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Potential hazards of *Laurus nobilis* essential oil on juveniles of a non-target mosquitofish: chemical characterization, acute toxicity and biomarker responses

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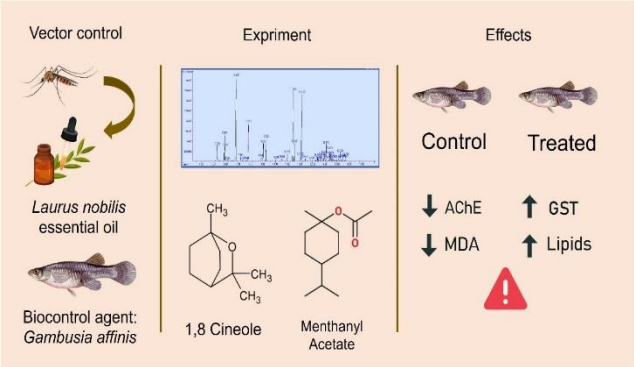
Graphical abstract



Abstract

The efficacy of essential oils against mosquito larvae has been highlighted, being used as an alternative to chemical insecticides in a vector control program. However, few works demonstrate the side effects of these substances on non-target organisms like *Gambusia affinis* (Baird and Girard 1853) (Actinopterygii: Poeciliidae) considered as a reference biological control agent. This study focuses on the potential side effects of an essential oil (EO) of *Laurus nobilis* L. (1753) on juveniles of *G. affinis*. The mosquitofish were collected from El Karma (in Annaba province). First, we analyzed the biochemical composition of the essential oil using gas chromatography-spectrometry (GC/MS). Then, acute toxicity tests (48 hours) were carried out, with the application of different concentrations of the EO added to the fish-rearing water of juveniles of *G. affinis,* and determination of the LC50/48h. Also, different morphometric parameters such as Head Weight, Body Weight, Full Weight, Head Length, Body Length and Full Length have been measured after acute exposure to the EO. Lastly, the acetylcholinesterase (AChE) and the glutathione-S-transferase (GST) activities, malondialdehyde (MDA) rate, and total lipid levels were measured in control and treated series. GC/MS analysis demonstrated the different biochemical constituents of the EO, with 1,8-cineole (21.9%) as the main compound followed by menthanyl acetate (17.15%) and methyl eugenol (17.11%). The determined LC50/48h was 49.48 mg/L. In addition, the morphometric measurements indicated that the treated series was characterized by lower weights compared to the control one. Regarding environmental biomarkers, the current results showed that individuals exposed to the LC50/48h caused a significant decrease in AChE and an increase in GST activities. Moreover, it generated a slight decrease in MDA rates, with an increase in total lipid contents. Regardless, juveniles of this non-target organism are sensitive to the high concentrations tested, compared with those obtained against some mosquito species, hence the need to take the necessary measures when using this EO as an alternative to chemical pesticides.

Keywords: *Gambusia affinis*, Acute toxicity, Botanical insecticides, Biochemical markers, Morphometrics, Essential oil

1. Introduction

Conventional insecticides have detrimental effects on the environment and human health (Benelli *et al.* 2021; Mavrogenis *et al.* 2023). As a result, there is a growing interest on exploring sustainable and eco-friendly alternatives for controlling vectors and crop pests (Benelli 2015a, 2015b; Benelli *et al.* 2015a, 2015b; Jeevitha *et al.* 2025). Plant-based products, such as botanical extracts and essential oils (EOs), constitute promising substitutes to synthetic pesticides (Karabörklü and Ayvaz 2023). They include rich sources and various bioactive components, many of which are selective and have little or no adverse effect on non-target animals and the environment (Govindarajan and Benelli 2016a). The bioactive components of essential oils have antioxidant, antibacterial, and larvicidal properties, with documented effects on fundamental metabolic, biochemical,

physiological, and behavioural processes of insects. Numerous plant extracts can function as toxicants, repellents, larvicides, or growth regulators, potentially serving as effective alternatives to conventional synthetic pesticides (Verheggen *et al.* 2022).

Laurus nobilis L. 1753 (Lauraceae), commonly known as the Noble Laurel, is an aromatic plant species native to the Mediterranean basin (Marzouki *et al.* 2011). Several studies conducted on laurel oil have revealed its antibacterial, antifungal, antioxidant, insecticidal, acaricidal and repellent properties (Aissaoui *et al.* 2023 ; Bouzidi *et al.* 2020 ; Fidan *et al.* 2019).

Oxidative stress occurs when ROS formation exceeds the capacity of cellular antioxidants to neutralize or remove them (Narra *et al.* 2017). This can affect macromolecules such as lipids, proteins, and nucleic acids. On the other hand, some biomarkers indicate potential neurotoxicity of xenobiotic molecules, using neurotransmitters such as acetylcholinesterase (AChE). Moreover, glutathione S-transferase enzymes (GSTs), another group of biomarkers, are cytosolic enzymes involved in phase II biotransformation of a vast range of contaminants (Mazari *et al.* 2023). Lipid peroxidation is a free-radical-mediated series of reactions that, once initiated, cause oxidative degradation of polyunsaturated lipids. Biological membranes' components are the most prevalent targets (Sule *et al.* 2022). For many years, malondialdehyde (MDA) has been employed as a convenient biomarker for lipid peroxidation, as it is one of the ultimate products of polyunsaturated fatty acid peroxidation in the cells (Zhang *et al.* 2019).

Teleost fish are often used as models in ecotoxicological studies, due to their relative abundance across many aquatic ecosystems (Cheghib *et al.* 2020). Western mosquitofish *Gambusia affinis* (Baird & Girard 1853 (Cyprinodontiformes, Poeciliidae), have been introduced in several countries and extensively used against mosquito larvae (Bendali *et al.* 2001; Zaidi and Soltani 2013). The mosquitofish *G. affinis* has been the subject of extensive research that has addressed a variety of topics (Denna *et al.* 2022 ; Huang *et al.* 2016). The data collected serves as an experimental foundation for the investigation of the adverse effects of xenobiotics on this non-target larvivorous fish species.

