dark photoperiod of 12:12h was maintained throughout the entire exposure.

Based on preliminary assays, EO was first dissolved in 1 mL of ethanol and then diluted in 100 mL of breeding water (collected from Lake Reghaïa) to obtain the desired test concentrations (5, 10, 20 and 30 ppm). Solutions were vortexed prior to dilution and gently homogenised after addition to test containers. All test solutions were freshly prepared immediately before exposure. For each concentration, 20 larvae were transferred into 100 mL of test solution. Negative controls contained only site water, while solvent controls contained ethanol at the corresponding concentration. Each treatment was

replicated three times. No food was provided to larvae during the exposure period (WHO 2005). Larval mortality was recorded after 24, 48 and 72 hours of exposure. Larvae were considered dead if they did not respond to probing of the siphon or cervical region with a fine needle. Mortality percentages were corrected using Abbott's formula (Abbott 1925), and lethal concentrations (LC_{50} and LC_{90}) with their 95% confidence limits (CL) were calculated.

In silico molecular docking analysis

Protein preparation

As a complementary approach, molecular docking was carried out to predict possible interactions between two major compounds of Cypress EO (α -pinene and 3-carene) and target enzymes associated with insecticidal activities (acetylcholinesterase and glutathione S-transferase). Pyriproxyfen was included as a reference insecticidal compound due to its documented efficacy against *Culex pipiens*. It was used for comparative docking purposes rather than as a specific inhibitor of AChE or GST.

Ligand preparation

The molecular docking study focused on the major constituents of Cypress EO, α -pinene and 3-carene (CIDs: 6654 and 26049, respectively). The three-dimensional structures of the selected ligands were downloaded from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>) in SDF format. The structures were converted to PDB format using the CACTUS Online SMILES Translator (<https://cactus.nci.nih.gov/translate/>). Ligand preparation was completed using AutoDockTools and saved in PDBQT format, making them suitable for docking simulations performed with AutoDock Vina.

Target protein preparation

Acetylcholinesterase (AChE) and glutathione S-transferase (GST) were selected as the target enzymes of this study. The crystallographic structures of these enzymes were retrieved from the RCSB Protein Data Bank (<https://www.rcsb.org/>) (Berman et al. 2000), using the PDB IDs 5X61 for acetylcholinesterase and 18GS for glutathione S-transferase.

Prior to molecular docking simulations using AutoDock Vina (Morris et al. 2009), the protein structures were prepared by removing water molecules, co-crystallized ligands, and other non-essential heteroatoms associated with the structures. Polar hydrogen atoms were added, and the prepared protein structures were then saved in PDBQT format, making them suitable for subsequent docking analysis.

Molecular Docking simulation

AutoDockTools version 1.5.6 was used to carry out the molecular docking analysis. A grid box covering the active site of each target enzyme was defined, with appropriate dimensions to allow sufficient conformational flexibility during docking. Binding affinities were calculated based on the docking pose exhibiting the lowest binding energy. Discovery Studio software was used to visualise and analyse the docking poses and protein-ligand interactions. This analysis allowed for the identification and characterisation of key intermolecular interactions, including hydrogen bonds, hydrophobic contacts, and other relevant ligand-receptor interactions (Baroroh et al. 2023).

Statistical analysis

Data are expressed as mean $\pm$ standard deviation (SD). The normality of the distributions was assessed using the Shapiro-Wilk test. The effects of the different concentrations were compared using a Kruskal-Wallis ANOVA, followed by a Dunn-Sidak post hoc test for multiple comparisons. The significance threshold was set at $p < 0.05$. The lethal concentrations LC_{50} and LC_{90} were estimated by Probit regression after logarithmic transformation (base 10) of the concentrations. Model parameters (slope, intercept), Pearson's chi-square (χ^2), and degrees of freedom (df) were calculated. A heterogeneity factor was applied when the χ^2 goodness-of-fit was significant ($p < 0.05$). The 95% confidence intervals (95% CI) of the LCs were obtained using the likelihood profile method. All analyses were performed using SPSS (version 26) and Origin (version 2018) software.

Results

Extraction yield

Steam distillation of *C. sempervirens* yielded a pale yellow EO with a strong odour and an extraction yield of 0.87 ± 0.21 % (w/w).

GC-MS analysis

The chemical profiling of *C. sempervirens* EO revealed a diverse chemical composition. A total of 46 distinct compounds were identified, representing 94.427% of the total composition of the oil. Identified compounds are listed in the order of elution using a nonpolar HP-5MS capillary column (Fig. 1, Table 1).

