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Biological Activities of *Chamaecyparis lawsoniana* (A.Murray bis) Parl. and *Thuja plicata* Donn ex D.Don Essential Oils: Toxicity, Genotoxicity, Antigenotoxicity, and Antimicrobial Activity

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Abstract: Essential oils (EOs) derived from evergreen conifer trees are recognized as complex sources of agents with various biological activities. In this study, the biological activities of the EOs derived from *Chamaecyparis lawsoniana* (A.Murray bis) Parl. (CLEO) and *Thuja plicata* Donn ex D.Don (TPEO) were investigated. The third instar larvae of the fruit fly *Drosophila melanogaster* were used as a model organism to determine the in vivo toxicity, genotoxicity, and antigenotoxicity of five different concentrations of CLEO and TPEO, ranging from 0.19% to 3%. *Artemia salina* was used for acute toxicity assessment and determination of LC_{50} after 24 h and 48 h. The antimicrobial activity of the CLEO and TPEO was tested against fourteen bacterial and two fungal strains using the microdilution method. The larvicidal activity and developmental time of *D. melanogaster* increased as the concentrations of the EOs rose. CLEO and TPEO produced a dose-dependent increase in DNA damage compared with the negative control. Both oils, at concentrations up to 1.5%, demonstrated the ability to reduce the genotoxic effect induced by ethyl methanesulfonate, with a reduction rate exceeding 60%. CLEO and TPEO were highly toxic against *Artemia salina* nauplii. The results indicate that the tested EOs act as antimicrobial agents against some pathogenic bacteria and fungi. CLEO and TPEO show promising potential as a source of antimicrobial and antigenotoxic agents and as potential biocides against pest insects and arthropods.

Keywords: essential oil; *Artemia salina*; toxicity; genotoxicity; antigenotoxicity; *Drosophila melanogaster*; biopesticides

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1. Introduction

Species of the family Cupressaceae Gray (redwood or cypress family) are considered an important source of specialized bioactive metabolites, such as essential oils [1–3]. *Chamaecyparis lawsoniana* (A.Murray bis) Parl. (Port Orford cedar, ginger-pine) and *Thuja plicata* Donn ex D.Don (western redcedar, giant arborvitae) are two members of the Cupressaceae family with a natural distribution area in western North America [4]. *C. lawsoniana* is mainly limited to the Pacific Coast, from southwestern Oregon to northwestern California, while *T. plicata* has a somewhat broader distribution along the Pacific Coast from southeastern Alaska to northwestern California as well as inland, along the Rocky Mountains from

British Columbia to Idaho and Montana [5]. It is known that *C. lawsoniana* essential oil has been widely used in aromatherapy [6], and *T. plicata* essential oil is used to treat a variety of upper respiratory ailments and superficial wounds and as a natural insect repellent and wood preservative [7].

Some biological activities of *C. lawsoniana* essential oil (CLEO) and *T. plicata* essential oil (TPEO) have recently been published, with a primary focus on their antibacterial and antifungal activities [7–12]. In addition, the inhibitory effect of CLEO and TPEO was also reported against plant-damaging fungi, specifically *Phytophthora* [6]. Studies that seek alternatives to conventional antibiotics are invaluable in the post-antibiotic era, given the growing issue of (multi)resistance to these agents. Thus, new knowledge about the antimicrobial properties of natural, plant-derived materials such as essential oils (EOs), which have demonstrated their efficacy as promising candidates in antimicrobial application, is essential. Fast screening of the biological activities of EOs facilitates quick access to knowledge, setting guidelines for more in-depth analyses. For this purpose, an array of bioassays can be employed [13] using very reliable model organisms, such as the common fruit fly *Drosophila melanogaster* Meigen (Drosophilidae) [14–18] and the crustacean species brine shrimp *Artemia salina* L. (Artemiidae) [19–21]. Both are eukaryotic model organisms with short life cycles and are very affordable for laboratory husbandry, allowing the rapid acquisition of experimental results [13,22,23].

Furthermore, extrapolating the results obtained on these model systems, especially in *D. melanogaster*, has great potential. In that manner, the similarities with human physiology [24], including gene homology between *D. melanogaster* and humans [25], make fruit flies an excellent model system. Furthermore, the results obtained in experiments using these models have the potential to be extrapolated for use as biocides against pest insects and arthropods. However, in the available literature, there are no studies related to the biological activities of CLEO and TPEO strictly on *D. melanogaster* and *A. salina*. The most approximate study is about the influence of the essential oil of *C. obtusa*, a related species to *C. lawsoniana*, on the model insect *D. melanogaster* [15]. The EO of species related to *T. plicata*, particularly *T. occidentalis*, was reported as toxic to *A. salina* [19]. Thus, this study aims to test CLEO and TPEO biological activities, particularly toxic, genotoxic, and antigenotoxic, in *D. melanogaster* larvae, as well as toxicity against *A. salina* and their antimicrobial activity against human bacterial and fungal pathogens.

2. Materials and Methods

2.1. Plant Material and Isolation of EOs

The plant material (twigs with leaves) of *C. lawsoniana* and *T. plicata* was collected from adult individuals in a forest nursery near Belgrade (Sremčica) in May 2020. The twigs with leaves from the lower third of the crown of 5 randomly selected individuals per species were collected and stored as bulk samples in polyethylene bags in a freezer (at −20 °C) until further use. Vaucher specimens of both taxon *C. lawsoniana* and *T. plicata* (acquisitions numbers 18324 and 18325, respectively) were deposited in the "Herbarium Moesiacum Niš", Department of Biology and Ecology, Faculty of Sciences and Mathematics, University of Niš, Republic of Serbia. The plant material was identified by coauthor Dr B. M. Nikolić.

The twigs with leaves from both species were first cut to a length of 2–3 mm. Hydrodistillation was then performed using a Clevenger-type apparatus for 2 h. Diethyl ether was used to extract the obtained EOs and anhydrous magnesium sulfate for drying. After filtration, diethyl ether was removed under a gentle stream of nitrogen at room temperature to prevent any loss of the EO.

2.2. Assays in D. melanogaster Model

2.2.1. Fly Strain

The flies and larvae of the wild-type strain of *D. melanogaster* (Oregon-R-C strain (strain number 5) available from the Bloomington Drosophila Stock center at Indiana University, USA) were cultured under standard laboratory conditions. They were maintained at a

temperature of 25 °C, relative humidity of approximately 60%, and a 12:12 h light/dark regime. The culture medium consisted of a standard cornmeal-based *D. melanogaster* medium containing agar, corn, sugar, and yeast, with the addition of a standard fungicide.

2.2.2. In Vivo Genotoxicity and Antigenotoxicity

Phosphate-buffered saline (PBS) without calcium and magnesium, normal melting point agarose (NMA), low melting point agarose (LMA), and collagenase were obtained from Alfatrade Enterprise D.O.O., Belgrade, Serbia; ethyl methanesulfonate (EMS) was from Sigma-Aldrich, St. Louis, MO, USA. All other chemicals were obtained locally and were of analytical reagent grade.