Many studies have investigated the potential hazards of some potent insecticides widely used for vector and pest control against *G. affinis*. Thus, inhibitors of chitin synthesis that are classified as benzoylphenylurea (Zaidi and Soltani 2013), or actara® a neonicotinoid insecticide (Cheghib *et al.* 2020) have been tested. In addition, recent studies have been conducted in our laboratory on the evaluation of the insecticidal activity of several EOs (essential oils) against mosquito vectors, among which a larvicidal activity of *L. nobilis* EO was found against two abundant mosquito species *Culiseta longiareolata* (Bouzidi *et al.* 2020) and *Culex pipiens* L. (Aissaoui *et al.* 2023). However, only a few studies have assessed their impacts on non-target organisms.

Therefore, the present study aimed to determine the chemical composition of EO from *L. nobilis* collected at a humid area (Northeast Algeria), to assess its toxicity and to evaluate its potential effect on different morphometric parameters as well as selected biochemical markers against a non-target mosquitofish *G. affinis* following an acute exposure. Prior to the implementation of this EO as a botanical insecticide against mosquitoes, knowledge on their potential impacts on this fish, considered as the best agent for biological control programs is needed.

2. Materials and methods

2.1. Sampling site

Juveniles of *G. affinis* were collected from a rather clean site, El Karma (El Hadjar) located in southern Annaba province (Northeast Algeria: 36°44.8080'N, 7°40.2960'E). Its area is around 600 square meters. Furthermore, there is no anthropogenic activities around the selected area (**Figure 1**).

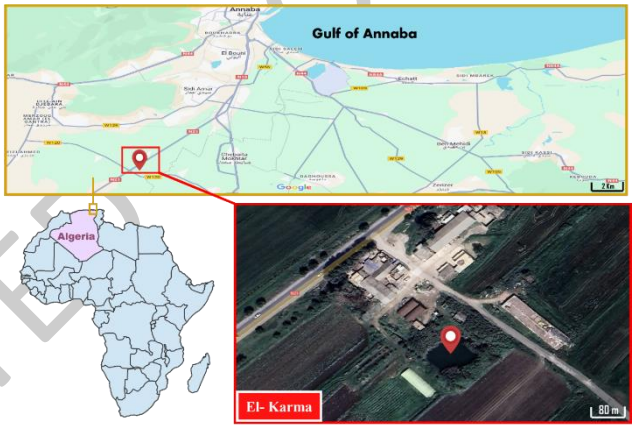


Figure 1. Localization of the sampling site of *G. affinis* juveniles at El Karma region in Annaba province (Algeria) (Google Earth Pro).

2.2. Plant material, essential oil extraction and yield calculation

Laurus nobilis leaves were gathered in the region of Seraïdi (36°55'N, 7°40'E) which is located in the north of the of Annaba province. The sampled leaves were authenticated at the herbarium that contains voucher specimens found in the Botany Department at the University of Badji-Mokhtar (Annaba, Algeria). The extraction of the essential oil was performed using a Clevenger-type hydrodistillation equipment for 3h. For each hydrodistillation, 200 g of the leaves were dipped in 1500 ml of distilled water. Once the essential oil and the aqueous phase separated, and in order to clear out any remaining water, the essential oil was dried over using anhydrous sodium sulfate. The latter was then stored in the darkness under refrigeration in order to be used for further analyses. According to the plant's dry matter content, the yield of the EO was calculated.

2.3. Gas chromatography – mass spectrometry analysis of the essential oil

The EO extracted from *L. nobilis* leaves was analyzed using gas chromatography-mass spectrometry (GC-MS) to

separate and figure out its constituents. The GC-MS was equipped with an HP-5MS column (5% phenyl-methylpolysiloxane; 30 m × 250 µm LD × 0.25 µm), added to an Agilent injection system (Agilent 19091S-433: 2169.66548). Helium (He) was used as a gas carrier with a flow rate of 0.9 ml/min, while the split ratio was set to 5:1. A volume of 0.2 µl of the oil sample was injected. The chromatogram was acquired by initially maintaining the oven temperature at 50°C for 1 minute, followed by a gradual increase to 300°C over 10 minutes at a rate of 10°C/min.

2.4. Fish collection and acute toxicity

The fish juveniles were captured and placed into proper buckets. They were then taken to the laboratory of Applied Animal Biology (Faculty of Science, University of Badji-Mokhtar Annaba, Algeria), to conduct the assays. Aquariums with a capacity of 80 liters were regularly aerated. Laboratory conditions like temperature, photoperiod and pH were constant. Individuals were kept for 10 to 15 days for acclimation, after which the acute toxicity tests were undergone. A control series consisting of untreated fish was used. After the EO was extracted, it was used to carry out preliminary acute toxicity tests at different concentrations (35; 45; 55; 65; 75 mg/L), and the lethal concentrations were determined following an exposure time of 48h. Three repetitions of every concentration were applied, with 10 individuals in each. Mortality was examined every day for 2 days (48h), with the cumulative mortality being calculated. The observed mortality rate was adjusted (Abbott 1925). Subsequently, the data underwent analysis of variance following the angular transformation of the observed mortality percentages. The different lethal concentrations as well as their confidence limits (95%) were determined. The Hill slope of the concentration-mortality curves was calculated.

2.5. Biochemical procedures

During the exposure, 15 treated and control individuals of *G. affinis* were used to quantify different biomarkers, in which we measured several morphometric parameters: Head Weight (Head. W), Body Weight (Body. W), Full Weight (Full. W), Head Length (Head L.), Body Length (Body L.) and Full Length (Full L.). These parameters and their correlations were assessed through the use of the Spearman's correlation coefficient and principal component analysis (PCA).

The enzymatic activity of AChE was measured using the technique described by Ellman *et al.* (1961), by using *G. affinis* juveniles' heads. In this context, once acetylcholinesterase, which is found in the brain's supernatant, combines with acetylthiocholine (Asch), there is a release of acetic acid and thiocholine (Sch). The specific activity of the biomarkers is expressed relative to the proteins, whose assay is performed using Bradford (1976) method, with absorbances measured at 595 nm wavelength.