According to their functional groups, the compounds of EO were classified into five chemical classes. The composition was dominated by monoterpene hydrocarbons, with α -pinene as the major component (21.367%), followed by 3-carene (18.355%), limonene (6.353%), sabinene (5.266%), α -terpinolene (4.295%), and o-cymene (3.549%). In addition to this dominant fraction, the oil also contained appreciable amounts of oxygenated monoterpenes (4-Terpineol with 2.656%), sesquiterpene hydrocarbons (Cedrene with 1.03%), oxygenated sesquiterpenes (Cedrol with 1.532%), and a minor fraction of diterpenes (Sclareol with 2.21%).

Mosquito larvicidal activity

The effect of *C. sempervirens* EO at different concentrations on the mortality of *C. pipiens* L4 larvae after 24, 48, and 72 hours of exposure are shown in Fig. 2 and Table 2. No mortality was observed in the control group, confirming the absence of natural deaths during the experiment.

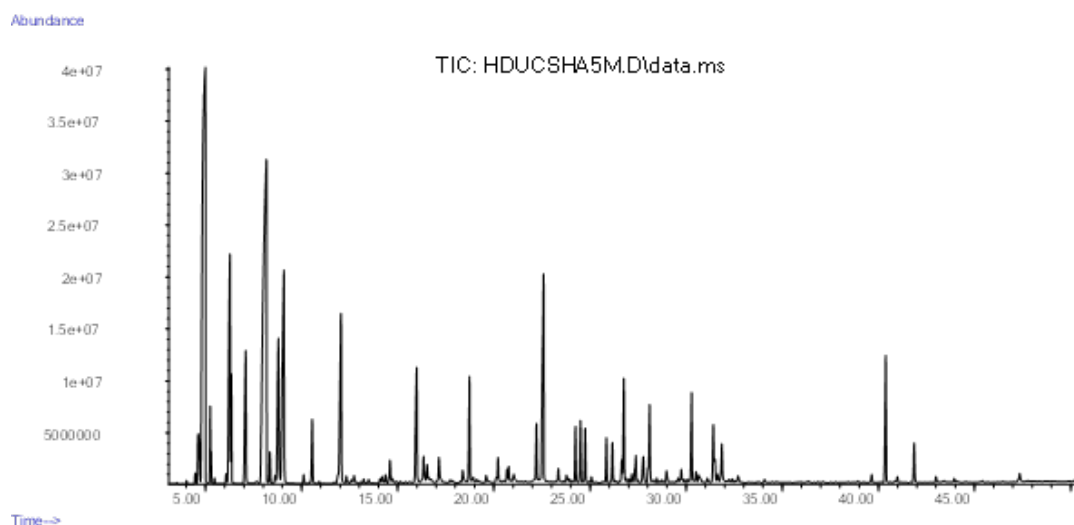


Fig. 1. Chromatogram of *C. sempervirens* L. essential oil.

Table 1. Chemical characterisation of *C. sempervirens* L. EO using GC–MS, showing retention time, compound group, and relative percentage composition.