The third instar larvae of *D. melanogaster* (74 ± 2 h) were placed in the standard feeding medium containing five different concentrations (0.19, 0.38, 0.75, 1.5, and 3%) of CLEO or TPEO dissolved in distilled water. They were allowed to grow on it for 24 h. Negative control larvae were grown on the standard feeding medium without EMS. EMS (1 mM in distilled water) was used as a positive control [26]. For the antigenotoxic study, essential oils in the same concentrations as for genotoxicity were co-administered with 1 mM EMS for 24 h. At 96 ± 2 h, the larvae were removed from the food and washed with 50 mM sodium phosphate buffer. The anterior midgut from the controls and treated larvae were removed from the media and dissected, and single-cell suspensions were prepared according to Howell and Taylor [27], modified by Mukhopadhyay et al. [28] and Siddique et al. [29].

The comet assay was performed according to Singh et al. (1988) [30] with minor modifications [28]. The images were visualized and captured with a 40× objective lens of a fluorescence microscope Nikon (Ti-Eclipse) attached to a CCD camera. One hundred randomly selected cells (50 cells per two replicate slides) were analyzed per treatment by a visual scoring method [31] and classified into five categories (T0—no or very low damage; T1, T2, and T3—low, medium and high DNA damage, and T4—highest level of DNA damage). The total comet score and percentage reduction (% R) in the total comet score were calculated according to Manoharan and Banerjee [32] and Waters et al. [33].

Data were analyzed using the SPSS statistical software package (version 13.0). One-way analysis of variance (ANOVA) followed by the T3 Dunnett test or with Bonferroni test for post hoc comparison between controls and treated groups was used. The results were statistically significant at $p < 0.05$.

2.2.3. Toxicity Assessment and Larvicidal Activity in *D. melanogaster*

Toxicity and larvicidal activity assessment of CLEO and TPEO against *D. melanogaster* larvae was conducted based on procedures described in previous studies [16,21]. Namely, young adult *D. melanogaster* flies, which had been previously mated, were allowed to lay eggs on a cornmeal-based feeding medium for a few hours, resulting in larvae of the same age. The larvae were collected and used in the experiment three days after hatching. Feeding media for the treated groups contained the tested EOs in final concentrations of 0.19%, 0.38%, 0.75%, 1.5%, and 3% (*v*/*v*). The experiment was carried out in three replicates for each concentration, with each replicate comprising 20 larvae. Daily observations were made during the developmental period, recording the number of pupated larvae and eclosed adults until all flies completed their development. The developmental time (DT) from larvae to pupae and larvae to adults (imago) was calculated using the formula $DT = \Sigma nd \times d/nt$, where 'd' represents the number of observation days after the larvae were transferred, 'nd' is the number of emerging flies or pupated larvae, and 'nt' represents the total number of individuals that were eclosed or pupated by the end of the experiment.

2.3. Acute Toxicity in *A. salina*

A. salina was hatched from lyophilized cysts (Dajana Pet, Bohuňovice, Czech Republic) in 48 h in an artificial seawater medium that was prepared according to Kester et al. [34]. Briefly, laboratory conditions were a strong airflow at a temperature of 28 °C with constant illumination. After hatching, larvae were transferred to Petri dishes containing 20 mL

of artificial seawater. EOs dissolved in dimethyl sulfoxide (DMSO) were also added in final concentrations (mg/mL) as follows: 0.0022; 0.0044; 0.0088; 0.0176; 0.0352; 0.0703; 0.1406; 0.2813, and 0.5625 for CLEO and 0.001; 0.002; 0.004; 0.0081; 0.0161; 0.0322; 0.0644; 0.1288; 0.2575, and 0.515 for TPEO. The final concentration of the solvent DMSO was less than 1% (*v*/*v*). During the treatment, the test organisms were not fed, the medium was not aerated, and they were reared at room temperature under constant illumination. The negative control was without EOs and contained only DMSO in final concentrations equal to the treatments, while sodium dodecyl sulfate (SDS) was used as a positive control. All tests were performed in three replicates. The effect of seawater on the nauplii (larvae) was checked by filling a Petri dish only with seawater in the same volume as for all the other Petri dishes. Immediately after treatment, 24 h and 48 h, survived and dead larvae were counted. The concentration lethal to 50% of brine shrimps (LC_{50}) was determined according to the Spearman–Karber method [35].

2.4. Statistical Analysis

For toxicity and larvicidal activity assessment in *D. melanogaster* and acute toxicity in *A. salina*, statistical data processing was performed by STATISTICA 8 software (Statsoft, Inc., Tulsa, OK, USA). The obtained results were analyzed with one-way ANOVA followed by Fisher's Least Significant Difference (LSD) post hoc test if significant.

2.5. Antimicrobial Activity

2.5.1. Microbial Strains

The antimicrobial activity of CLEO and TPEO was tested against fourteen Gram-positive and Gram-negative bacterial and two fungal strains. These strains belonged to the American Type Culture Collection (ATCC) reference strains or were isolated from human material of different origins, including nose, throat, ear swabs, sputum, or aspiration material. The strains belonging to the reference group were the following: *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella enterica subsp. enterica* (ATCC 13076), *Bacillus cereus* (ATCC 11778), *Escherichia coli* (ATCC 8739) and *Candida albicans* (ATCC 24433). The tested isolates were four strains of *S. aureus*, three strains of *P. aeruginosa*, two strains of *E. coli*, and one strain of *C. parapsilosis*.

2.5.2. Microdilution Method

Antimicrobial testing was performed using the microdilution methodology as described in our previous research [18]. Briefly, suspensions of bacterial or fungal cells were prepared from overnight cultures in a sterile physiological saline solution. The suspensions were adjusted to 0.5 McFarland units on a turbidimeter (DEN-1 McFarland Densitometer, Biosan, Riga, Latvia). The adjusted suspensions were then used to inoculate wells that contained double dilutions of CLEO or TPEO. Mueller Hinton Broth (MHB) was used for bacteria, and Sabouraud Dextrose Broth (SDB) was used for yeast cells. Stock solutions of the oils were prepared in DMSO (100%), and the final concentrations of the oils ranged from 0.001 to 20.0 mg/mL. Wells containing MHB/SDB + DMSO and MHB/SDB + DMSO + the test EO were also included as the growth and sterility controls, respectively. Positive control wells contained serial double dilutions of streptomycin for bacteria or nystatin for yeasts. Inoculated plates were incubated for 24 h at 37 °C. Following incubation, growth was visualized by adding triphenyl tetrazolium chloride (TTC, 0.5%). The wells with the lowest concentration of the oils where no growth (red-colored pellet) was observed were considered to contain minimal inhibitory concentrations (MICs) of CLEO and TPEO.