Following the technique provided by Habig *et al.* (1974), the activity of the GST was measured using the whole bodies of *G. affinis* juveniles. In order to homogenize the tissues, 1 ml of phosphate buffer (0.1 M; pH 6) was used. To quantify the protein levels, Bradford's (1976) method was followed with bovine albumin as a standard. The absorbances were red at 340 nm wavelength.

Quantifying malondialdehyde rates was used to indicate a potential lipid peroxidation. The latter was measured following the technique provided by Draper & Hadley (1990), using entire bodies of *G. affinis* juveniles. The reaction between malondialdehyde and a component, thiobarbituric acid (TBA), was highlighted according to the amount of color generated. The absorbances were measured at a wavelength of 532 nm.

The lipid levels were determined following the vanillin-based technique described by the Goldsworthy et al (1972) method. Lipids from entire bodies of *G. affinis* juveniles were extracted according to the method provided by Shibko et al (1966). After each sample was homogenized in 1 ml of TCA (20%), they underwent centrifugation (5000 g for 10 min). The pellet was retrieved and then placed into a solution of ether and chloroform (v/v) before being centrifuged again (5000 g for 10 min). The obtained supernatant was used to quantify lipids, with absorbances being read at a wavelength of 532 nm.

2.6. Statistical Analysis

In this work, all statistical analyses were conducted using R, version 4.4.1 (R Core Team 2024; Ihaka and Gentleman 1996), and RStudio (Posit Team 2024) for Windows, except toxicity tests were performed using GraphPad Prism version 9 (GraphPad software, La Jolla, CA, USA). The Shapiro-Wilk test was employed to assess the normality of the variables. Data were presented as mean ± standard error (SE). The comparisons for each variable between groups were conducted using the nonparametric Kruskal-Wallis and Mann-Whitney rank sum tests. The Kruskal-Wallis test was succeeded by the nonparametric pairwise Dunn's test (with Bonferroni-adjusted p-values) to identify post-hoc statistical differences at a significance threshold of $\alpha = 0.05$. Correlations among the investigated variables were computed using Spearman's nonparametric correlation coefficient. To characterize both control and treated groups based on their morphometric parameters, assessing the potential effects of essential oil on the growth of *G. affinis* juveniles, we conducted a multivariate analysis utilizing principal component analysis (PCA) as the ordination technique. Our statistical analyses and data visualisation employed several R packages, including 'FactoMineR' (Lê *et al.* 2008), 'ggplot2' (Wickham 2016), 'dunn.test' (Dinno 2024), 'factoextra' (Kassambara and Mundt 2020), 'Hmisc' (Harrell 2025), 'ggcorrplot' (Kassambara 2023a), 'ggpubr' (Kassambara 2023b), 'psych' (Revelle 2023), and 'PMCMRplus' (Pohlert 2024).

3. Results

3.1. Yield and GC/MS analysis of L. nobilis EO

The hydrodistillation of *L. nobilis* EO had a yield percentage of 0.52 ± 0.04%. After hydrodistillation, the EO

was analyzed by GC/MS. The different constituents of the EO were identified according to their retention times (RT) using the MS library (**Figure 2**). The analysis identified 38 chemical compounds, constituting a total of 100.001% of the whole EO composition. The main constituents that were identified were namely: 1,8-cineole (21.900%), menthanyl acetate (27.150%), methyl eugenol (17.112%), linalool (8.410%) and sabinene (3.936%) (**Table 1**).

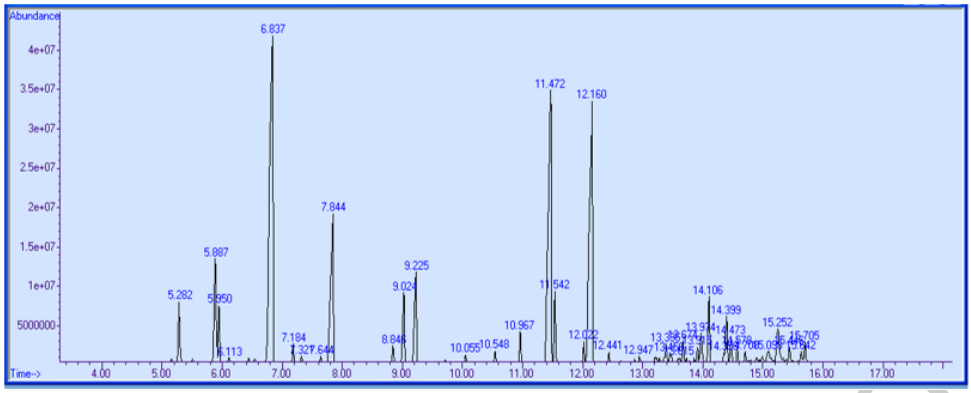


Figure 2. Gas-chromatogram identifying the different components of L. nobilis EO according to their retention times and their abundance.

Table 1. Chromatography-spectrometry (GC/MS) analysis of the chemical composition of *L. nobilis* essential oil.

N°	Retention time (Min)	Chemical compound	Concentration (%)
1	5.282	α-Pinene	2.208
2	5.887	Sabinene	3.936
3	5.950	β -Pinene	1.836
4	6.113	β -Myrcene	0.287
5	6.837	1,8 Cineole	21.900
6	7.184	γ-Terpinene	0.615
7	7.321	Trans Sabinene hydrate	0.282
8	7.644	α-Terpinolene	0.280
9	7.844	Linalool	8.410
10	8.846	Endo-Borneol	0.607
11	9.024	4- Terpineol	2.412
12	9.225	α-Terpineol	3.505
13	10.055	Lynalyl Acetate	0.255
14	10.548	Bornyl Acetate	0.370
15	10.967	Delta Terpinyl Acetate	0.927
16	11.472	Menthanyl Acetate	17.150
17	11.542	Eugenol	2.195
18	12.022	β- Elemene	0.610
19	12.160	Methyleugenol	17.112
20	12.441	Caryophyllene	0.362
21	12.947	Cyclodecene	0.314
22	13.395	Bicyclogermacrene	0.689
23	13.456	Phenol, 2,6-di-tert-butylphenol	0.514
24	13.615	Cubedol	0.310
25	13.674	Delta- Cadinene	0.722
26	13.915	α -Copaen-11-ol	0.474
27	13.974	Elemicin	1.156
28	14.106	Nerolidol	1.955
29	14.356	Germacrene D	0.340
30	14.399	(+) Spathulenol	1.477
31	14.473	Caryophyllene oxide	0.911
32	14.578	Veridiflorol	0.620
33	14.708	Ledol	0.403
34	15.098	T-Muurolol	0.721
35	15.252	α- Cadinol	2.140
36	15.446	Naphtalene	0.754
37	15.642	1(10),4-Cadinadien-8.α.-ol	0.446
38	15.705	Shyobunol	0.796
Total			100.001