N°	Compound ^a	Rt (min)	Molecular formula	Calculated IK ^b	Theoretical IK ^c	% ^d
Monoterpene hydrocarbons (68.285%)						
1	Tricyclene	4.469	C ₁₀ H ₁₆	914	918	0.202
2	α-Thujene	4.635	C ₁₀ H ₁₆	918	918	1.491
3	α-Pinene	4.997	C ₁₀ H ₁₆	927	925	21.367
4	d-Camphene	5.250	C ₁₀ H ₁₆	934	948	0.907
5	Camphene	5.294	C ₁₀ H ₁₆	935	937	0.298
6	Sabinene	6.274	C ₁₀ H ₁₆	960	964	5.266
7	β-Pinene	6.349	C ₁₀ H ₁₆	962	964	1.355
8	β-Myrcene	7.097	C ₁₀ H ₁₆	981	983	2.919
9	α-Phellandrene	7.645	C ₁₀ H ₁₆	996	998	tr
10	3-Carene	8.173	C ₁₀ H ₁₆	1007	1009	18.355
11	α-Terpinene	8.354	C ₁₀ H ₁₆	1011	1012	0.496
12	β-Cymene	8.625	C ₁₀ H ₁₄	1016	1021	0.143
13	o-Cymene	8.798	C ₁₀ H ₁₄	1020	1022	3.549
14	Limonene	9.081	C ₁₀ H ₁₆	1026	1026	6.353
15	β-cis-Ocimene	10.108	C ₁₀ H ₁₆	1047	1046	0.163
16	τ-Terpinene	10.555	C ₁₀ H ₁₆	1056	1056	1.126
17	α-Terpinolen	12.051	C ₁₀ H ₁₆	1086	1086	4.295
Oxygenated monoterpenes (11.698%)						
18	α-Pinene epoxide	12.332	C ₁₀ H ₁₆ O	1092	1094	0.092
19	Thujone	13.229	C ₁₀ H ₁₆ O	1111	1110	tr
20	Camphor	14.367	C ₁₀ H ₁₆ O	1135	1138	0.165
21	4-Terpineol	15.992	C ₁₀ H ₁₈ O	1169	1174	2.656
22	p-Cymen-8-ol	16.352	C ₁₀ H ₁₄ O	1177	1175	0.531
23	α-Terpineol	16.535	C ₁₀ H ₁₈ O	1181	1185	0.218
24	cis-Verbenol	17.148	C ₁₀ H ₁₆ O	1194	1188	0.565
25	Thymol methyl ether	18.737	C ₁₁ H ₁₆ O	1237	1237	1.681
26	Borneol, acetate	20.221	C ₁₂ H ₂₀ O ₂	1279	1277	0.399
27	4-Terpineol acetate	20.777	C ₁₂ H ₂₀ O ₂	1295	1304	0.241
28	α-Terpineol acetate	22.594	C ₁₂ H ₂₀ O ₂	1348	1348	5.15
Sesquiterpene hydrocarbons (9.053%)						
29	Copaene	23.357	C ₁₅ H ₂₄	1370	1371	0.254
30	Junipene	24.252	C ₁₅ H ₂₄	1395	1396	0.86
31	Cedrene	24.515	C ₁₅ H ₂₄	1403	--	1.03
32	Caryophyllene	24.775	C ₁₅ H ₂₄	1410	1416	0.889
33	α-Caryophyllene	25.850	C ₁₅ H ₂₄	1445	1445	0.666
34	(+)-Epi-bicyclosquiphellandrene	26.167	C ₁₅ H ₂₄	1455	1471	0.622
35	τ-Murolene	26.652	C ₁₅ H ₂₄	1470	1471	0.362
36	Germacrene D	26.761	C ₁₅ H ₂₄	1473	1474	1.73
37	α-Murolene	27.382	C ₁₅ H ₂₄	1493	1494	0.524
38	τ-Cadinene	27.771	C ₁₅ H ₂₄	1505	1506	0.6
39	δ-Cadinene	28.102	C ₁₅ H ₂₄	1517	1515	1.516
Oxygenated sesquiterpenes (3.181%)						
40	Caryophyllene oxide	29.747	C ₁₅ H ₂₄ O	1573	1576	0.228
41	Cedrol	30.287	C ₁₅ H ₂₆ O	1592	1596	1.532

Table 1. Continuation.

N°	Compound ^a	Rt (min)	Molecular formula	Calculated IK ^b	Theoretical IK ^c	% ^d
42	1,5,5,8-Tetramethyl-12-oxabicyclo[9.1.0]dodeca-3,7-diene	30.514	C ₁₅ H ₂₄ O	1599	1607	0.174
43	Cubenol	31.112	C ₁₅ H ₂₆ O	1621	1625	0.06
44	tau.-Muurolol	31.493	C ₁₅ H ₂₆ O	1635	1635	0.414
45	α-Cadinol	31.858	C ₁₅ H ₂₆ O	1648	1651	0.773
Diterpene (2.21%)						
46	Sclareol	40.379	C ₂₀ H ₃₆ O ₂	1981	--	2.21
					Total	94.427

^a Identified compounds are listed in the order of elution using a nonpolar HP-5MS capillary column ; ^b Calculated retention indices obtained on a nonpolar HP-5MS capillary column ; ^c Literature retention indices reported for columns of similar polarity (HP-5MS or DB-5) ; ^d Relative percentages of identified compounds, calculated from GC–MS peak areas using an HP-5MS column ; tr: trace amounts (<0.05%) ; % percentage ; Rt (min) : Retention time (minutes).