3. Results

3.1. Genotoxicity and Antigenotoxicity Assessment in D. melanogaster

The present study aimed to investigate the genotoxic effect and potential DNA protective activity of CLEO and TPEO against ethyl methanesulfonate (EMS)-induced DNA damage in the anterior midgut of third instar *D. melanogaster* larvae using the alkaline

comet assay. As a positive control, EMS induced a statistically significant increase in the total comet score compared to the negative control (Tables 1 and 2, group II vs. group I), as can be seen in the frequency of damaged cells that were classified into five types of comets. Out of the total number of observed cells, 12.48% were type T0, 81.23% were types T1, T2, and T3, and 6.29% exhibited the highest level of DNA damage (T4).

Table 1. Genotoxic and antigenotoxic effects of different concentrations of CLEO on *D. melanogaster*.

Groups [a]	Comet Types [b]					Total Comet Score	%R [c]
	T0	T1	T2	T3	T4		
I	79.59 ± 0.24	20.41 ± 0.51	/	/	/	20.41 ± 0.34 *	/
II	12.48 ± 0.23	30.80 ± 0.38	29.36 ± 0.21	21.07 ± 1.20	6.29 ± 1.54	177.89 ± 0.81 †	/
III	77.48 ± 0.57	22.52 ± 0.91	/	/	/	22.52 ± 0.51 †	/
IV	76.47 ± 0.12	23.53 ± 0.84	/	/	/	23.53 ± 0.37 †	/
V	78.46 ± 0.41	16.19 ± 0.80	5.35 ± 0.24	/	/	26.89 ± 0.41 *†	/
VI	72.18 ± 0.31	20.52 ± 0.41	6.18 ± 0.51	1.12 ± 0.81	/	36.24 ± 0.85 *†	/
VII	71.50 ± 0.28	19.32 ± 0.13	6.77 ± 0.40	2.41 ± 0.54	/	40.09 ± 0.23 *†	/
VIII	55.42 ± 0.17	33.35 ± 0.38	11.23 ± 0.16	/	/	55.81 ± 0.12 *†	77.52
IX	54.09 ± 0.35	32.50 ± 0.14	13.41 ± 0.23	/	/	59.32 ± 0.51 *†	75.29
X	52.47 ± 0.21	27.31 ± 0.18	18.72 ± 0.61	1.50 ± 1.21	/	69.25 ± 0.37 *†	68.98
XI	48.39 ± 0.12	25.30 ± 0.12	21.51 ± 0.27	4.80 ± 0.71	/	82.72 ± 0.81 *†	60.43
XII	32.75 ± 0.27	43.33 ± 0.73	16.51 ± 0.65	7.41 ± 0.51	/	98.58 ± 0.83 *†	50.36

[a] Values represented mean ± SD from three independent experiments; [b] Comet types defined as T0, T1, T2, T3, and T4 (no or very low damage, low, medium, and long DNA migration, and the highest level of DNA damage, respectively); [c] %R, percentage reduction in DNA damage; CLEO-Essential oils of *Chamaecyparis lawsoniana* (A.Murray bis) Parl.; I—Negative control. II—Ethyl methanesulfonate (EMS), 1 mM; III—CLEO 0.19%; IV—CLEO 0.38%; V—CLEO 0.75%; VI—CLEO 1.5%; VII—CLEO 3%; VIII—CLEO 0.19% + 1 mM EMS; IX—CLEO 0.38% + 1 mM EMS; X—CLEO 0.75% + 1 mM EMS; XI—CLEO 1.5% + 1 mM EMS; XII—CLEO 3% + 1 mM EMS; * $p < 0.05$ compared to negative control. † $p < 0.05$ compared to positive control.

Table 2. Genotoxic and antigenotoxic effects of different concentrations of TPEO on *D. melanogaster*.

Groups [a]	Comet Types [b]					Total Comet Score	%R [c]
	T0	T1	T2	T3	T4		
I	79.59 ± 0.24	20.41 ± 0.51	/	/	/	20.41 ± 0.34 *	/
II	12.48 ± 0.23	30.80 ± 0.38	29.36 ± 0.21	21.07 ± 1.20	6.29 ± 1.54	177.89 ± 0.81 †	/
III	78.46 ± 0.31	21.54 ± 0.61	/	/	/	21.54 ± 0.90 †	/
IV	76.32 ± 0.62	23.68 ± 0.82	/	/	/	23.68 ± 0.64 †	/
V	74.13 ± 0.23	25.87 ± 0.64	/	/	/	25.87 ± 0.47 *†	/
VI	71.74 ± 0.36	26.72 ± 0.23	1.54 ± 0.80	/	/	29.78 ± 0.57 *†	/
VII	75.04 ± 0.23	15.84 ± 0.82	7.32 ± 1.20	1.80 ± 1.03	/	35.88 ± 0.61 *†	/
VIII	68.57 ± 0.83	23.93 ± 0.33	7.58 ± 0.16	/	/	39.09 ± 0.22 *†	88.14
IX	64.35 ± 0.23	25.84 ± 0.34	9.81 ± 0.17	/	/	45.46 ± 0.52 *†	84.09
X	62.64 ± 0.82	24.82 ± 0.12	12.54 ± 0.22	/	/	49.89 ± 0.68 *†	81.28
XI	54.15 ± 0.37	36.91 ± 0.81	7.42 ± 0.34	1.52 ± 0.21	/	56.25 ± 0.21 *†	77.24
XII	42.30 ± 0.51	37.09 ± 0.34	14.40 ± 1.03	6.21 ± 1.12	/	84.52 ± 0.71 *†	59.29

[a] Values represented mean ± SD from three independent experiments. [b] Comet types defined as T0, T1, T2, T3, and T4 (no or very low damage, low, medium, and long DNA migration, and the highest level of DNA damage, respectively). [c] %R, percentage reduction in DNA damage; TPEO-Essential oils of *Thuja plicata* Donn ex D.Don; I—Negative control. II—Ethyl methanesulfonate (EMS), 1 mM; III—TPEO 0.19%; IV—TPEO 0.38%; V—TPEO 0.75%; VI—TPEO 1.5%; VII—TPEO 3%; VIII—TPEO 0.19% + 1 mM EMS; IX—TPEO 0.38% + 1 mM EMS; X—TPEO 0.75% + 1 mM EMS; XI—TPEO 1.5% + 1 mM EMS; XII—TPEO 3% + 1 mM EMS; * $p < 0.05$ compared to the negative control. † $p < 0.05$ compared to the positive control.