3.2. Acute toxicity

Acute toxicity assays were conducted and different concentrations of *L. nobilis* EO were applied (35; 45; 55; 65; 75 mg/L) in 3 tanks, containing 10 juveniles each. The EO exposure for 24h to the aforementioned concentrations, revealed an LC_{50} = 55.93 mg/L. The exposure for 48h induced fish mortality, which exhibited a dose-response relationship (**Figure 3**). A significant effect of the concentration was determined by the statistical analysis. Different toxicological parameters such as lethal concentrations, their 95% confidence limits, and their slopes were determined (**Table 2**).

Table 2. Toxicological parameters of *L. nobilis* EO against juveniles of *G. affinis* after different exposure times: lethal concentration (LC, mg/L), 95% confidence interval (CI.), Hill slope, and coefficient of determination (R^2)

Exposure time (hours)	Slope	LC_{50} (mg/L)	LC_{50} CI (95%)	R^2
1	3.791	71.72	65.08 – 88.17	0.91
24	5.466	55.93	49.25 – 62.42	
48	5.581	49.48	41.93 – 56.46	

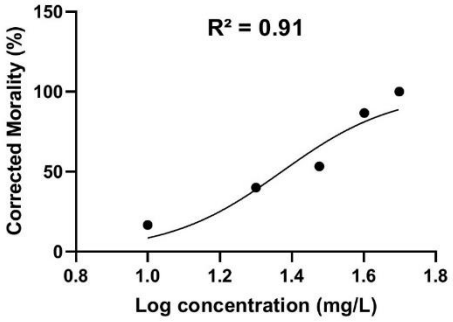


Figure 3. Toxicity of *L. nobilis* EO on juveniles of *G. affinis* at different concentrations (mg/L): sigmoidal dose-dependent response curve of corrected mortality (%) as a function of the decimal logarithm of concentrations.

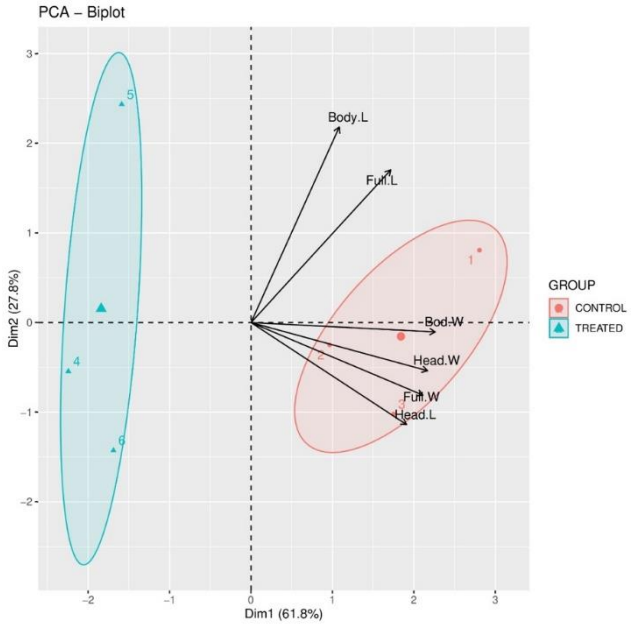


Figure 4. Principal component analysis (PCA) biplot of acute toxicity (control and treated series) and explanatory morphometric variables (*n* = 15). The biplot shows the PCA scores of the explanatory variables as vectors (in colors) and the toxicity experimental apparatus as points. Points aligned with a specified variable should be regarded as exerting a significant influence on it. The magnitude of the vectors indicates the intensity of their contribution to each axis. Colored concentration ellipses (0.95 probability level) illustrate observations pertaining to toxicity types categorized by mark class (Dim 1: 61.8% and Dim 2: 27.8%).

3.3. Morphometric parameters

In this study, we measured the following morphometric parameters: Head Weight (Head. W), Body Weight (Body. W), Full Weight (Full. W), Head Length (Head L.), Body Length (Body L.) and Full Length (Full L.) for each individual in both control and treated groups, as a way of determining the possible effects of the exposure of *L. nobilis* EO on *G. affinis* juveniles. The morphometric parameters were measured using graph paper and a precision balance (precision of 0.1°mg).

We used the Principal Component Analysis (PCA) because it is a real versatile statistical method. In our study we applied it as an exploratory and ordination technique and it allowed us to reduce the dimensionality of our morphometric dataset (based on 6 weight and linear variables) and to preserve the most important patterns of relationships between the analyzed variables measured on the mosquitofish individuals of *G. affinis* (Full.L, Head.L, Body.L, Full.W, Head.W and Body.W) according to the control and treated groups. The PCA outcomes provided an approximation of our original data on the morphological variation within and between treated and control groups. In a synthetic way, the PCA outcomes indicate that the first two axes explain 89.6% of the total inertia (data variance) present in our dataset, indicating that the biplot represents the almost of the morphometric variability (**Figure 4**). On the one hand, the 1st axis (Dim 1) alone, explains 61.8% of the total variation and show strongly positive correlation especially with: Body.W (r = +0.93; cos^2 = 0.86), Head.W (r = +0.89; cos^2 = 0.79), Full.W (r = +0.87; cos^2 = 0.75) and Head.L (r = +0.78; cos^2 = 0.61). On the other hand, the 2nd axis (Dim 2) precisely explains 27.8% of the total variation and it was also positively correlated with the two remaining linear variables: Body.L (r = +0.89; cos^2 = 0.80) and Full.L (r = +0.70; cos^2 = 0.49). Moreover, PCA show different patterns of correlations between the analyzed variables and the biplot revealed an important weight differentiation between the control group of *G. affinis* and the treated one; which was treated by an essential oil extracted from *Laurus nobilis* L. (Family Lauraceae). In conclusion, the 1st axis clearly distinguishes the control group from the treated one and reveals that the treated individuals are mainly characterized by weak weights. This result could be explained by the impact of our selected EO on the mosquitofish growth process.

