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Lethal Toxicity of *Thymus mastichina* and *Helichrysum italicum* Essential Oils to Non-Target Aquatic Organisms: Tools to Screen Environmental Effects?

Sandra Afonso [1,*], Juliana Nogueira [2], Carlos Cavaleiro [3,4], Fernanda M. L. Ferreira [2] and Matilde Moreira-Santos [1]

[1] CFE—Centre for Functional Ecology—Science for People and the Planet, Associate Laboratory TERRA, Department of Life Sciences, University of Coimbra, 3000-456 Coimbra, Portugal; matilde.santos@zoo.uc.pt
[2] CFE—Centre for Functional Ecology—Science for People and the Planet, Associate Laboratory TERRA, Department of Agricultural Sciences and Technologies, Coimbra College of Agriculture, Polytechnic Institute of Coimbra, 3045-601 Bencanta, Portugal; juliana_vnogueira@outlook.pt (J.N.); fferreira@esac.pt (F.M.L.F.)
[3] Faculty of Pharmacy, Health Sciences Campus, University of Coimbra, 3000-548 Coimbra, Portugal; cavaleir@ff.uc.pt
[4] Chemical Process Engineering and Forest Products Research Centre, University of Coimbra, 3030-790 Coimbra, Portugal
* Correspondence: safonso@uc.pt

Abstract: Essential oils (EOs) from *Thymus mastichina* (EO-thyme) and *Helichrysum italicum* (EO-curry) have wide commercial applications, but little is known about their ecotoxicity to aquatic life. We evaluated the lethal toxicity of both EOs toward standard freshwater (*Daphnia. magna* and *Thamnocephalus platyurus)* and saltwater (*Artemia* sp.) species. Dimethylsulfoxide was used as a solvent after establishing a maximum safe but effective concentration of 1% (*v*/*v*). EO-curry was significantly more toxic than EO-thyme (24–48 h LC50 values of 15.93–55.80 and of 84.78–153.0 mg L^{-1}, respectively) for all species; sensitivity ratios ranged from threefold for *D. magna* (48 h) and *Artemia* sp. (24 h) to fivefold for *T. platyurus* (24 h). *Artemia* sp. was the least sensitive, and *T. platyurus* was the most sensitive species, although significantly more so than *D. magna* only to EO-curry. The second major compound in EO-thyme, β-pinene (5%), is more toxic to aquatic life than major compound 1,8-cineole (62%), although 1,8-cineole facilitates penetration of other EO constituents into crustaceans' epidermis. Among the main compounds of EO-curry, only α-pinene (13%) is known to be toxic to aquatic organisms. However, minor compounds present in both EOs, like *p*-cymene (0.3–1.1%), also cause synergistic effects by enhancing the penetration of other EO constituents. Before any of these standard tests can be recommended for the ecotoxicity characterization and environmental management of EOs, their sensitivity to a wider range of EOs, at least from closely related families, needs to be assessed.

Keywords: aromatic and medicinal plants; water quality; terpenes; ecotoxicity; aquatic invertebrates

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1. Introduction

Essential oils (EOs) comprise a mixture of volatile compounds with aromatic characteristics, low molecular weight, and different structures. They are produced by the secondary metabolism of aromatic and medicinal plants and have a wide range of applications, such as in the food industry; in personal care products; for pharmacological, therapeutic, and veterinary purposes; and as biopesticides [1–4]. *Thymus mastichina* and *Helichrysum italicum* are aromatic plants native to the Mediterranean region, and their EOs (EO-thyme and EO-curry, respectively) are among those recently receiving more attention due to their multiple biological properties and uses [5,6].

Thymus mastichina (Lamiaceae) is a perennial herb indigenous to the Iberian Peninsula and known by various common names, such as "bela-luz", "sal-puro", "tomilho-alvadio-

do-Algarve", "mastic thyme", "white thyme", and "Spanish marjoram" [6,7]. There are over 220 species of this genus, and great diversity can be found in the Mediterranean region, particularly in the Iberian Peninsula [8,9]. The name thyme originates from the Greek word "thymon", which means to fumigate, reflecting its historical use by ancient Greeks in burnt sacrifices; it is also associated with courage, elegance, and grace [10]. Traditional uses of *T. mastichina* include in culinary applications to replace salt and, due to its flavoring properties, and as herbal medicine to treat digestive, respiratory, and rheumatic disorders [6,9]. Owing to its aromatic compounds, *T. mastichina* is also used to extend the shelf life of food products as an alternative to synthetic additives [1,11]. The EOs and/or extracts derived from o *T. mastichina* have demonstrated a wide range of biological activities, including antibacterial, antifungal, insecticidal, repellent, herbicidal, antioxidant, anticancer, antiviral, anti-Alzheimer, and anti-inflammatory effects [7,12,13]. The EOs are usually obtained by hydrodistillation from the aerial parts of the plant [7], and the main constituents consist of 1,8-cineole (or eucalyptol), linalool, limonene, camphor, borneol, and α-terpineol [7,9]. However, the composition may vary depending on the origin, and three main subtypes are recognized based on the predominant compounds: 1,8-cineole, linalool, and 1,8-cineole/linalool [7].