The CLEO did not exhibit a genotoxic effect at concentrations of 0.19% and 0.38% when compared to the negative control (Table 1, groups III and IV vs. group I). Notably, the CLEO demonstrated significant DNA protective potential when co-administered at these concentrations with EMS, leading to a substantial decrease in the total comet score

from 177.89 (EMS alone) to 55.81 and 59.32 (Table 1, group II vs. groups VIII and IX) with percentage reductions of 77.52% and 75.29%, respectively. At lower CLEO concentrations (0.19% and 0.38%), a high incidence of comets with no or very low damage (types T0 and T1) was observed, while comets with high DNA damage (type T3) were only seen at concentrations of 1.5 and 3%. Comets with the highest DNA damage (type T4) were not observed at any of the tested concentrations. Furthermore, treatment of third instar *D. melanogaster* larvae with 0.75%, 1.5%, and 3% of CLEO resulted in a slight but significant increase in the total comet score compared to the negative control (Table 1, groups V, VI, and VII vs. group I). However, these same concentrations significantly reduced DNA damage in the anterior midgut induced by co-treatment with EMS (Table 1, groups X, XI, and XII vs. group II) with percentage reductions of 68.98%, 60.43% and 50.36%, resp.

The TPEO did not induce DNA damage at concentrations up to 0.75%, as evidenced by the total comet scores, which were not significantly different from the negative control (Table 2, groups III and IV vs. group I). However, at the tested concentrations of 0.75%, 1.5%, and 3%, TPEO did result in a total comet score that was slightly different from the negative control (Table 2, groups V, VI, and VII vs. group I), but significantly less than the DNA damage induced by EMS alone (Table 2, groups V, VI, and VII vs. group II).

The statistically significant DNA protective potential was shown for the same EO applied at concentrations of 0.19%, 0.38%, and 0.75% with a percentage reduction of 88.14, 84.09, and 81.28%, resp. (Table 2, groups VIII, IX, and X) followed by treatment with a concentration of 1.5% with a percentage reduction of 77.24% (Table 2, group XI), whereas the 3% concentration exhibited weaker DNA protective activity with a percentage reduction level above 50%.

3.2. Toxicity and Larvicidal Activity in D. melanogaster

At a concentration of 0.19%, the percentage of pupated larvae in the CLEO treatment group was significantly lower ($p < 0.05$) compared to the control group, and at concentrations of 1.5% and 3%, it was drastically lower ($p < 0.001$) than in both the control group and the TPEO treatment group (Table 3). TPEO induced a significantly lower ($p < 0.001$) percentage of pupated larvae compared to the control only at the highest concentration of 3%. In the cases of the highest concentrations (3% and 1.5%) of both EOs, dead larvae became visible in the feeding medium a few days after the transfer. The trend of significance for the results regarding the percentage of eclosed adults is similar to that given for the percentage of pupated larvae. CLEO induced a significantly lower percentage of eclosed adults at 0.19% and 0.75% ($p < 0.05$) than the control group, as well as at 1.5% and 3% ($p < 0.001$) compared to both the control group and the group treated with TPEO. Conversely, TPEO resulted in a significantly lower percentage of eclosed adults compared to the control group at 1.5% and 3% ($p < 0.001$). Furthermore, in treatments with TPEO at all concentrations, apart from live adults, a few dead but fully eclosed adults were detected. This was more pronounced at the highest concentrations of 1.5% and 3%.

Developmental time (DT) needed for larvae pupation is given in days (see *Material and Methods* for details). At the 0.19% concentration, CLEO and TPEO both induced significantly prolonged DT compared to the control group ($p < 0.05$). This trend continued at concentrations of 0.38% and 0.75%, where both CLEO and TPEO resulted in significantly prolonged DT ($p < 0.01$). TPEO caused a significant delay ($p < 0.05$) in DT at a concentration of 1.5% compared to the control group. Similarly, the DT required for larvae to reach the adult stage was recorded in days. At concentrations of 0.19% and 0.38%, only TPEO caused a significant delay in DT compared to the control group ($p < 0.05$). This delay was more pronounced at concentrations of 0.75% and 1.5% ($p < 0.01$). CLEO caused a significant delay in DT at 0.38% ($p < 0.05$) and 0.75% ($p < 0.01$). However, due to extremely high larvicidal activity in the CLEO treatment at concentrations of 1.5% and 3%, as well as in the TPEO treatment at 3% concentration (as shown in % of pupated larvae in Table 3), there were insufficient data for further statistical analyses. Consequently, the statistical analyses of DTs for these concentrations were not conducted.

Table 3. Statistical analysis of the influence of CLEO and TPEO on *D. melanogaster* developmental time, eclosed adults and pupated larvae.

D. melanogaster Stage	EO Concentration	F [1]	*p* [2]	CLEO	TPEO	Control
Pupated larvae (mean in % ± SD)	0.19%	7.0	*	85.00 ± 5.00 ^{a}	90.00 ± 5.00 ^{ab}	98.33 ± 2.89 ^{b}
	0.38%	1.3	ns	91.67 ± 7.64	88.33 ± 10.41	98.33 ± 2.89
	0.75%	2.2	ns	81.67 ± 15.28	80.00 ± 13.23	98.33 ± 2.89
	1.5%	93.0	***	18.33 ± 10.41 ^{a}	83.33 ± 7.64 ^{b}	98.33 ± 2.89 ^{b}
	3%	38.3	***	6.67 ± 11.55 ^{a}	61.67 ± 18.93 ^{b}	98.33 ± 2.89 ^{c}
Eclosed adults (mean in % ± SD)	0.19%	6.4	*	78.33 ± 10.41 ^{a}	90.00 ± 5.00 ^{ab}	98.33 ± 2.89 ^{b}
	0.38%	1.4	ns	86.67 ± 11.55	86.67 ± 12.58	98.33 ± 2.89
	0.75%	3.7	*	76.67 ± 10.41 ^{a}	78.33 ± 15.28 ^{ab}	98.33 ± 2.89 ^{b}
	1.5%	79.0	***	13.33 ± 10.41 ^{a}	78.33 ± 10.41 ^{b}	98.33 ± 2.89 ^{c}
	3%	84.6	***	1.67 ± 2.89 ^{a}	56.67 ± 15.28 ^{b}	98.33 ± 2.89 ^{c}
Larvae to pupae DT (mean in days † ± SD)	0.19%	9.4	*	2.86 ± 0.13 ^{b}	3.32 ± 0.79 ^{b}	1.7 ± 0.18 ^{a}
	0.38%	15.0	**	2.61 ± 0.44 ^{b}	3.08 ± 0.26 ^{b}	1.7 ± 0.18 ^{a}
	0.75%	12.0	**	3.46 ± 0.84 ^{b}	3.42 ± 0.15 ^{b}	1.7 ± 0.18 ^{a}
	1.5%	3.5	*	n/a	3.94 ± 0.51 ^{b}	1.7 ± 0.18 ^{a}
	3%	1.4	n/a	n/a	n/a	1.7 ± 0.18
Larvae to imago DT (mean in days † ± SD)	0.19%	5.4	*	8.41 ± 0.28 ^{ab}	9.03 ± 0.94 ^{b}	7.51 ± 0.10 ^{a}
	0.38%	10.0	*	8.35 ± 0.65 ^{b}	9.03 ± 0.28 ^{b}	7.51 ± 0.10 ^{a}
	0.75%	13.4	**	9.55 ± 0.88 ^{b}	9.45 ± 0.33 ^{b}	7.51 ± 0.10 ^{a}
	1.5%	17.3	**	n/a	10.24 ± 0.42 ^{b}	7.51 ± 0.10 ^{a}
	3%	3.0	n/a	n/a	n/a	7.51 ± 0.10