In addition, a nonparametric correlation analysis was carried out to appraise potential relationships among the previously mentioned morphometric parameters measured on *Gambusia affinis* individuals following acute exposure to *Laurus nobilis* EO. In general, the two correlograms indicate that the calculated Spearman's correlation coefficients, between the morphometric parameters, are higher in the treated group compared to those calculated in the control group (**Figure 5**). As instance, in the treated series, Body W. was strongly correlated with Full W. (r = +0.95) whereas in the control group this correlation is slightly less (r = +0.83). This result could explain the growth imbalance (lack of proportion) between the control and treated individuals.

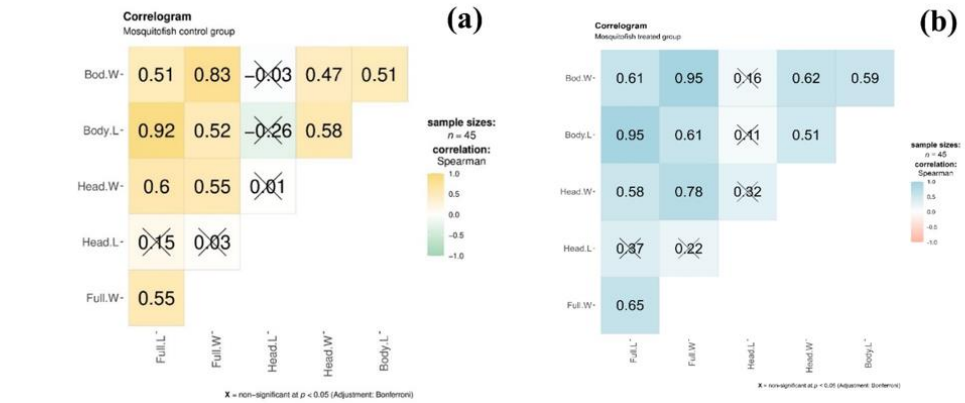


Figure 5. Spearman's correlation carried out on the morphometric parameters (weight and length) in control (a) and treated (b) juveniles of *G. affinis* after acute exposure to *L. nobilis* essential oil.

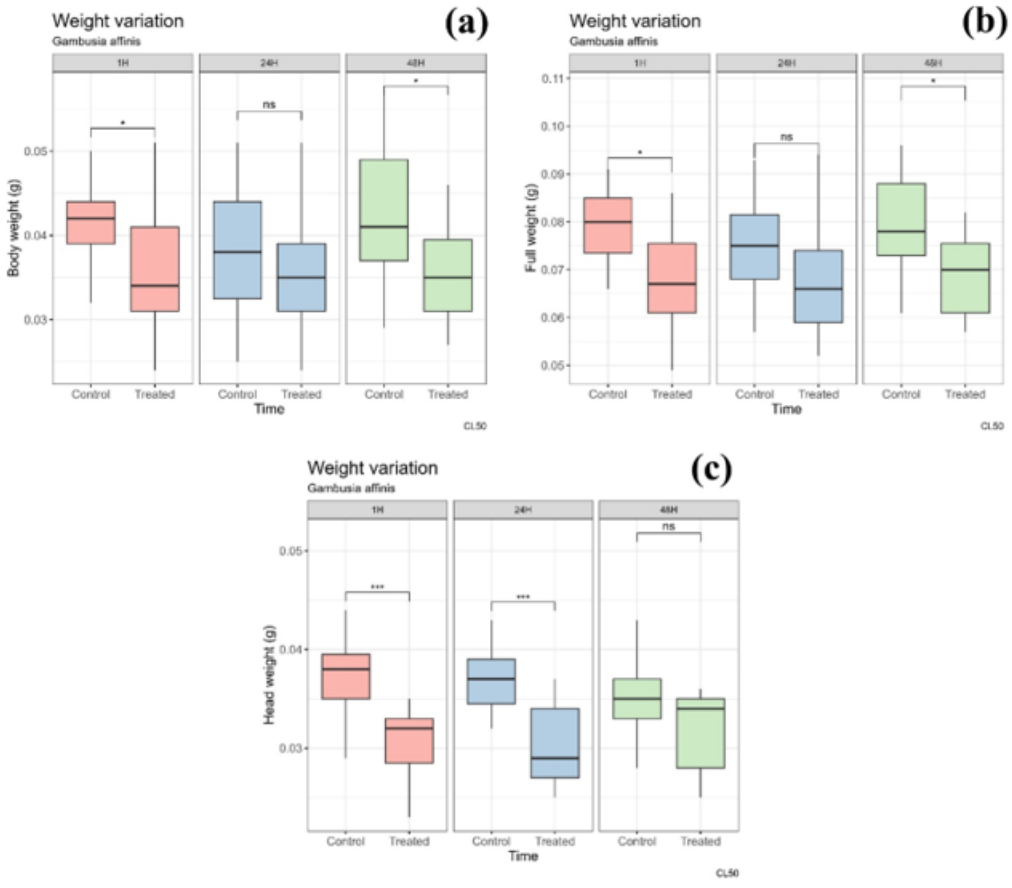


Figure 6. Variation in body weight (a), full weight (b) and head weight (c) parameters (g) after acute exposure to *Laurus nobilis* essential oil (n = 15, asterisks show the significant difference level reported by Mann-Whitney rank sum test). Box plots describe minimum and maximum values, median (thick central bar); central box limits show the interquartile range (IQR) with the first (lower box bound) and third (upper box bound) quartiles.

3.4. Weight variation

According to the PCA results; it was illustrated that the control individuals were characterized by higher weights compared to the treated ones. Regarding the Body W., a significant difference was found between the control and treated groups after 1H and 48H (p < 0.05). By contrast,

no significant difference was reported after 24H of exposure (p > 0.05, **Figure 6a**). The Full W. parameter showed the same variation throughout the experiment (**Figure 6b**). Meanwhile, the Head W. parameter showed highly significant differences between the control and the treated series after 1H and 24H of exposure (p < 0.05), although it showed no significant difference after 48H of exposure (**Figure 6c**). Moreover, Kruskal-Wallis test showed no significant differences between different treatment times (1H, 24H and 48H) in both control and treated series (for each variable).