Helichrysum italicum (Asteraceae) is a flowering plant native to the Mediterranean region. It is commonly known as "perpétua-das-areias", "curry plant", "everlasting", and "immortelle" (the inflorescences maintain their form and color, even when dried) and belongs to the genus *Helichrysum* (Miller), which comprises over a thousand taxa [5,14]. This species name is derived from the Greek words "helios", meaning sun, and "chryos", meaning gold, alluding to the color of its inflorescences [5]. The plant and particularly its EO are used in traditional medicine to treat conditions mostly related to skin, digestive, and respiratory issues, being highly valued in aromatherapy [5,14]. The EO is rich in valuable antioxidant compounds and has been proposed as a flavoring agent and a bioactive ingredient in food products [5,15]. Its compounds have demonstrated several biological properties, such as antimicrobial, anti-inflammatory, antiviral, antioxidant, antihyperglycemic, anti-inflammatory, antineoplastic, and insecticidal actions [15–17]. The EO is obtained from the inflorescences usually by steam distillation, and its composition exhibits high variability depending on environmental factors, with monoterpenes and sesquiterpenes being the major constituents [5,16]. In effect, several chemotypes have been attributed to EO-curry based on the main constituents and their concentrations [15].

The available data suggest more than 104,000 tonnes per year of global EO production based on estimates for the top 20 EOs, with the US and China being the main producers [18]. Global EO production seems to largely target the flavor and fragrance industries, specifically cosmetics, perfumes, soft drinks, and food [18]. Although North America appears to dominate the global EO market, the European market is on the rise and is expected to increase at a faster annual rate of 9.6% from 2022 to 2029 [19]. Given the widespread marketing and broad application range of EOs, triggered by the rising demand for sustainable natural products, particularly from plants [20], their increasing use can potentiate adverse effects on the organisms that inhabit ecosystems [21–23]. This can be particularly threatening to aquatic systems, which serve as receiving environments for the industrial and domestic effluents [24] in which EOs can be found. According to research on water contamination by several classes of products, the rate of removal of volatile organic compounds and fragrances from wastewater is often high (>70%) during conventional treatments [25,26]. However, certain fragrances are suspected to be persistent and accumulative due to their chemical structure and lipophilicity; thus, more knowledge on the physicochemical properties of these products is crucial in order to establish efficient methods for their wastewater removal [27]. Although the literature on environmental water concentrations of EO constituents is scarce, a recent study by Musee et al. [25] reported the occurrence of several aromatic compounds, including from plant sources, used in sanitizers and disinfection products in several environmental matrices, including effluents, surface and ground water, rivers, and marine waters. The concentrations reported from available

data were mostly below 100 μg L^{-1} but, at times, close to 100 mg L^{-1}, indicating that environmental concentrations may be currently low, although it is essential to evaluate the effects on aquatic ecosystems. The vast majority of studies on the toxic potential of EOs have focused on their cytotoxicity, using cell lines to evaluate the potential for inhibition of cell proliferation or damage to cells [4,28], with fewer studies on their ecotoxicity on aquatic biota [21]. Therefore, in line with the worldwide increase in the use of aromatic and/or medicinal plant extracts or EOs, further investigation is necessary to assess their ecotoxicity to aquatic organisms. It is also essential to conduct toxicity testing using key functional/taxonomic organisms to obtain a comprehensive understanding of the potential ecological impacts. Non-target aquatic organisms used in ecotoxicity screening to evaluate the adverse effects of herbal products on aquatic ecosystems typically include crustaceans *Daphnia magna* (Anomopoda) and *Artemia salina* (Anostraca) and fish *Danio rerio* (Cypriniformes) [21,29,30], with the ranking of organism sensitivity depending not only on the tested product but also on the test organism functional group, species, and strain [31,32]. In effect, the available data are not clear on the sensitivity of aquatic organisms to EOs, although generally, *Daphnia* species are known to be highly sensitive indicators of aquatic system health [33] and have been reported to be sensitive to plant products [21,34], whereas *Artemia* species are commonly considered the least sensitive organism among aquatic invertebrates [30]. *Danio rerio* embryotoxicity testing seems to hold potential for toxicity screening of a wide range of herbal products [29]. Few studies have used *Thamnocephalus platyurus* (Anostraca) to assess the toxicity of plant products in the aquatic environment, although Mayorga et al. [35] found *T. platyurus* to be more sensitive in detecting the lethal toxicity of plant extracts compared to *A. salina*.

The mode of action and toxicity of EOs to aquatic organisms can be attributed to specific interactions of their compounds with physiological processes of the organisms [36,37]. Different chemical constituents within EOs can exhibit varying degrees of toxicity and act through different mechanisms, such as inhibition of acetylcholinesterase and carboxylesterase enzymes, as reported in fish [36], or by affecting the cell lipid profile, increasing cell membrane permeability, and inducing DNA damage, as reported in microorganisms [38,39]. Furthermore, the presence of multiple compounds within EOs can result in synergistic or antagonistic interactions that influence the overall toxicity [40,41]. Understanding the mode of action and toxicity of EO compounds in aquatic organisms is essential for assessing their ecological risks and implementing appropriate management strategies. For instance, limited research is available on the toxicity of EO-thyme or its constituents to aquatic organisms, with a study on its toxicity to *A. salina* [42] and another on the toxicity of monoterpene 1,8-cineole (a common major compound) to the guppy fish (*Poecilia reticulata*) [36]. Regarding the toxicity of EO-curry, little information is available, with studies evaluating its toxicity to crustaceans *D. magna* [43] and *A. salina* [44] generating contradictory results. Furthermore, based on the disclosed (scarce) information and/or data exploration, it is generally difficult—or virtually impossible—to establish a connection between the ecotoxicity of EOs and their compositions.