[1] F: ANOVA F-test. [2] *p*: level of significance (ns: not significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$). Means with different superscript letters within the same row (^{a}, ^{b}, ^{c}) differ significantly (LSD post hoc test). The 0.19%–3% treatment concentrations *v*/*v*. †—represents the number of observation days after the larvae transfer. n/a—stands for not available due to insufficient data for analysis caused by high larvicidal activity. CLEO—*Chamaecyparys lawsoniana* (A.Murray bis) Parl. essential oil; TPEO—*Thuja plicata* Donn ex D.Don essential oil.

The influence of CLEO and TPEO on the DTs from larvae to pupae and from pupae to adult (imago) in *D. melanogaster* is presented in Figure 1. Both groups treated with CLEO and TPEO exhibited a similar pattern. As the concentrations increased, there was a noticeable delay in DTs, both from larvae to pupae and from pupae to adult (imago), as illustrated in Figure 1. However, due to the high larvicidal activity observed, there was insufficient data for the analysis of DTs at 3% and 1.5% concentrations for CLEO and 3% concentration for TPEO.

3.3. Acute Toxicity in *A. salina*

The assessment of acute toxicity involved determining the lethal concentration that kills 50% of the test organism (LC_{50} value) in µg/mL after 24 h and 48 h for both tested EOs using the model organism *A. salina* (Table 4). The results revealed significant differences in toxicity among the tested groups and between the tested groups and the positive control. Compared to CLEO, TPEO was much more toxic at the 24 h point. TPEO exhibited a slightly higher LC_{50} value but significantly greater toxicity than the positive control during the 24 h incubation period. After 48 h of incubation, TPEO remained much more toxic than CLEO and was slightly less toxic than the positive control.

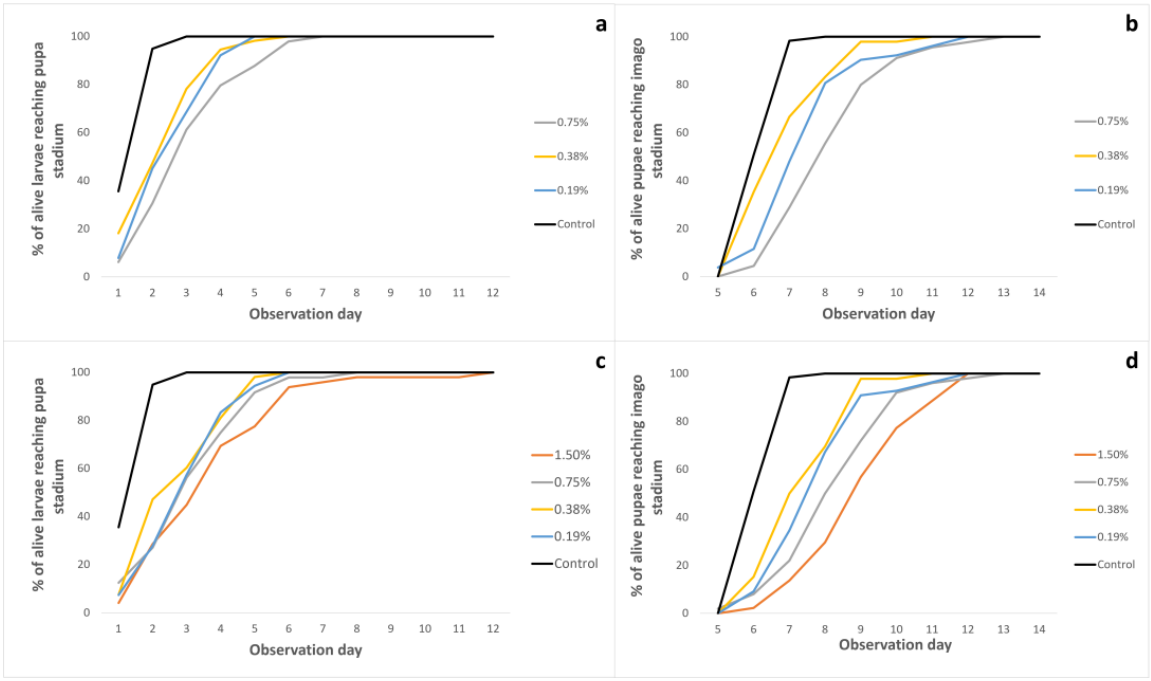

Figure 1. The influence of CLEO—*Chamaecyparis lawsoniana* (A.Murray bis) Parl. essential oil; TPEO—*Thuja plicata* Donn ex D.Don essential oil on *D. melanogaster* developmental time (DT). (**a**,**c**)—DT larvae to pupae; (**b**,**d**)—DT pupae to imago. (**a**,**b**)—0.19%–0.75% CLEO; (**c**,**d**)—0.19%–1.5% TPEO treatment. The control did not contain any tested EOs.

Table 4. Acute toxicity of CLEO and TPEO on *A. salina*.

	F [1]	*p* [2]	CLEO	LC_{50} (Mean in µg/mL ± SD)	
				TPEO	SDS [3]
after 24 h	1,103,101.6	***	53.29 ± 0.05 [c]	13.13 ± 0.03 [a]	13.30 ± 0.03 [b]
after 48 h	652,851.6	***	39.15 ± 0.06 [c]	10.67 ± 0.02 [a]	8.28 ± 0.02 [b]

[1] F: ANOVA F-test. [2] *p*: Level of significance (***: $p < 0.001$). Means with different superscript letters within the same row ([a], [b], [c]) differ significantly (LSD post hoc test). [3] SDS: sodium dodecyl sulfate (positive control). CLEO—*Chamaecyparis lawsoniana* (A.Murray bis) Parl. Essential oil; TPEO—*Thuja plicata* Donn ex D.Don essential oil.