3.5. Effects on environmental stress biomarkers

For the biomarker approach, it was found that AChE activity decreased in the treated series, compared to the control one, starting from 1H, up until 48h of exposure to the EO. In fact, the lowest values were observed after 1H. Mann-Whitney test revealed significant differences (p < 0.05, **Figure 7a**) between treated and control individuals for each exposure time.

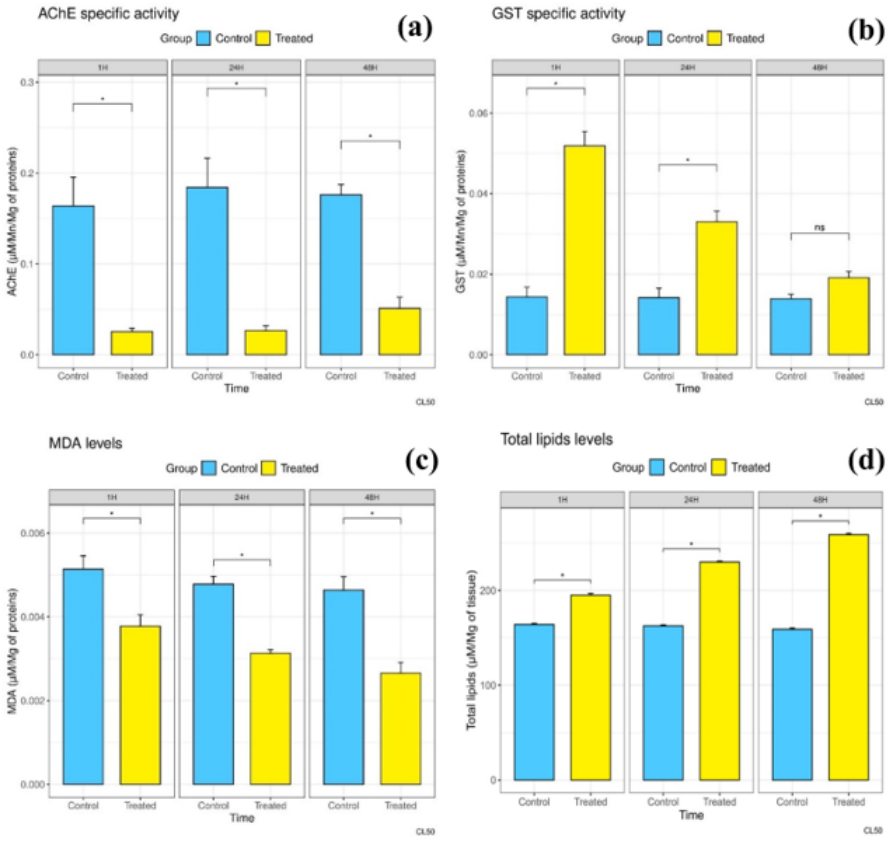


Figure 7. Bar plots showing the effect of *L. nobilis* essential oil on the specific activity of acetylcholinesterase (AChE) (µM/Mn/mg of proteins) (a), GST (µM/Mn/mg of proteins) (b), MDA (µM/mg of proteins) (c) and total lipids (µM/mg of tissue) (d) on *G. affinis* juveniles following different exposure times (m ± se, n = 5) for the same exposure time; asterisks above show the significant difference level between control and treated series reported by Mann-Whitney rank sum test at p < 0.05).

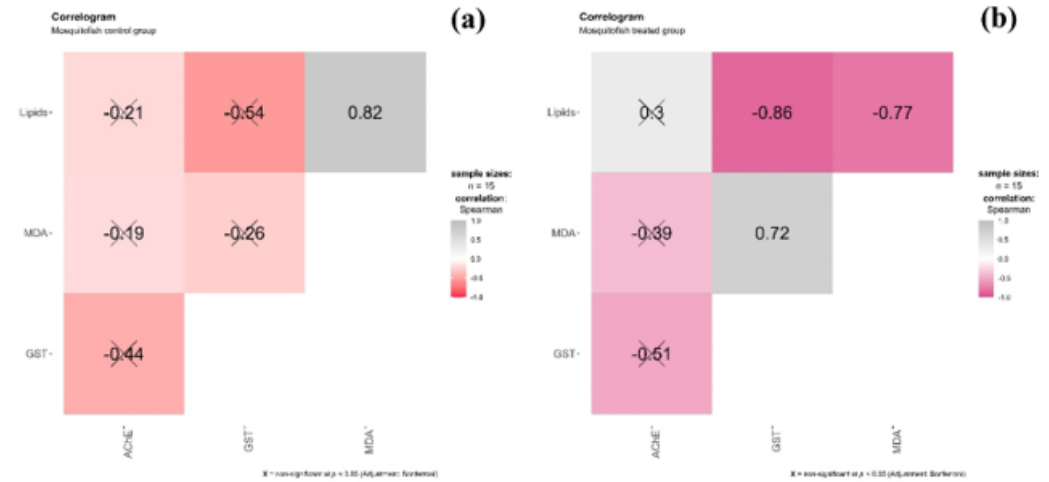


Figure 8. Correlograms showing the Spearman's correlation analysis carried out on biochemical biomarkers (AChE, GST, MDA and lipids) in control (a) and treated (b) juveniles of *G. affinis* exposed to *L. nobilis* EO.

With regard to the GST activity*;* after 1H as exposure time, it was reported that the GST activity difference in *G. affinis* juveniles was significant between control and treated groups ($p < 0.05$). Then, the GST activity gradually decreased over time, reaching its lowest level after 48H of exposure, indicating no significant differences between them ($p > 0.05$, **Figure 7b**).

Overall, a time-dependent gradual decrease in MDA levels was found in the treated series compared to the control one, starting from 1H of exposure, up until the end of the experiment. A significant difference was observed between treated and control individuals during the entire exposure period ($p < 0.05$, **Figure 7c**).