Table 2. LC₅₀ and LC₉₀ of *C. sempervirens* L. EO against fourth-instar larvae of *C. pipiens* after 24 h of exposure

LC ₅₀ (ppm) CI 95% (LCL-UCL)	LC ₉₀ (ppm) CI 95% (LCL-UCL)	Slope ± SD	Intercept ± SD	X ² (Pearson)	Df
6.59 (4.65–8.29)	23.40 (17.74–37.76)	2.329± 0.151	-1.908± 0.160	53.64	10

LC₅₀ lethal concentration that kills 50% of larvae (ppm), LC₉₀ lethal concentration that kills 90% of larvae (ppm), 95% CI confidence interval of 95%, LCL lower confidence limit, UCL upper confidence limit (95% fiducial limit), X² Pearson's chi-square, Df degrees of freedom.

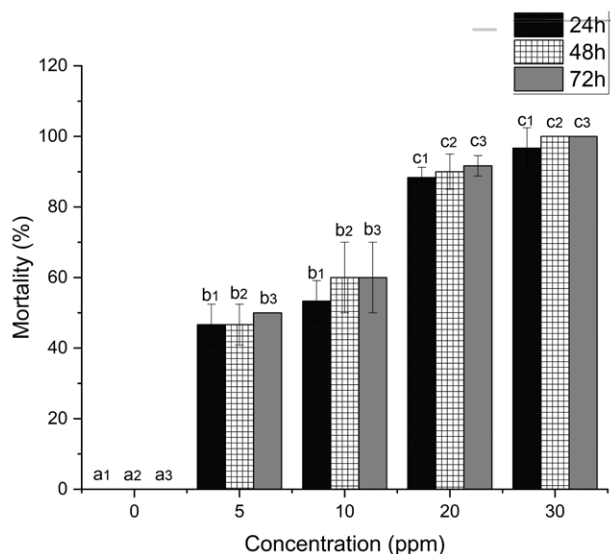


Fig. 2. Mortality rate of *C. pipiens* L4 in response to different concentrations of *C. sempervirens* L. EO. Different letters (a, b, c) for each exposure time (subscripts 1, 2, 3 for 24h, 48h, and 72h, respectively) indicate a significant difference according to the Dunn-Sidak test ($P < 0.05$).

The results indicate a clear dosedependent increase in larval mortality at all exposure times, but no significant timedependent effect was observed ($p > 0.05$). Indeed, at 24 hours, mortality increased with concentration, reaching $46.7 \pm 5.8\%$ (5 ppm), $53.3 \pm 5.8\%$ (10 ppm), $88.3 \pm 2.9\%$ (20 ppm), and $96.7 \pm 5.8\%$ (30 ppm). After 48 hours, mortality further increased to $46.7 \pm 5.8\%$ (5 ppm), $60 \pm 10\%$ (10 ppm), $90 \pm 5\%$ (20 ppm), and 100% (30 ppm). By 72 hours, mortality rates reached 50% (5 ppm), $60 \pm 10\%$ (10 ppm), $91.7 \pm 2.9\%$ (20 ppm), and 100% (30 ppm). According to the Dunn–Sidak test, differences between concentrations were globally significant ($p < 0.05$), except for the pairs 5 ppm vs 10 ppm and 20 ppm vs 30 ppm which did not differ statistically.

Probit regression analysis confirms significant insecticidal activity after 24 hours of exposure, with an LC₅₀ of 6.59 ppm (95% CI: 4.65–8.29 ppm) and an LC₉₀ of 23.40 ppm (95% CI: 17.74–37.76 ppm). The dose-response relationship is characterised by a significant positive slope (2.329 ± 0.151) and an intercept of -1.908 ± 0.160 , indicating a steady increase in mortality as concentration rises. The model

fit is statistically significant (Pearson's $\chi^2 = 53.643$, $df = 10$, $p < 0.001$); a heteroscedasticity factor was applied in the calculation of confidence intervals, ensuring the robustness of the estimates.

Molecular docking results

According to the molecular docking analysis, the major compounds of Cypress EO, α -pinene and 3-carene, exhibited favourable binding affinities toward target enzymes acetylcholinesterase (AChE; PDB ID: 5X61) and glutathione S-transferase (GST; PDB ID: 18GS), with high binding affinities (Fig. 3, Table 3).

For glutathione S-transferase (18GS), the binding energies of both ligands were similar, with a value of -5.3 kcal/mol, suggesting comparable potential affinity toward the enzyme. 3-Carene binds to the enzyme through hydrophobic interactions, which include alkyl interactions with Arg282 and π -sigma interactions with Tyr287, as well as other

hydrophobic interactions involving Ala294. Similarly, α -pinene was involved in multiple hydrophobic interactions with GST, which include π -alkyl interactions with Tyr287 and Tyr79 and an alkyl interaction with Arg282. The predominance of hydrophobic interactions is consistent with the non-polar nature of α -pinene and may contribute to its stabilisation within the binding pocket. Pyriproxyfen, used here as a reference insecticidal compound, displayed a more negative binding energy (-6.4 kcal/mol) through a hydrophobic π -alkyl interaction with Arg74.