Given the above, the main aim of the present study was to assess the short-term lethal ecotoxicity of two widely commercialized EOs to aquatic organisms in order to contribute to the knowledge of potential environmental adverse impacts of their use, which is exponentially increasing. To fully attain the study objective, *T. mastichina* and *H. italicum* EO toxicity was evaluated using both freshwater and marine standard test species so that sensitivity differences could be explored, as well as the potential of such tests for future use in the ecotoxicity characterization and environmental impact studies of EOs with closely related compositions. The test species included *D. magna* and *T. platyurus* (both freshwater species) and saltwater species *Artemia* sp. *Daphnia* sp., particularly *D. magna*, are recommended organisms at the regulatory level for ecotoxicity evaluations of chemicals and other substances in aquatic systems [45–48]. Together with *D. magna*, *T. platyurus* and *Artemia* sp. have the considerable advantage of being test organisms that can be obtained from stored dormant eggs, making them a cost-effective and easily accessi-

ble option for the preliminary toxicity screening of toxic products, including EOs [29,35]. Therefore, the short-term lethal toxicity of both EOs was determined on *D. magna* after 48 h of exposure and on *T. platyurus* and *Artemia* sp. after 24 h of exposure. Additionally, the compositions of the EOs were determined by a combination of gas chromatography (GC-FID) and gas chromatography–mass spectrometry (GC/MS) to explore the association between the toxicity and chemical compositions of both EOs while providing valuable insights for future studies. In addition, toxicity tests with the three species were first carried out with organic solvent dimethyl sulfoxide (DMSO) to establish the maximum non-lethal concentrations to be used in the EO-thyme and EO-curry tests and even in future lethal studies with these organisms, particularly with EOs.

2. Materials and Methods

2.1. Essential Oils Origin and Chemical Composition

The EO-thyme and EO-curry used in the present study were produced and supplied by a Portuguese company ([a].TM010718 and HI010718, respectively; Planalto Dourado, Pinhel, Portugal). According to the information provided by the supplier, both EOs presented a density of 0.9 g mL^{-1} and were extracted by steam distillation—the EO-thyme from the aerial parts of *T. mastichina* and the EO-curry from the inflorescences of *H. italicum*.

The qualitative and quantitative characterization of the composition of the EOs were determined both in the original products to interpret toxicity results more comprehensively and in the stock solutions prepared using DMSO solvent (see Section 2.2). The rationale for the latter analysis was to ensure a closer estimation of the actual concentrations of the major compounds to which the organisms were exposed, although these results also reveal the bias introduced by the errors propagated in the analysis of the stock solutions. The characterizations were conducted by a combination of GC-FID and GC-MS. GC-FID analysis were performed on a Hewlett-Packard 6890 (Agilent Technologies, Palo Alto, CA, USA) gas chromatograph, with a single injector and two flame ionization detectors (FID) for simultaneous sampling on two different columns (SPB-1: polydimethylsiloxane 30 m × 0.20 mm i.d., film thickness 0.20 μm; and SupelcoWax-10: polyethyleneglycol 30 m × 0.20 mm i.d., film thickness 0.20 μm) (both from Supelco, Bellefonte, PA, USA). An HP GC ChemStation Rev. A.05.04 data system was used for operation and data handling. The determinations were conducted under the following conditions: oven temperature program, 70–220 °C (3 °C/min) and 220 °C (15 min); injector temperature, 250 °C; carrier gas, helium (adjusted to a linear velocity of 30 cm s^{-1}); detector temperature, 250 °C. GC-MS analysis was performed with an Agilent 6890 gas chromatograph interfaced with an MSD 5973 mass-selective detector (Agilent Technologies), both operated using HP Enhanced ChemStation software, version A.03.00. An HP1 fused silica column (polydimethylsiloxane 30 m × 0.25 mm internal diameter; film thickness, 0.25 μm) was used. GC parameters were as described above, whereas MSD parameters were set as follows: interface temperature, 250 °C; MS source temperature, 230 °C; MS quadrupole temperature, 150 °C; ionization energy, 70 eV; ionization current, 60 μA; scan range, 35–350 units; scans, 4.51 s^{-1}.

Samples of the original EOs were diluted (1:8) in *n*-pentane and injected (0.2 μL) in split mode (1:40). Identifications of the EO components were achieved by considering the following concurrently: (1) the acquired retention indices on both SPB-1 and SupelcoWax-10 columns determined by linear interpolation relative to the retention times of C8–C24 of *n*-alkanes compared with reference data from authentic products (available in the laboratory database of the Faculty of Pharmacy, University of Coimbra) and literature data; and (2) the acquired mass spectra compared with reference data from the literature [49–51]. Relative amounts (%) of each component of the original EO products were calculated from GC-FID raw data without any correction, and results were also expressed in mg L^{-1} considering the oil density provided by the supplier (0.9 g mL^{-1}).