Furthermore, the total lipid content measured in *G. affinis* whole body remains significantly constant in control individuals. In contrast, a slight significant increase was recorded in treated groups with significant differences between control and treated values at all exposure times, with the higher content value at 48H ($p < 0.05$, **Figure 7d**).

Another Spearman's correlation analysis was performed between the different biochemical biomarkers measured in control and treated individuals. In the control series, a strongly positive correlation was indicated between MDA levels and total lipid contents ($r = +0.82$). Simultaneously, there was a non-significant negative correlation between GST and AChE levels ($r = -0.44$) (**Figure 8a**).

On the other hand, in the treated series, the correlation analysis showed a strongly negative correlation, firstly, between MDA levels and total lipid contents ($r = -0.77$), and secondly, between total lipids and GST rates ($r = -0.86$). Besides this result, a positive correlation was calculated between MDA and GST rates ($r = +0.72$) (**Figure 8b**).

4. Discussion

The hydrodistillation extraction of the essential oil from dry leaves of *L. nobilis* harvested in Seraïdi (Algeria) had an average yield of 0.52% (w/w). The estimated yield of the same plant species varies depending on the area from which the plant is collected. Bouzidi ***et al.*** (2020) reported a yield of 0.96% for the same plant species collected at Tébessa a semi-arid region (East Algeria). Aissaoui ***et al.*** (2023) also observed a yield of 0.79% (w/w) in the leaves of *L. nobilis* collected from Setif located in a semi-arid area (Algeria). Meanwhile, Haddadi ***et al.*** (2022) reported an average yield of 0.2% in Chlef (Center Algeria) and Tlemcen (Northwest Algeria). In fact, several factors including climate conditions, harvesting season, plant parts, soil quality, period of the extraction and even the extraction method itself can influence not only the yield but also the chemical constitution of *L. nobilis* EO (Bekhti ***et al.*** 2020). The current research identified 38 chemical constituents in *L. nobilis* EO collected in a humid area (Northeast Algeria). The main constituents identified were namely: 1,8-Cineole (21.900%), Menthanyl Acetate (27.150%), Methyl Eugenol (17.112%), Linalool (8.410%) and Sabinene (3.936%). Aissaoui ***et al.*** (2023) reported a similar result concerning *L. nobilis* growing at Setif (Center Algeria) located in a semi-arid area, with a total of 39 constituents. However, the main components identified were 1.8-Cineole (22.41%), Isolongifolene (10.22%), 3-carene (7.74%), alpha-zingiberene (6.64%) among others. Bekhti ***et al.*** (2020) identified 27 constituents in *L. nobilis* leaves collected from the west northern Algeria region. Similar to our results, the major components were mainly 1,8-cineole (30.1%), a-terpynil acetate (21.6%), methyl eugenol (16.9%) and others. A study conducted by Fidan ***et al.*** (2019) on *L. nobilis* EO in Bulgaria pointed out the main constituents being 1.8-cineole (21.2%), linalool (12.2%) and methyl eugenol (4.6%). Studies carried out on *L. nobilis* EO in some areas of Algeria revealed 1.8-cineole as the most dominant component with 22.41% in Setif (Center Algeria) area, and 31.61% in Constantine (Northeastern Algeria) (Chibani ***et al.*** 2024).

4.1. Acute toxicity of L. nobilis essential oil on juveniles of G. affinis

Laurus nobilis EO was reported to exhibit an insecticidal activity following a 24H exposure according to standard procedures recommended by the WHO (1996) in abundant mosquito species like *Culex pipiens* (Aissaoui *et al.* 2023; Menakh *et al.* 2024). Based on the LC observed against fourth instar larvae, Aissaoui *et al.* 2023 reported that *L. nobilis* EO showed insecticidal activity on *Culex pipiens* with an LC50-24H= 7.1 mg/L, while Menakh *et al.* 2024 found an LC50-24H= 31.94 mg/L. Regarding our results, juveniles of *G. affinis* seem to be less sensitive than fourth instar larvae of *Culex pipiens*, exhibiting a LC50-24H of 55.93 mg/L.

The acute toxicity of *L. nobilis* EO on the mosquitofish *G. affinis* juveniles revealed the following LC50 at different exposure times: LC50-24H= 55.93 mg/L and LC50-48h of 49.48 mg/L. Several other studies investigated the acute impacts of EO towards non-target organisms. Studies on the acute toxicity of *Thymus mastichina* and *Helichrysum italicum* EO on some freshwater species like *Daphnia magna* and *Thamnocephalus platyurus* (Arthropoda, Anostraca) and saltwater species like *Artemia sp*., were carried out, revealing their respective LC50. *D. magna* exposed to *T. mastichina* showed an LC50-24H of 153.0 mg/L, and a LC50-24H of 55.80 mg/L, after being exposed to *Helichrysum italicum* (Afonso *et al.* 2024). Govindarajan *et al.* (2016a) investigated the 48h toxicity of *Pinus kesiya* EO on three non-target organisms: *Anisops bouvieri* (Arthropoda: Notonectidae), *Diplonichus indicus* (Arthropoda: Notonectidae) and *G. affinis*, reporting LC50 values of 4135, 4545 and 8390 mg/mL, respectively. On the other hand, *Syzygium zeylanicum* EO toxicity was tested against *G. affinis*, revealing a LC50 value of 20,374 mg/L (Govindarajan and Benelli, 2016b). Govindarajan & Benelli (2016a) evaluated the toxicity of *Artemisia absinthium* EO on non-target organisms like *Chironomous circumdatus, Anisops bouvieri* and *G. affinis*, indicating LC50 values of 1040, 3132.70 and 4385 µg/ml, respectively. De Oliveira et al (2022) reported low toxicity of the EO of *Tetradenia riparia* against *G. affinis* with LC_{50} reaching 924.89 ppm. A study was conducted on the toxicity of *Kaempferia galanga* EO on some non-target organisms, revealing LC_{50} values varying between

2173.48 mg/L on *A. bouvieri* and 4773.04°mg/L on *G. affinis* (AlSalhi *et al.* 2020).