Regarding acetylcholinesterase (5X61), the predicted binding energies were slightly lower than those observed for GST, suggesting a possible stronger interaction with this target. The binding energy of 3-Carene was calculated at -6.3 kcal/mol. The ligand interacted mainly through hydrophobic contacts with the aromatic residues Tyr494, Phe490, Phe449, and Trp441, together with alkyl interac-

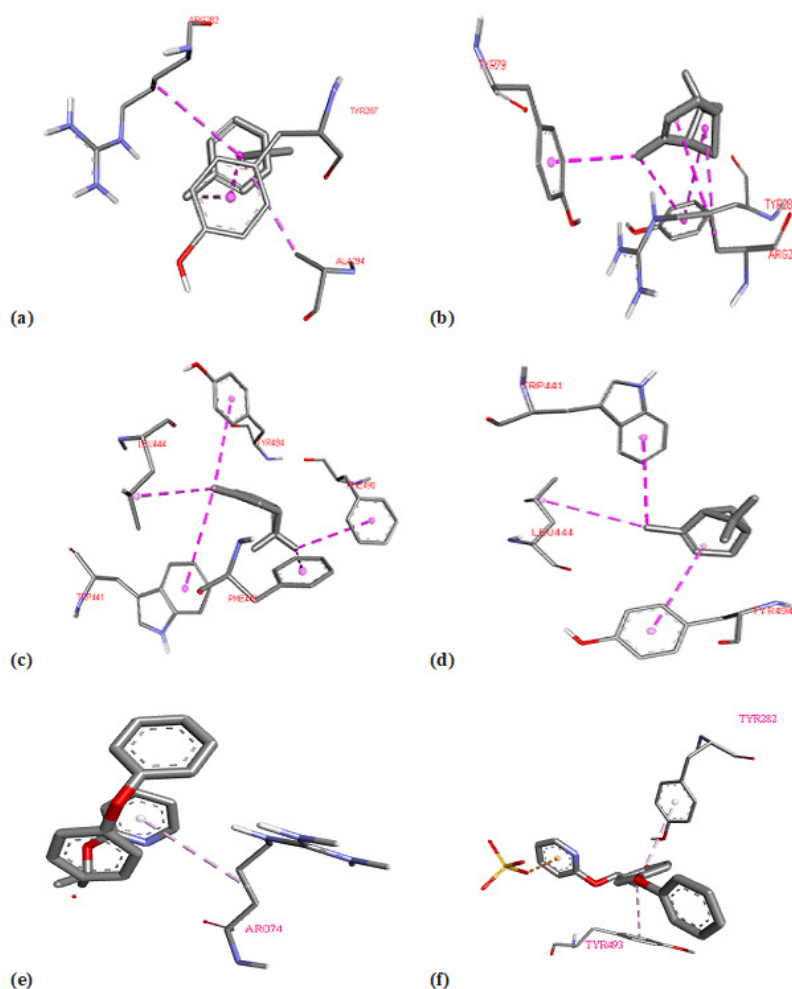


Fig. 3. Molecular interactions of 3-carene and α -pinene with 18GS and 5X61 proteins. (a) – 18GS-3-Carene ; (b) – 18GS- α -Pinene ; (c) – 5X61-3-Carene ; (d) – 5X61- α -Pinene ; (e) – 18GS- Pyriproxyfen ; (f) – 5X61- Pyriproxyfen.

Table 3. Molecular docking results of major Cypress EO constituents against AChE and GST

Target enzyme (PDB ID)	Ligand	Binding affinity (kcal/mol)	Key interacting residues	Interaction type
GST (18GS)	3-Carene	-5.3	Arg282, Tyr287, Ala294	Alkyl, π -sigma, hydrophobic
	α -Pinene	-5.3	Tyr287, Tyr79, Arg282	π -alkyl, alkyl
	Pyriproxyfen	-6.4	Arg74	hydrophobic Pi-alkyl bond
AChE (5X61)	3-Carene	-6.3	Tyr494, Phe490, Phe449, Trp441, Leu444	π -alkyl, alkyl
	α -Pinene	-6.2	Tyr494, Trp441, Leu444	π -alkyl, alkyl
	Pyriproxyfen	-8.1	Tyr282, Tyr493	hydrophobic Pi-alkyl bond