For the quantitative determination of nine of the major compounds in the EO stock solutions (in DMSO), a calibration curve (R^2 = 0.9428) was first generated using DMSO solutions of 1,8-cineole at five levels of concentration (ranging from 0.00352 to

0.07033 mg mL^{-1}), with 10 µL mL^{-1} of cyclohexanol added as an internal standard. The same concentration of cyclohexanol (10 µL mL^{-1}) was also added to the samples. Quantitative results are expressed as mg mL^{-1} equivalent 1,8-cineole (MW = 154.249 g mol^{-1}). The limit of detection and limit of quantification for 1,8-cineole were 1.35×10^{-4} mgL^{-1} and 4.50×10^{-4} mgL^{-1} calculated with respect to signal-to-noise ratios of 3:1 and 10:1, respectively. For comparative purposes (with levels in the original products and with literature data), final values are expressed according to the concentration of each component in the DMSO stock solution (as % v/v) and in mg L^{-1} (considering the density of the EO).

2.2. Dimethyl Sulfoxide Testing

Toxicity tests were conducted to assess the toxicity of the DMSO solvent, i.e., to establish the maximum non-lethal concentration that could be used for each test organism in the toxicity tests with the EOs. DMSO is a commonly used and effective solvent for evaluating cytotoxicity in cell line studies [52] and, recently, has been more frequently used in ecotoxicity tests with invertebrates [53,54]. Given the polarity and poor solubility of EOs, it was initially assumed that it would be necessary to use concentrations of DMSO higher than those usually recommend (below 0.01% v/v) for the dissolution of poorly water-soluble substances [55]. The concentrations tested in previous studies that included the evaluation of the lethal effects of DMSO on aquatic species were between 0.06 and 10% (v/v), and the species evaluated included crustaceans *D. magna*, *A. franciscana*, and *Allorchestes compressa* and fish *Danio rerio* (embryos and larvae) [53,54]. Nonetheless, information regarding the lethal effects of DMSO on *T. platyurus* is not available, and previous studies on the evaluation of the lethal effects of EOs on *D. magna* and embryos of *D. rerio* reported the use of DMSO solvent at 0.1% concentration [43,56]. Thus, it was necessary to evaluate DMSO toxicity to all species using a broad range of concentrations, allowing us to define the maximum tolerable concentration for each species and confirm the findings of previous research.

For all three test species (*D. magna*, *T. platyurus*, and *Artemia* sp.), the highest concentration of DMSO (99.7%; Sigma-Aldrich, Steinheim, Germany) selected to be tested was 10 mL L^{-1} (1%, v/v). This concentration was prepared in each species' respective control medium (see Sections 2.4.2–2.4.4), followed by preparation of a range of four to nine concentrations of DMSO by serial dilution, using the control medium as dilution water. The following ranges of DMSO concentrations were tested: 6.0, 7.0, 8.0, 9.0, and 10.0 mL L^{-1} for *D. magna*; 7.0, 8.0, 9.0, and 10.0 mL L^{-1} for *T. platyurus*; and 2,0, 3.0, 4,0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 mL L^{-1} for *Artemia* sp.

2.3. Thymus mastichina and Helichrysum italicum Essential Oil Testing

In order to conduct the toxicity tests with the EO-thyme and EO-curry and due to their low solubility, a stock solution was first prepared by dissolving each EO in the DMSO solvent at a concentration of 100 mL of EO L^{-1} of DMSO. Then, serial dilutions of each stock solution were prepared to conduct the toxicity tests with the three invertebrate test species, using each species' respective control medium as dilution water (see Sections 2.4.2–2.4.4). The ranges of tested concentrations for the EO-thyme and EO-curry, as well as the respective maximum concentration of DMSO to which each test species was exposed, are presented in Table 1. The stock solutions of each EO in DMSO were prepared once at the start of the study and preserved at room temperature in darkness until the chemical analyses, which were conducted after completion of all toxicity tests, while all ranges of tested concentrations were prepared the same day as the test.

Table 1. Ranges of tested concentrations (with total number of concentrations; dilution factor) of the essential oils (EOs) of *Thymus mastichina* (EO-thyme) and *Helichrysum italicum* (EO-curry) in the toxicity tests with test organisms *Daphnia magna*, *Thamnocephalus platyurus*, and *Artemia* sp., with indication of the maximum (Max.) concentration of dimethyl sulfoxide (DMSO) used in each toxicity test. Tested concentrations are expressed both in ml L^{-1}, which was used during testing, and in mg L^{-1} (based on the known EO density; 0.9 mg L^{-1}) for the purpose of comparison with literature data.

Essential Oil	Test Organism	Tested Concentration		Max. DMSO Concentration (mL L^{-1})
		(ml L^{-1})	(mg L^{-1})	
EO-thyme	*D. magna*	0.0198–0.100 (5; 1.50)	17.82–90.00	0.900
	T. platyurus	0.0198–0.100 (5; 1.50)	17.82–90.00	0.900
	Artemia sp.	0.0107–0.100 (5; 1.75)	9.630–90.00	0.900
EO-curry	*D. magna*	0.0148–0.100 (6; 1.50) [a]	13.32–90.00	0.900
	T. platyurus	0.0148–0.100 (6; 1.50) [a]	13.32–90.00	0.900
	Artemia sp.	0.0109–0.350 (6; 2.00)	9.810–315.0	3.15

Note: [a] due to a minor fault, the two lowest concentrations were 0.0148 and 0.0227 instead of 0.0132 and 0.0198 mL L^{-1}, respectively.