4.2. Effects of L. nobilis essential oil on morphometric parameters

The current study indicated that this EO had an impact on the morphometrics of *G. affinis*' juveniles, mainly the weight parameters (Body W.; Head W. and Full W.). Different results were reported by Roldan-Juarez *et al.* (2023), claiming that fish supplemented with a mixture of EOs exhibited a 16.9% increase in final body weight and a 10.43% increase in length in comparison to the control category. Another study showed that using *Cinnamon cassia* EO in silver catfish *Rhamdia quelen* diets demonstrated significant growth improvements, exhibiting significantly higher weight gain and length in the treated group (Bandeira-junior *et al.* 2022). Although EO and plant extracts are being used for their safety, very few studies reported their potential negative effects on non-target organisms. An investigation into the effects of garlic EO on *Heros severus* freshwater fish revealed that higher dietary levels of garlic EO led to a reduction in growth performance parameters, including final weight and length (Campelo *et al.* 2020). The negatively impacted weight parameters obtained in our results could be explained by the high concentration of the EO.

4.3. Effects of L. nobilis essential oil on biomarker responses

When organisms are exposed to pollutants, biochemical biomarkers are frequently evaluated, which initiates a series of biological responses that are induced by stress (Keles *et al.* 2023). The results obtained in this study revealed that *L. nobilis* EO caused the inhibition of AChE activity in the treated series of *G. affinis* juveniles, compared to the control one. This could be explained by the presence of monoterpenes in this EO. In fact, 1.8-cineole-rich EOs seemingly reduce AChE activity (Capatina *et al.* 2020). This agrees with the study conducted by Bullangpoti *et al.* (2018) which also indicated a strong inhibition of AChE activity due to 1.8-cineole exposure. An inhibition in AChE activity in zebrafish *D. rerio* after exposure to EO of *Thymus vulgaris* (Capatina *et al.* 2020) and *Citrus reticulata* (Brinza *et al.* 2024) was also noticed. The EO of *Ocimum gratissimum* on the pink shrimp *Farfantepenaeus paulensis* caused similar effects (Becker *et al.* 2021).

Glutathione S-transferases (GSTs) are one of the most abundant and ubiquitous enzyme families as they're engaged in various physiological activities (Tang *et al.* 2020). They perform an essential role in eliminating toxic compounds like heavy metals, pesticides or other pollutants (Mazari *et al.* 2023). Our study revealed that *L. nobilis* EO enhanced the activity of GST in treated juveniles, compared to control series starting the beginning of exposure. Our findings are in accordance with those of Brandão *et al.* (2022), observed with *Mentha piperita* EO on the fish *Colossoma macropomum*, and Saccol *et al.* (2016) in *C. macropomum* exposed to *Myrcia sylvatica* and *Curcuma longa* EOs. Studies carried out on silver catfish *Rhamdia quelen*, and the gilthead sea bream *Sparus aurata* also reported an induction in GST activity following an exposure to *Lippia alba* EO (Salbego *et al.* 2014; Toni *et al.* 2015). An experiment conducted on silver catfish *R. quelen* also indicated similar results after an exposure to *Melaleuca alternifolia* EO (Souza *et al.* 2018b).

Lipid peroxidation (LPO) is a complex chemical chain reaction that occurs due to the damaging effect of free radicals on polyunsaturated fatty acids within cellular membranes. It has been recognized as an essential biomarker of cell damage and dysfunction (Cordiano *et al.* 2023). Malondialdehyde (MDA), a by-product of LPO, is toxic and can therefore damage DNA and proteins. MDA levels are often used to reflect not only oxidative stress and LPO, but also the ability to remove free radicals. Our results showed that the exposure to *L. nobilis* EO led to a decrease in MDA levels and an increase in lipid levels in the treated series compared to the control one. Saccol *et al.* (2016) reported similar results, in *C. macropomum* after an exposure to *M. sylvatica* and *C. longa* EOs. Similarly, *Cymbopogon citratus* and *Pelargonium graveolens* EOs decreased MDA rates within the Nile tilapia (Al-Sagheer *et al.* 2018). On the other hand, Sönmez *et al.* (2015) observed comparable effects after exposing the rainbow trout *Oncorhynchus mykiss* to *Salvia officinalis*, *Mentha spicata* and *Thymus vulgaris* EOs. Moreover, exposing individuals of the silver catfish *R. quelen* to *Aloysia triphylla* EO, presented a lower LPO (Zeppenfeld *et al.* 2014). In fact, studies on fish exposed to EOs or alternative lipid sources, show changes related to lipogenesis and lipid storage. These changes often lead to increased triglycerides and adipose tissue hypertrophy, which are signs of lipid accumulation (Xu *et al.* 2022). The inverse relationship between malondialdehyde (MDA) levels and total lipid accumulation may result from reduced lipid peroxidation due to increased fat storage and enhanced antioxidant defenses. Higher lipid levels, particularly in adipose tissue, limit the exposure of polyunsaturated fatty acids to oxidative stress, while antioxidants like vitamin E and glutathione further mitigate peroxidation (de Paz *et al.* 2024). Similar studies in Nile Tilapia fish (*Oreochromis niloticus*) treated with *Laurus nobilis* EO found that antioxidant enzyme activities, including superoxide dismutase (SOD) and catalase (CAT), increased with essential oil supplementation, while malondialdehyde (MDA) levels decreased, indicating reduced oxidative stress (Shehata *et al.* 2025).

5. Conclusions

The *L. nobilis* EO tested against juveniles of the mosquitofish *Gambusia affinis* showed an impact on morphometrics, indicating weaker weights in the treated individuals. Also, there was an inhibition in AChE activity explained by the neurotoxicity of the EO and an induction in GST activity, manifesting oxidative stress. Meanwhile, it improved lipid peroxidation by reducing MDA levels and increasing total lipid contents. The results show that this EO has a slight impact on the growth process and biochemical biomarkers of *G. affinis* juveniles which were sensitive to the high concentrations of the EO. The

analyzed parameters could be suitable indicators for predicting the EO potential toxicity in aquatic ecosystems, which should be used carefully if applied as a biopesticide. For environmental risk assessment, further investigations are needed to assess the effect of this EO on reproductive events.

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