EO : essential oil; AChE : acetylcholinesterase; GST : glutathione S-transferase; PDB : Protein Data Bank; PDB ID : Protein Data Bank identification code; kcal/mol : kilocalories per mole; Arg : arginine; Tyr : tyrosine; Ala : alanine; Phe : phenylalanine; Trp : tryptophan; Leu : leucine.

tions with Leu444. Similarly, α -pinene had a binding energy of -6.2 kcal/mol and interacted primarily through hydrophobic interactions with Tyr494, Trp441, and Leu444. Pyriproxyfen, included as a reference insecticidal compound, demonstrated the highest predicted binding affinity (-8.1 kcal/mol) through hydrophobic Pi-alkyl interactions with Tyr282 and Tyr493.

Overall, the docking analysis suggests that α -pinene and 3-carene may exhibit greater potential affinity to acetylcholinesterase compared to glutathione S-transferase. The interaction profiles show a predominance of hydrophobic and π -alkyl interactions, which may contribute to the stabilisation of ligand-enzyme complexes. However, these docking findings remain predictive and do not by themselves confirm specific binding or enzymatic inhibition.

Discussion

Natural products, particularly plant EOs, have attracted growing interest as alternative larvicidal agents due to their chemical complexity, rapid biodegradability, and multiple biological targets (Pavela 2015). This interest is further reinforced by the increasing resistance of mosquito populations to conventional synthetic insecticides and their associated environmental risks. Previous studies have reported variable EO yields depending on the geographical origin of the plant and the extraction conditions. For example, hydrodistillation of dried aerial parts of *C. sempervirens* collected in Saudi Arabia produced a brownish EO with a yield of 2.6% (v/w) (Selim et al. 2014). In the region of Blida (Algeria), a lower yield of 0.41% was reported (Amara & Boughérara 2017), suggesting that local environmental conditions and plant material may influence oil recovery. These authors also reported that steam distillation

produced a slightly higher yield than conventional hydrodistillation, emphasising the impact of the extraction method on oil efficiency. Such variability is commonly observed and can be attributed to several factors, including botanical variety, pedoclimatic conditions, physiological state at harvest, and extraction parameters such as water-to-plant ratio, distillation time, and temperature. These factors affect not only the quantity of volatile compounds obtained but also the overall chemical composition of the EO (Herzi et al. 2013).

GC-MS is a powerful analytical technique widely used for the qualitative and quantitative characterisation of EO constituents, enabling the identification of major and minor compounds potentially responsible for biological activity (Onwujiogu et al. 2025). The GC-MS profiling of *C. sempervirens* EO revealed a chemically diverse composition, with 46 identified compounds accounting for 94.43% of the total oil content, indicating a high level of analytical coverage and reliable compound identification. The oil was predominantly composed of monoterpene hydrocarbons, with α -pinene (21.37%) and 3-carene (18.36%) as the major constituents, followed by limonene, sabinene, α -terpinolene, and o-cymene. The chemical composition of *C. sempervirens* EO shows a strong predominance of hydrocarbon monoterpenes (68.285%), particularly α -pinene, 3-carene, limonene, sabinene, and α -terpinolene, which make up the majority of the analysed EO. This chemical pattern is consistent with previous reports describing *C. sempervirens* EO as a monoterpene-rich system dominated by α -pinene and carene-type compounds (Herzi et al. 2013, Giatropoulos et al. 2013, Selim et al. 2014, Almadiy & Nenaah, 2022, Sriti et al. 2024). Studies on the mosquito-specific insecticidal activity of *C. sempervirens* EOs in Algeria, however, remain limited. Existing literature

shows that Algerian populations are characterised by a chemical profile dominated by α -pinene and δ -3-carene (Zouaghi et al. 2015, Amara & Boughérara 2017).

Comparable dominance of α -pinene has been reported in EOs from Mediterranean and Middle Eastern populations of *C. sempervirens*. Selim et al. (2014) reported α -pinene as a major compound in Saudi Arabian samples, while Herzi et al. (2013) demonstrated that hydrodistillation and supercritical CO₂ extraction yielded oils with distinct quantitative profiles, despite qualitative similarities. Similar findings have been reported in Tunisia (Sriti et al. 2024) and Lebanon (Anka et al. 2020). However, other studies have reported different dominant constituents, with (–)-terpinen-4-ol identified as the major compound (10.62%) (Baz et al. 2025) and cedrol reaching a high relative abundance (22.64%) (Hamid et al. 2021), (8.3%) (Mazari et al. 2010), whereas both compounds were detected only as minor components in our sample (<3%).