3.4. Antimicrobial Activity

The present research aimed to assess the antimicrobial potential of CLEO and TPEO against a spectrum of human opportunistic pathogens, including Gram-positive and Gram-negative bacteria and fungi (Table 5). The microdilution assay results revealed that TPEO exhibited higher activity, with an average MIC of 10.5 mg/mL, inhibiting the growth of 93.7% of the tested microbial strains. Notably, only one strain of *P. aeruginosa* demonstrated resistance to the highest tested concentration of TPEO. Furthermore, TPEO displayed its highest potential against one *S. aureus* strain, with a 2.5 mg/mL MIC. On the other hand, CLEO exhibited antimicrobial action within the concentration range of 5–20 mg/mL with an average MIC of 15.9 mg/mL, but inhibitory action was found against 68.7% of the tested strains. Five strains that showed resistance to the highest tested concentration belonged to different species, including both Gram-positive and Gram-negative bacteria (Table 5). Nevertheless, the highest activity was detected against the yeast *C. albicans*. According to results from Table 5, the antimicrobial activity of both oils cannot be considered as selective based on cell type (bacteria or fungi), cell wall structure (Gram-positive or Gram-negative), or species.

Table 5. Minimum inhibitory concentration (MIC) of the CLEO—*Chamaecyparis lawsoniana* (A.Murray bis) Parl. essential oil; TPEO—*Thuja plicata* Donn ex D.Don essential oil.

Microbial Species	Origin	CLEO	TPEO	AB [1]/AM [2]
		MIC (mg/mL)	MIC (mg/mL)	MIC (µg/mL)
Staphylococcus aureus	ATCC 6538	>20	2.5	0.0031
Staphylococcus aureus	nose	20	10	0.0031
Staphylococcus aureus	nose	20	5	0.0016
Staphylococcus aureus	nose	20	10	0.0016
Staphylococcus aureus	nose	20	5	0.0016
Pseudomonas aeruginosa	ATCC 9027	20	20	0.0004
Pseudomonas aeruginosa	throat	10	>20	0.1000
Pseudomonas aeruginosa	sputum	>20	5	0.0016
Pseudomonas aeruginosa	sputum	>20	5	0.0031
Salmonella enteritidis	ATCC 13076	>20	10	0.0016
Bacillus cereus	ATCC 11778	>20	10	0.0016
Escherichia coli	ATCC 8739	20	20	0.0016
Escherichia coli	aspiration material	20	5	0.0016
Escherichia coli	aspiration material	10	20	0.0016
Candida albicans	ATCC 24433	5	20	0.0016
Candida parapsilosis	ear	10	10	0.0031

[1] AB—antibiotic streptomycin. [2] AM—antimycotic nystatin (used only for *Candida* strains).

4. Discussion

The chemical composition of CLEO and TPEO that were used in the present study and that were isolated from plant material of the same origin (forest nursery near Belgrade, May 2020) was previously analyzed by Nikolić et al. [6]. To summarize, CLEO consists of monoterpenes, sesquiterpenes, and diterpenes (40.8%, 30.3%, and 19.1%, respectively), with dominant compounds such as limonene (16.7%), oplopanonyl acetate (14.5%), beyerene (10.1%), and 13-epi-dolabradiene (6.7%). TPEO was found to be rich in monoterpenes (96.4%), with dominant components including α-thujone (76.9%), β-thujone (5.3%), sabinene (4.5%), and terpinene-4-ol (3.2%) [6]. Considering that elevated concentrations of plant-derived active components can exhibit toxicity in zoological systems [13] and the potential toxicity of the EOs against non-target vertebrates [36], it is imperative to establish their toxicity to determine potentially harmful doses for humans and other non-target vertebrates that may be exposed where EOs are to be used as biopesticides. In this context, and based on the results obtained, we will discuss the potential of the CLEO and TPEO as biocidal agents against tree or crop arthropod/insect pests. Additionally, we will explore the capacity of CLEO and TPEO as potential sources of antimicrobial and antigenotoxic agents with potential benefits for humans.

4.1. Genotoxicity and Antigenotoxicity of CLEO and TPEO in D. melanogaster

Neither CLEO nor TPEO caused significant DNA damage at concentrations of 0.19% and 0.38%. At higher concentrations (from 0.75% to 3%), they induced slightly higher DNA damage than the negative control but significantly lower than the positive control, suggesting a mild genotoxic effect only at higher doses. On the other hand, a significant DNA protective effect was observed at all tested concentrations, although the level of protection decreased as the concentration of EOs increased. Based on these data, neither of the EOs at concentrations of 0.19% and 0.38% demonstrated genotoxicity, and they had the highest DNA protective effect in *D. melanogaster* larvae. This notable DNA protective capability may be attributed to the dominant compounds in the selected essential oils.

The most dominant compounds of CLEO and TPEO were limonene and α-thujone, resp., as reported by Nikolić et al. [6], and these two EO-derived monoterpene compounds have been extensively studied for their activities in the available literature. In one study, limonene's capability to scavenge free radicals was supported [37], which may contribute to its DNA protective potential. Moreover, another study reported that limonene (+) demon-

strated in vitro and in vivo antioxidant activity [38], as well as anti-inflammatory properties [39]. However, a more recent study showed weak antioxidant activity of CLEO [2], suggesting that the antioxidant activity of this compound may differ when it is in isolation versus when it is present in a mixture with other components in EO. Relevant studies in the *D. melanogaster* model have shown that limonene possesses antimutagenic properties in addition to its antioxidant effects [40], corroborating the obtained antigenotoxicity results. Most of the available literature data indicate that limonene is not genotoxic. Bacanli et al. [37] observed no genotoxic potential in the lowest concentrations and noted limonene's DNA protective effect of limonene in human lymphocytes and Chinese hamster fibroblast cells using micronucleus and alkaline comet assays. Limonene was found to be non-genotoxic at low concentrations in the somatic mutation and recombination test (SMART) on *D. melanogaster* and exhibited DNA protective activity against H_2O_2 [41], consistent with our results. Additionally, CLEO demonstrated cytotoxic activity against human breast, colon, lung, and hepatocellular cancer cell lines by inhibiting cell proliferation and inducing apoptosis [2].

As the dominant compound in TPEO, α-thujone may play a role in the observed activities. Previous studies by Nikolić et al. [42,43], showed no genotoxic effects of thujone at the lowest concentrations and indicated its protective potential against 4NQO-induced DNA damage in a Vero cell line using the alkaline comet assay in post- and pretreatment procedures. Additionally, Pelkonen et al. [44] reported no genotoxicity of thujone in the micronucleus test on peripheral blood erythrocytes in male 6C3F1 mice. Pavlidou et al. [45] reported no genotoxic effects of thujone in the somatic mutation and recombination (SMART) test on *D. melanogaster*. Furthermore, lower concentrations of TPEO have been deemed safe for humans as they were found to be non-cytotoxic and did not induce DNA damage in human HEL cells [8]. However, at higher concentrations, TPEO can cause DNA damage and cytotoxicity in human HEL cells [8], which supports the results obtained in the present study. The literature also indicates that monoterpenes such as thujone exhibit cytotoxic activity against colorectal cancer cell lines [43], which is significant given that thujone is the dominant compound in TPEO. In a review by Jasuja et al. [46], thujone from *Thuja* species was highlighted as a highly promising anticancer compound with potential application in chemotherapy, corroborating the DNA protective capacity of TPEO in our results. Hence, concentrations of 0.19% and 0.38% of both CLEO and TPEO can be considered safe and non-genotoxic, with strong antigenotoxic potential for potential use in humans. However, confirmation through preclinical and clinical studies is necessary. On the other hand, the higher concentrations, from 0.75% to 3% had minor genotoxic effects (significantly below that of the positive control), which is consistent with the understanding that higher doses of essential oils or their components can potentially exhibit genotoxicity. Additionally, thujone has been reported to have mild genotoxic effects at higher doses, but at lower doses, it appears to have an antigenotoxic effect and enhances DNA repair mechanisms (NER, recombination, and MMR) [43].