2.4. Ecotoxicity Tests

2.4.1. General Procedures

All tests were performed following standard international guidelines and/or standard operating procedures available for short-term lethal toxicity testing with the invertebrate species selected to conduct the present study, with minor modifications to conform with the unique characteristics of the EOs. These included (i) the use of only glass material to prepare the test concentrations and expose the test organisms due to the organic nature of the EOs, (ii) the addition of a small flake of cetyl alcohol (Panreac, Darmstadt, Germany) to each test replicate to decrease the surface tension of the test solutions due to the lipophilic nature of the EOs, and (iii) the covering of each test replicate with Parafilm (American National Can, Menasha, WI, USA) to decrease the rate of evaporation and volatilization of the EOs. For all tested species, mortality was measured indirectly using organism immobilization for a minimum period of 15 s after gentle agitation of the test replicate. Tests were considered valid only if the percentage of mortality in the control was equal to or less than 10%.

Measurements of physicochemical parameters pH (WTW pH537, Wissenschaftlich Technische Werkstätten, Weilheim, Germany) and conductivity (WTW LF92) were taken in each test solution at the start of each toxicity test, except in the *Artemia* sp. test with EO-thyme. Salinity was also measured (WTW LF92) but only in the *Artemia* sp. EO-curry test. Dissolved oxygen was not measured because it was ensured that all test solutions were well oxygenated at the start of the tests. Due to the low volumes of test solutions used to expose the test organisms, it was not possible to measure the physicochemical parameters at the end of the tests.

2.4.2. Survival of *Daphnia magna*

Toxicity tests with freshwater crustacean *D. magna* were carried out according to OECD standard 202 [48]. Synthetic hard reconstituted water [57] was used as the test medium, both for the control and as dilution water. Test organisms were third- to fifth-brood neonates less than 24 h old obtained from cultures maintained according to the procedures described by Rosa et al. [58]. In short, the culture medium was test medium enriched with vitamins (B_1, B_{12}, and biotin) and seaweed extract (Glenside, Stirling, UK), cultures were fed daily with green microalgae *Raphidocelis subcapitata* (3×10^5 cells mL^{-1}), and the medium was renewed three times a week on alternate days. For each control and test concentration, four replicates were set up in 60 mL vials, each filled with 50 mL of test solution and inoculated with five neonates. Tests were incubated under the same conditions used for culturing,

i.e., at 20 to 22 °C under a 14 h light (cold-white fluorescent at an intensity of 30 μEm^{-2} s^{-1}):10 h dark photoperiod for 48 h. At the end of the 48 h of exposure, the total number of dead organisms per test treatment was counted (dead organisms after 24 h of exposure were removed).

2.4.3. Survival of *Thamnocephalus platyurus*

Toxicity tests with freshwater crustacean *T. platyurus* were performed according to ISO standard 14380 [59] and the Thamnotoxkit F standard operating procedure (https://www.microbiotests.com/wp-content/uploads/2019/07/thamnocephalus-toxicity-test_thamnotoxkit-f_standard-operating-procedure.pdf; accessed on 30 April 2023). Synthetic moderately hard reconstituted water [57] was used as test medium, both for the control and as dilution water. Organisms for testing were freshly hatched *nauplii* obtained by hatching cysts (Microbiotests, Gent, Belgium) in test medium diluted eight times with distilled water at a temperature of 24 to 26 °C with continuous top illumination (cold-white fluorescent light at an intensity of 50 μEm^{-2} s^{-1}) for 22 h. For each control and test concentration, three replicates were set up in 5 mL glass tubes, each filled with 1 mL test solution and inoculated with 10 organisms. Test samples were incubated at the same temperature used for cyst hatching but in darkness for 24 h. At the end of the 24 h exposure, total dead organisms per test treatment were counted.

2.4.4. Survival of *Artemia* sp.

The toxicity tests with marine crustacean *Artemia* sp. were conducted according to the Artoxkit M standard operating procedure (https://www.microbiotests.com/wp-content/uploads/2019/07/artemia-toxicity-test_artoxkit-m_standard-operating-procedure.pdf; accessed on 30 April 2023). Synthetic reconstituted marine water was employed as test medium for controls and as dilution water [60]. Organisms for testing were instar II-III *nauplii* obtained by hatching cysts (Ocean Nutrition Europe, Essen, Belgium; Batch: 0N13280) in test medium at a temperature of 24 to 26 °C with continuous top illumination (cold-white fluorescent light at an intensity of 50 μEm^{-2} s^{-1}) for 30 h. The remaining test procedures and incubation conditions were the same as described for the *T. platyurus* tests.

2.5. Data Analysis

At the end of all toxicity tests, the lethal concentrations that cause mortalities of 50 (LC50) and 20% (LC20) in *D. magna*, *T. platyurus*, and *Artemia* sp., as well as the respective 95% confidence limits (CL), were estimated from the concentration–response curves. The estimations were performed using PriProbit 1.63 software, applying probit transformation to the mortality percentage and logarithmic transformation to the concentration (http://ars.usda.gov/Services/docs.htm?docid=11284; acceded on 31 July 2023). Differences in species sensitivity within each EO and between the toxicity of the EOs within each species were evaluated by comparing lethal concentrations according to the difference between two means in a *t*-test (computed from the standard deviation and the n = number of concentrations) using Statistica 7.0 software. Analyses were conducted separately for the LC50 and LC20 values, and results were considered significant at a p level < 0.05.