The relative chemotypic variation within this species, can be attributed to environmental conditions, genetic background, phenological stage, growth conditions and extraction technique (Sriti et al. 2024). Accordingly, the variations observed in the composition and biological activity of essential oils between studies can be partly explained by differences in extraction techniques. Extraction procedures can significantly influence the qualitative and quantitative composition of volatile compounds by affecting the recovery of monoterpenes, sesquiterpenes, and oxygenated compounds. Previous studies on *C. sempervirens* have reported differences in the main constituents and bioactivities depending on the extraction conditions and analytical approaches (El-Sheikh et al. 2011, Batiha et al. 2022, Baz et al. 2025). These variations may contribute to the differences in larvicidal activity observed between studies, highlighting the importance of considering methodological factors when comparing the biological activities of essential oils.

The obtained LC₅₀ value (6.59 ppm) is well below the thresholds reported by Cheng et al. (2003) and Dias & Moraes (2014) who classified EOs with an LC₅₀ below 100 ppm as effective larvicides and those below 50 ppm as highly active. Based on the observed mortality rates at each concentration, the calculated lethal concentrations (LC₅₀ and LC₉₀) further support the classification of this EO as a potent larvicidal agent.

To the best of our knowledge, few studies have combined the larvicidal evaluation of *C. sempervirens* essential oil against *C. pipiens* with an ex-

ploratory molecular docking analysis of its major constituents. The only closely related report is the work conducted in Egypt by Baz et al. (2025), which focused exclusively on the insecticidal effects of the same species without incorporating an *in silico* approach. In Algeria, available studies have been largely limited to chemical profiling and the evaluation of biological activities of *C. sempervirens* extracts (Boussoussa et al. 2018) and EOs (Zouaghi et al. 2014, Amara & Boughérara 2017, Mazari et al. 2010, Fadel et al. 2021). In comparison with the present findings, Baz et al. (2025) reported a lower larvicidal efficacy of *C. sempervirens* EO against *C. pipiens* larvae (LC₅₀ = 96.38 ppm; LC₉₀ = 298.01 ppm). Such differences in bioactivity are likely related to variations in EO composition, as their oil was dominated by (–)-terpinen-4-ol, ethylene brassylate, 1,1-oxy-bis-2-propanol, o-cymene, and γ -terpinene. The significant presence of hydrocarbon monoterpenes such as α -pinene and 3-carene is particularly relevant for insecticidal activity. These compounds are known for their ability to rapidly penetrate the insect cuticle, disrupting neuronal transmission through acetylcholinesterase (AChE) inhibition, leading to paralysis and eventually the death of mosquito larvae or adults (Rants'o et al. 2022). For instance, similar terpene profiles have been correlated with high insecticidal toxicity of *C. sempervirens* against *Tribolium castaneum* (LC₅₀ = 164.3 μ L/L air after 24 h exposure) (Sriti et al. 2024) and the Asian tiger mosquito *Aedes albopictus* (LC₅₀ = 54.7 mg/L ; LC₉₀ = 78.1 mg/L) (Giatropoulos et al. 2013). Several other studies on different mosquito species (*Aedes aegypti* and *Aedes albopictus*) also indicate that monoterpenes such as limonene and sabinene contribute to toxic activity via interactions with insect neurological and metabolic enzymes such as acetylcholinesterase and α -amylase (Huong et al. 2020, Ali et al. 2025). These observations are supported by broader evidence that monoterpenes can disrupt key physiological functions, including enzyme inhibition and neurotoxicity, leading to paralysis and mortality in insect targets (Şengül Demirak & Canpolat 2022). Oxygenated monoterpenes, although often present in lower proportions, may also contribute synergistically to insecticidal effects by disrupting cell membranes and interfering with detoxification and metabolic enzyme systems (Galeano et al. 2025). Furthermore, hydrocarbon and oxygenated sesquiterpenes such as α -cedrol have been reported as insecticidal and repellent agents in cypress and other conifer EOs, notably against *Culex quinquefasciatus* and *Aedes albopictus* (Giatropoulos et al. 2013, Almadiy & Nenaah 2022). Herein, *C. sempervirens*

EO exhibited strong larvicidal activity against *C. pipiens* larvae. Previous studies have frequently reported synergistic interactions between major and minor constituents of EOs. Such synergism may occur either among different EO mixtures or among components within the same oil, where each compound, even when present in minor proportions, can contribute to cellular penetration, interaction with biomembranes, and intracellular distribution, ultimately enhancing the overall bioactivity (Caesar & Cech 2019, Almadiy & Nenaah 2022).