4.2. Toxicity of CLEO and TPEO in D. melanogaster

Dietary exposure of *D. melanogaster* larvae to concentrations ranging from 0.19% to 3% of CLEO and TPEO revealed their potent toxic activity, particularly at higher concentrations. In the case of CLEO, larvicidal activity was strikingly evident, with over 70% and 95% reduction in pupated larvae observed at the two highest concentrations of 1.5% and 3%, respectively. TPEO, on the other hand, exhibited significant larvicidal activity only at a concentration of 3%, resulting in approximately 45% fewer pupated larvae compared to the control. Notably, qualitative differences in the effects of CLEO and TPEO on *D. melanogaster* were observed. CLEO displayed higher toxicity in terms of larval mortality than TPEO. However, TPEO treatment led to a more pronounced delay in developmental times, both from larvae to pupae and from larvae to adults. Additionally, adult mortality immediately after eclosion was observed only in the TPEO treatments. Consequently, fewer adults were recorded at these concentrations in both CLEO- and TPEO-treated groups. The

qualitative differences between the effect of CLEO and TPEO were also observed in a recent study involving the pest insect *Lymantria dispar* (gypsy moth), where CLEO exhibited a slight phagostimulatory effect at lower concentrations, resulting in a higher consumption rate, and TPEO had an antifeedant effect and lower relative consumption rate [6]. In the present study, the percentage of eclosed adults is directly related to the percentage of pupated larvae, and it is expected to follow a similar trend. At a concentration of 3%, CLEO exhibited the most vigorous larvicidal activity compared to TPEO and the control. These results collectively suggest a potent larvicidal activity against *D. melanogaster* larvae when they were exposed to dietary treatments of CLEO and TPEO at concentrations of 1.5% and 3%. In addition to larvicidal activity, pupal mortality (the difference between pupated larvae and eclosed adults) was more pronounced in the CLEO treatment than in the TPEO treatment. However, a few dead eclosed adults were observed only in the TPEO treatment (in all concentrations), particularly pronounced at the highest concentration. In line with recent studies of *Pinus* [16,47], *Pseudotsuga* [21], and *Abies* [18], EOs bioactivity against *D. melanogaster*, the CLEO and TPEO showed the highest larvicidal activity so far. The main difference between the mentioned EOs and the CLEO and TPEO is in EO composition, implying a significant contribution to the toxicity of thujones and limonene as major components in CLEO and TPEO. Notably, the results from this study indicate a clear trend of increasing developmental delays from larvae to pupae and from pupae to adult (imago) with rising concentrations in dietary treatments in both CLEO- and TPEO-treated groups.

EOs are known to target various physiological processes in insects, potentially disrupting their normal functioning. These mechanisms include the Regnault disruption of the GABA synapse, inhibition of P450 cytochromes, interference with the cholinergic system, and modulation of the octopaminergic system [36,48–50]. EOs and their components, including tetraterpenoids and monoterpenes, can disrupt the endocrine balance and act as neurotoxins in insects by inhibiting acetylcholinesterase activity [49]. One mechanism of neurotoxic activity, observed with some EO constituents such as the monoterpene thymol, involves disrupting GABA synapses in *D. melanogaster* [50]. Both CLEO and TPEO are rich in monoterpenes, especially TPEO (comprising 96.4%) [6], suggesting that this might be a potential mode of action in *D. melanogaster*. According to the results of this study, TPEO exhibited higher larvicidal activity compared to the control, although it was not significant up to a concentration of 1.5%. In contrast, CLEO showed pronounced larvicidal activity for most tested concentrations, peaking at 1.5% and 3% concentrations. Related studies have reported that at lower concentrations, CLEO was non-toxic to mice [51], and TPEO was non-cytotoxic and did not induce DNA damage in human HEL cells; however, toxicity was observed at higher concentrations [8]. Additionally, one study reported the potential therapeutic biological activity of TPEO on human skin cells [52]. (R)-(+)-limonene and (−)-α-thujone were tested in *D. melanogaster* as well as in *D. suzukii* (a soft crop insect pest) and were found to act as repellents against these insects [53]. Limonene has also been reported to cause morphological and physiological deformities in *D. suzukii* [54]. The inhibition of acetylcholinesterase activity by (−)-limonene in two pest insects, *Sitophilus oryzae* and *Tribolium castaneum*, has been documented [55], which might explain its action mode against *D. melanogaster*. CLEO has been reported as an effective repellent against other dipteran insects, such as mosquitoes [56]. This suggests that neither limonene nor α-thujone are attractive to fruit flies and may have toxic bioactivity against them. A highly relevant study concluded that the EO of the related species *C. obtusa* EO has insecticidal, repellent activity and affects the development of *D. melanogaster* [15], which is in line with the findings of our study. The compound α-thujone is a known neurotoxic compound for insects that acts as a modulator of GABA type A receptors [57], and it likely contributes to the observed bioactivity of higher concentrations of TPEO. This corroborated with a study by Enan [58], who reported that some components of plant EOs, such as the monoterpenoid phenol derivatives thymol and carvacrol, exhibit insecticidal activity in *D. melanogaster* by mediating through the tyramine receptor. Tyramine is a neuroactive ligand and a direct

precursor to octopamine, which is also a neuroactive ligand [58]. These mechanisms could potentially explain the action mode of the tested EOs and their components in our study.

Numerous studies have evaluated natural, plant-derived compounds intended for pest control [59–62]. In this regard, results obtained on the *D. melanogaster* model can be acknowledged, especially as a potential biocide, but extrapolation is also possible on the insect crop pests. The insecticidal effects of plant-derived EOs [14,17,63,64] and the mechanisms of action of the constituents of plant-derived EOs [50,58] have been frequently evaluated in the *D. melanogaster* model; thus, the fast screening of EO activity in this model organisms is of great importance because it contributes valuable new insights to this field.