3. Results

3.1. Chemical Composition of Essential Oils and Dimethyl Sulfoxide Stock Solutions

The chemical analysis of the pure EOs (supplied by Planalto Dourado, Pinhel, Portugal) allowed for the identification of 36 compounds in the EO-thyme (Table 2) and 41 compounds in the EO-curry (Table 3). EO-thyme showed a predominance of monoterpene compounds—mostly oxygen-containing (73.7%; 0.66 mg L^{-1}) but also hydrocarbons. The latter consisted mostly of monoterpenes (19.1%; 0.17 mg L^{-1}) but also sesquiterpenes (4.4%; 0.040 mg L^{-1}). Overall, the major constituent of this EO was, by far, 1,8-cineole (61.5%; 0.55 mg L^{-1}), followed by β-pinene (5.1%; 0.046 mg L^{-1}) and only three more compounds with percentages above 3%, namely linalool (3.8%), α-pinene, and sabinene

(both with 3.6%). EO-curry showed a predominance of sesquiterpenes (54.5%)—either hydrocarbons (45.2%; 0.41 mg L^{-1}) or oxygen-containing (9.3%; 0.084 mg L^{-1})—followed by monoterpenes (38.8%)—both oxygen-containing (24.0%; 0.22 mg L^{-1}) and hydrocarbons (14.8%; 0.13 mg L^{-1}). The main compound found in EO-curry was γ-curcumene, which accounted for 15.7% (0.14 mg L^{-1}) of the oil composition, followed by α-pinene (13.2%; 0.12 mg L^{-1}) and neryl acetate (11.6%; 0.10 mg L^{-1}), with seven more compounds with percentages between 3.2 and 7.2% (in increasing order: ar-curcumene, italicene, 1,8-cineole, cis-α-bergamotene, eudesm-5-en-11-ol, trans-α-bergamotene, and neryl propionate).

Table 2. Chemical composition of the essential oil of aerial parts of *Thymus mastichina* (Planalto Dourado, Pinhel, Portugal), with the quantification of the 36 identified compounds listed in order of their elution on the SPB-1 column. Qualitative and quantitative composition determined by GC-FID and GC-MS. GC-FID analyses were performed on a Hewlett-Packard 6890 gas chromatograph with a single injector and two flame ionization detectors (FIDs) for simultaneous sampling on two columns with different stationary phases, i.e., polydimethylsiloxane and polyethyleneglycol. GC-MS analysis was performed with an Agilent 6890 gas chromatograph equipped with a polydimethylsiloxane column interfaced with an MSD 5973 mass-selective detector operating at 70 eV ionization energy. Identifications of the EO components were achieved considering (1) the acquired retention indices compared with reference data and (2) the acquired mass spectra compared with reference data. Relative amounts (%) of each component were calculated from GC-FID raw data without any correction (for details, see Materials and Methods Section 2.1).

Exp. RI-SPB-1 [a]	Ref. RI-np [b]	Exp. RI-SW-10 [c]	Ref. RI-p [d]	Compound	%	mg L^{-1}
921	922	1028	1028	α-Thujene	0.3	0.0027
928	929	1028	1030	α-Pinene	3.6	0.0324
941	943	1075	1073	Camphene	0.4	0.0036
962	964	1126	1124	Sabinene	3.6	0.0324
967	969	1117	1116	β-Pinene	5.1	0.0459
979	980	1162	1162	Myrcene	1.9	0.0171
995	997	1174	1171	α-Phellandrene	0.1	0.0009
1000	1010	n.d.	1081	α-Terpinene	t	t
1011	1011	1274	1275	*p*-Cymene	1.1	0.0099
1019	1020	1205	1205	Limonene	0.8	0.0072
1019	1020	1216	1215	1,8-Cineole	61.5	0.5535
1023	1025	1233	1235	*Z*-β-Ocimene	0.7	0.0063
1034	1035	1250	1253	*E*-β-Ocimene	0.6	0.0054
1045	1046	1250	1249	γ-Terpinene	0.7	0.0063
1051	1050	1461	1462	*E*-Sabinene hydrate	0.7	0.0063
1058	1058	n.d.	-	*Z*-Linalool oxide (THF)	0.1	0.0009
1075	1076	1286	1288	Terpinolene	0.2	0.0018
1082	1082	1545	1542	Linalool	3.8	0.0342
1094	1094	n.d	-	Oct-1-en-3-yl acetate	0.1	0.0009
1120	1118	n.d.	1515	Camphor	0.1	0.0009
1142	1144	1695	1695	Borneol	2.0	0.0180
1157	1158	1599	1597	Terpinen-4-ol	0.4	0.0036
1167	1169	1694	1692	α-Terpineol	3.9	0.0351
1174	1176	n.d.	1786	Myrtenol	0.4	0.0036
1221	1224	1604	1601	Carvacryl methyl oxide	0.4	0.0036
1238	1240	1559	1555	Linalyl acetate	t	t

Table 2. *Cont.*

Exp. RI-SPB-1 [a]	Ref. RI-np [b]	Exp. RI-SW-10 [c]	Ref. RI-p [d]	Compound	%	mg L^{-1}
1273	1275	2212	2215	Carvacrol	0.4	0.0036
1371	1369	n.d.	-	α-Copaene	0.3	0.0027
1378	1376	n.d	1517	β-Bourbonene	0.1	0.0009
1443	1442	n.d.	-	α-Humulene	0.2	0.0018
1462	1461	n.d.	-	Selina-4,11-diene	0.9	0.0081
1478	1470	1734	1735	Bicyclogermacrene	1.8	0.0162
1493	1497	1731	1723	β-Bisabolene	1.0	0.0090
1504	1501	n.d.	-	δ-Cadinene	0.1	0.0009
1548	1551	n.d.	-	Spathulenol	0.2	0.0018
1565	1557	n.d.	-	Caryophyllene oxide	0.3	0.0027
		Monoterpene hydrocarbons			19.1	0.1719
		Oxygen-containing monoterpenes			73.7	0.6633
		Sesquiterpene hydrocarbons			4.4	0.0396
		Oxygen-containing sesquiterpenes			0.5	0.0045
		Other compounds			0.1	0.0009
		Total identified			97.8	0.8802