Due to their central biological roles, both enzymes are widely employed as molecular targets to evaluate the insecticidal potential of EOs and their major constituents (Regnault-Roger et al. 2012). Acetylcholinesterase is an enzyme that regulates the transmission of nerve signals through the hydrolysis of acetylcholine at cholinergic synapses. Inhibition of this enzyme results in neurotoxicity, which ultimately kills the insect. Therefore, this enzyme is a target of insecticides and bioinsecticides (Casida & Durkin 2013). In contrast, glutathione S-transferases play a critical role in the detoxification process of insecticides through the conjugation of glutathione with foreign compounds, thereby contributing to insect tolerance to insecticides (Enayati et al. 2005). Recent experimental evidence has shown that EOs exhibiting strong larvicidal activity against *C. pipiens* are often associated with pronounced acetylcholinesterase inhibition and a concomitant, though moderate, inhibition of glutathione S-transferase, highlighting the complementary involvement of both enzymes in larval mortality (Toubal et al. 2025). In our simulations, these two ligands were found to have a stronger binding affinity for Acetylcholinesterase (AChE, 5X61) than for glutathione S-transferase (GST, 18GS), which indicates that Acetylcholinesterase may be a more favourable target for these monoterpenes (Reynoso-García et al. 2025). This view is supported by numerous reports highlighting the role of AChE inhibition in the insecticidal activity of essential oils (EOs) (Guarino et al. 2025). Although the interaction with glutathione S-transferase was less pronounced, it remains biologically relevant, as GSTs play a central role in insect detoxification and their partial inhibition may reduce the ability of insects to neutralise toxic compounds, thereby increasing susceptibility (Enayati et al. 2005), which is consistent with the enzyme-inhibitory patterns reported for EOs active against *C. pipiens* larvae (Toubal et al. 2025).

Although molecular docking provides useful insights into the possible interactions between the major compounds and the target enzymes (acetylcho-

linesterase and glutathione S-transferase), it remains a predictive computational approach. Therefore, it does not allow definitive conclusions regarding enzyme inhibition or the exact mechanism of action. In the absence of experimental enzymatic assays, the observed interactions should be considered as hypotheses of potential molecular mechanisms that require further biochemical validation.

The dominance of hydrophobic and π -alkyl interactions in the docking analysis is consistent with the nonpolar nature of monoterpenes and their binding preference for the hydrophobic parts of protein active sites. The same interaction patterns have been reported for other constituents of EOs binding to acetylcholinesterase (Regnault-Roger et al. 2012, Pavela & Benelli 2016). These findings support the idea that EO constituents can act through multiple complementary mechanisms, affecting both neural and detoxification pathways, which contributes to their effectiveness as natural insecticidal agents. Moreover, these *in silico* findings are consistent with and support the experimental results, reinforcing the proposed insecticidal potential of the studied compounds.

Despite the encouraging results obtained from this work, several limitations should be considered. First, the effects on non-target aquatic organisms have not been assessed, although monoterpenes can impact invertebrates, fish, amphibians, and microbial communities, representing a significant gap in the environmental safety assessment. Furthermore, the volatility and hydrophobicity of the essential oils may limit their stability and efficacy in the field, highlighting the need for appropriate formulations such as emulsions or encapsulation systems. Moreover, future research should focus on standardising the oil composition, conducting trials under semi-natural and field conditions, evaluating residual activity, and comparing performance with conventional larvicides within the framework of integrated vector management (IVM). Finally, the results of molecular modelling should be interpreted with caution, as they only provide predictive indications. Without enzymatic assays, the proposed mechanisms remain hypothetical.

Conclusion

C. sempervirens EO from Algeria, dominated by α -pinene and 3-carene, exhibited strong larvicidal activity against fourth-instar *C. pipiens* larvae (LC_{50} = 6.59 ppm; LC_{90} = 23.40 ppm), with a clear dose-dependent effect. Molecular docking suggested potential interactions of the major monoterpenes with

AChE and GST, providing preliminary mechanistic hypotheses that may contribute to the observed larvicidal activity. These findings highlight the EO's potential as an eco-friendly, multi-target natural larvicide, offering a sustainable alternative to synthetic insecticides, particularly in areas challenged by insecticide-resistant mosquito populations.

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