4.3. Toxicity against A. salina

Toxicity in the brine shrimp assay was determined following the methodology described by Meyer et al. [13], where LC_{50} values less than 1000 µg/mL were considered toxic [13]. It was observed that TPEO exhibited significantly higher toxicity compared to CLEO after 24 h and 48 h. The lower dose of TPEO induced an LC_{50} value comparable to the control after 24 h, but after 48 h, the LC_{50} value induced by TPEO was slightly weaker than the positive control. Similar studies indicated the toxicity of the related *T. occidentalis* against brine shrimp [19,20]. The toxic activity of TPEO may be attributed to its specific chemical composition, which mainly consists of monoterpenes (96.4%) with dominant components α-thujone (76.9%) and β-thujone (5.3%) [6]. Thujone is considered a toxic component, and it likely contributes to the toxicity against *A. salina*. Furthermore, thujone, combined with other EO constituents, might be more toxic than thujone alone [20]. On the other hand, CLEO exhibited lower toxicity than the positive control at both the 24 h and 48 h checkpoints. It is worth noting that EOs from conifers, such as three Balkan *Abies* species [18] or *Pseudotsuga menziesii* [21], display significant toxicity against *A. salina*, often attributed to dominant constituents such as pinenes and sesquiterpenes. CLEO contains 30.3% sesquiterpenes [6], which could explain its strong toxicity against *A. salina*. Comparing the LC_{50} values induced by EOs from *Abies* species [18] and TPEO in the present study, it can be concluded that TPEO is more toxic against *A. salina*. Both tested essential oils had LC_{50} values much below 1000 µg/mL, indicating strong toxicity against *A. salina*. Given that *A. salina* is an arthropod species, these tested EOs should be considered potentially toxic against other arthropod pests.

EOs hold promise as botanical pesticides, offering the potential to improve the quality and safety of food production; however, specific challenges need to be kept in mind [36]. The global use of synthetic pesticides in treating pest arthropods and insects can potentially cause environmental and human hazards [49]. Additionally, issues related to the resistance of pathogens and insects to some pesticides are also well-known [36,65]. It is well-established that plants have naturally evolved self-defense mechanisms against insects and microbes, including the production of various plant metabolites and compounds found in plant essential oils [49]. In this context, the tested EOs or their individual components could be suitable candidates for arthropod and insect pest control in forestry and agriculture. However, it is important to emphasize that further studies are needed to validate this hypothesis.

4.4. Antimicrobial Activity

EOs have been established as significant sources of antimicrobial compounds. What sets them apart is their ability to target diverse cellular structures and processes, making it challenging for resistance to develop. Therefore, these natural mixtures of compounds represent fundamental research subjects in the realm of antimicrobial activity. The activity of CLEO and TPEO appears to lack selectivity based on cell type (bacteria or fungi), cell wall structure (Gram-positive or Gram-negative), or microbial species. Both tested EOs exhibited a moderate antimicrobial effect, with TPEO displaying greater activity and lower average MIC values. Considering previous studies on CLEO, the authors of [10] reported antifungal activity against zoospore germination and hyphal growth of *Phytophtora ramorum*. Palá-Paúl

et al. [12] detected higher activity of the oil from plants grown in Spain to Gram-positive strains. The oil mentioned (from a study by Palá-Paúl et al. [12]) exhibited inhibitory action against *B. subtilis*, *S. aureus*, and *Micrococcus luteus*, as well as against *C. albicans*, but the methodology (disk diffusion) differed from ours, and therefore, a comparison of the active concentrations is not possible. The oil from Spain [12] was rich in limonene (77.7%), *p*-Cymen-7-ol (3%), and myrcene (2.4%), which is a strikingly different profile from the main terpene compounds in the CLEO we tested, in which limonene (16.7%), sabinene (5.2) and terpinen-4-ol (5.6%) are the major compounds [6]. However, this oil showed very high activity against *C. albicans*, which correlates to our results here in which the CLEO demonstrated the highest potential against the same species. The effectiveness against candida may be attributed to the limonene content in the EOs. Limonene has been reported to have excellent activity against planktonic cells by inducing apoptosis, exhibiting inhibition across all stages of candidal biofilms, influencing the morphogenesis of this species, and having a synergistic effect when combined with fluconazol [66]. The isolated diterpene beyerene, which is one of the dominant components in CLEO, has been isolated and showed antimicrobial and antifungal activity as well [67]. The study of Smith et al. [11] showed that compounds isolated from immature cones' CLEO possess high antibacterial activity against *S. aureus* and that these compounds can modulate antibacterial resistance by inhibiting the NorA efflux pump activity. However, the action was highly variable among strains of the same species, which was notable in the case of both oils.

The first research on the antimicrobial potential of *T. plicata* reported the isolation of thujaplicins and their chelating action in *S. typhimurium* [68]. A later study reported antibacterial and antifungal activity of the TPEO from foliage samples collected in Poland, with MICs ranging from 0.50 to 1.25 mg/mL and 0.87 to 1.12 mg/mL, respectively [67]. Notably, this activity was higher than that exhibited by the TPEO we tested. The variation in chemical compositions between the two essential oils, including differences in major compounds, such as α-thujone, β-thujone, terpinen-4-ol, and sabinene [6,67], may account for these discrepancies. Additionally, differences in the microbial strains used in the studies could contribute to variations in sensitivity to the tested oils. Another study investigated the population dynamics of TPEO, revealing substantial efficacy against both capacity and growth rate in a dose-dependent manner against *S. aureus* and *E. coli* strains; however, *P. aeruginosa* demonstrated resistance to the oil's action [69]. In the study by Hudson et al. [7], concentrations from 0.1% to 1% were tested using different methodologies (liquid and vapor phase) against a panel of bacterial strains. The authors found good activity of this oil against vegetative cells, bacterial spores, and the fungus *Aspergillus niger*, but the chemical profile of the EO was not provided. As expected for natural products, the activities of both oils were much lower compared to the conventional antibiotic (streptomycin) or antimycotic (nystatin) used as the control in this study.

5. Conclusions

To our knowledge, this is the first record of toxicity, antigenotoxicity, and genotoxicity of CLEO and TPEO in the *D. melanogaster* model and acute toxicity assay in the *A. salina* model. The low LC_{50} values in the *A. salina* model suggest that both CLEO and TPEO exhibited significant acute toxicity against this arthropod. Additionally, the high larvicidal activity and delayed developmental time observed in *D. melanogaster*, especially at higher concentrations, indicate that CLEO and TPEO could be potential biopesticides for controlling insect pests. This could have implications for pest management in agriculture and forestry. Furthermore, the significant DNA protective potential demonstrated by both essential oils, up to a concentration of 1.5%, suggests that they may serve as a source of antigenotoxic agents. The observed antimicrobial activity further underscores the potential of CLEO and TPEO as viable sources of antimicrobial agents against pathogens in humans. Finally, the mild genotoxic effect observed at higher concentrations in the *D. melanogaster* model is important information for assessing the safety of these EOs. This study provides a foundation for future studies and potential applications of CLEO and TPEO as antigeno-

toxic and antimicrobial agents in insect and/or arthropod pest control. Further research and testing are needed to validate and optimize their use in proposed applications.

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