Note: [a] Experimental retention indices on the SPB-1 column relative to C8–C24 *n*-alkanes. [b] Reference retention indices on a non-polar column [61]. [c] Experimental retention on the SupelcoWax-10 column relative to C8–C23 *n*-alkanes. [d] Reference retention indices on a polar column [61]. t: traces < 0.05%.

Table 3. Chemical compositions of the essential oil of inflorescences of *Helichrysum italicum* (Planalto Dourado, Pinhel, Portugal), with the quantification of the 41 identified compounds listed in order to their elution on the SPB-1 column. Qualitative and quantitative composition determined by GC-FID and GC-MS. GC-FID analyses were performed on a Hewlett-Packard 6890 gas chromatograph with a single injector and two flame ionization detectors (FIDs) for simultaneous sampling on two columns with different stationary phases, i.e., polydimethylsiloxane and polyethyleneglycol. GC-MS analysis was performed with an Agilent 6890 gas chromatograph equipped with a polydimethylsiloxane column interfaced with an MSD 5973 mass-selective detector operating at 70 eV ionization energy. Identifications of the EO components were achieved considering (1) the acquired retention indices compared with reference data and (2) the acquired mass spectra compared with reference data. Relative amounts (%) of each component were calculated from GC-FID raw data without any correction (for details, see Materials and Methods Section 2.1).

Exp. RI-SPB-1 [a]	Ref. RI-np [b]	Exp. RI-SW-10 [c]	Ref. RI-p [d]	Compound	%	mg L^{-1}
928	929	1028	1030	α-Pinene	13.2	0.1188
941	943	1075	1073	Camphene	t	t
967	969	1117	1116	β-Pinene	0.3	0.0027
979	980	1162	1162	Myrcene	t	t
997	999	n.d.	-	Methylanisole	0.6	0.0054
1011	1011	1274	1275	*p*-Cymene	0.3	0.0027
1019	1020	1205	1205	Limonene	0.6	0.0054
1019	1020	1216	1215	1,8-Cineole	6.0	0.5535
1023	1046	1250	1249	γ-Terpinene	0.2	0.0018
1051	1076	1286	1288	Terpinolene	0.2	0.0018
1058	1082	1545	1542	Linalool	0.6	0.0054
1119	1117	1651	1653	Pinocarveol	0.1	0.0009
1120	1158	1599	1597	Terpinen-4-ol	0.4	0.0036
1142	1169	1694	1692	α-Terpineol	0.7	0.0063
1207	1209	1801	1797	Nerol	0.9	0.0081
1167	1224	1604	1601	Carvacryl methyl oxide	0.5	0.0045

Table 3. *Cont.*

Exp. RI-SPB-1 [a]	Ref. RI-np [b]	Exp. RI-SW-10 [c]	Ref. RI-p [d]	Compound	%	mg L^{-1}
1340	1342	1724	1722	Neryl acetate	11.6	0.1044
1345	1342	1452	1457	α-Cubebene	0.3	0.0027
1362	1374	1493	1497	*iso*-Italicene	1.8	0.0162
1389	1397	1537	1536	Italicene	6.7	0.0603
1401	1411	1563	-	*cis*-α-Bergamotene	4.7	0.0423
1409	1410	1591	1591	*E*-Caryophyllene	1.7	0.0153
1423	1424	1579	1586	*trans*-α-Bergamotene	3.2	0.0288
1429	1428	1785	1782	Neryl propionate	3.2	0.0288
1442	1442	1662	1662	α-Humulene	0.6	0.0054
1448	1446	1664	1664	α-Acoradiene	0.6	0.0054
1448	1446	1668	1674	β-Acoradiene	0.8	0.0072
1463	1473	1768	1772	*ar*-Curcumene	7.2	0.0648
1465	1475	1684	-	γ-Curcumene	15.7	0.1413
1469	1461	n.d.	-	Selina-4,11-diene	1.1	0.0099
1493	1491	1720	1724	β-Bisabolene	0.4	0.0036
1508	1505	1750	1751	δ-Cadinene	0.4	0.0036
1542	1562	2890		Geranyl butirate	0.7	0.0063
1161	1557	n.d.	-	Caryophyllene oxide	0.8	0.0072
1571	1575	2111	-	Eudesm-5-en-11-ol	3.3	0.0297
1579	2584	2080	2077	Guaiol	2.2	0.0198
1590	1592	n.d.	-	10-epi-γ-Eudesmol	0.2	0.0018
1603	1603	2153	2150	γ-Eudesmol	1.9	0.0171
1627	1630	2212	2203	α-Eudesmol	0.4	0.0036
1636	1630	2202	2200	Bulnesol	0.5	0.0045
1733	1725	n.d.	-	Geranyl caproate	0.2	0.0018
		Monoterpene hydrocarbons			14.8	0.1332
		Oxygen-containing monoterpenes			24.0	0.2160
		Sesquiterpene hydrocarbons			45.2	0.4068
		Oxygen-containing sesquiterpenes			9.3	0.0837
		Other compounds			1.5	0.0135
		Total identified			94.8	0.8532

Note: [a] Experimental retention indices on the SPB-1 column relative to C8–C24 *n*-alkanes. [b] Reference retention indices on a non-polar column [61]. [c] Experimental retention on the SupelcoWax-10 column relative to C8–C23 *n*-alkanes. [d] Reference retention indices on a polar column [61]. t: traces < 0.05%.

The results of the chemical analysis of the EO stock solutions prepared in the DMSO solvent are shown in Table 4 together with the percentages of difference in the concentration of the nine main compounds quantified relative to their concentration in the original oil. The variation between nominal and real concentrations was generally low, ranging from 0.810 to 23.08% for EO-thyme compounds and from 0 to 40.13% for EO-curry compounds. In the case of EO-thyme, only two compounds showed variations greater than 20%, namely linalool (21.05%) and α-terpineol (23.08%), and in the case of EO-curry, only the compounds *ar*-curcumene (25.00%) and γ-curcumene (40.13%) showed variations greater than 20%. These four compounds were detected in the respective EOs at low concentrations ranging from 3.8 to 15.7% (Tables 2 and 3).

3.2. Toxicity of the Dimethyl Sulfoxide Solvent

The results of the toxicity tests conducted with the three invertebrate species to evaluate the potential toxicity of the DMSO solvent revealed that none of the tested solvent concentrations, i.e., 6.0 to 10.0 mL L^{-1} for *D. magna*, 7.0 to 10.0 mL L^{-1} for *T. platyurus*, and 2.0 to 10.0 mL L^{-1} for *Artemia* sp., caused lethal toxicity in either of the tested species. The percentage of mortality was 0% in practically all treatments, including the controls, and values up to a maximum of 6.7% were rarely observed. The limit value of mortality in the control treatment established in the standards of the three performed tests is 10%, serving as a criterion of validity. Hence, DMSO did not show lethal toxicity up to the highest tested

concentration of 10 mL L^{-1} (1%, *v*/*v*). However, at the highest tested concentration, it was observed that some organisms were not as active as in the control treatment or even as with the lowest DMSO concentrations, suggesting that it is not appropriate to use a DMSO concentration higher than—or even as high as—10 mL L^{-1}; in accordance, Table 1 (Section 2.2) depicts that the highest DMSO concentration used in the toxicity tests with the EOs was 3.15 mL L^{-1} in the EO-curry test with *Artemia* sp.

Table 4. Chemical composition of the stock solutions of the essential oils of *Thymus mastichina* (aerial parts; EO-thyme) and *Helichrysum italicum* (inflorescences; EO-curry) prepared in the dimethyl sulfoxide (DMSO) solvent with respect to the nine main quantified compounds. Results are expressed as mg mL^{-1} equivalent of 1,8-cineole and in mg L^{-1} for presentation of the percentage of difference (% Dif.) in concentration relative to the concentration in the original oil (O-oil) (see Tables 2 and 3).

EO-Thyme				EO-Curry			
Compound	**Concentration**			**Compound**	**Concentration**		
	mg mL^{-1} (1,8-Cineole)	**mg L^{-1}**	**% Diff (O-Oil)**		**mg mL^{-1} (1,8-Cineole)**	**mg L^{-1}**	**% of Diff (O-Oil)**
α-Pinene	0.003	0.027	16.7	α-Pinene	0.012	0.108	9.09
Sabinene	0.003	0.027	16.7	1,8-Cineole	0.006	0.054	0.00
β-Pinene	0.005	0.045	1.96	Neryl acetate	0.011	0.099	5.17
Myrcene	0.002	0.018	5.26	Italicene	0.008	0.072	19.4
p-Cymene	0.001	0.009	9.09	*cis*-a-Bergamotene	0.004	0.036	14.9
1,8-Cineole	0.061	0.549	0.81	*trans*-a-Bergamotene	0.003	0.027	6.25
Linalool	0.003	0.027	21.1	Neryl propionate	0.003	0.027	6.25
Borneol	0.002	0.018	0.00	*ar*-Curcumene	0.009	0.081	25.0
α-Terpineol	0.003	0.027	23.1	γ-Curcumene	0.022	0.198	40.1

3.3. Ecotoxicity of Essential Oils

All toxicity tests fulfilled the validity criteria established in the adopted guidelines for the control treatment, namely that the percentage of mortality should be ≤10%. The physicochemical measurements of pH, conductivity, and salinity (only in the EO-curry *Artemia* sp. test) recorded at the start of each toxicity test in the control and at all tested concentrations showed that the overall range between the minimum and maximum values of pH (7.73–8.13), conductivity (274–281 µS cm^{-1} for freshwater and 34.2–34.5 mS cm^{-1} for *Artemia* sp.), and salinity (23.7 to 24.0) were within ranges considered optimal for the test organisms [48,59].

The results of the toxicity tests with EO-thyme and EO-curry for all three invertebrate species are presented in Table 5, both as LC50 and LC20 values with respective 95% CL values. Both EOs were toxic for all tested species, with the LC50 values of EO-thyme ranging from 84.78 to 153.0 mg L^{-1} and those of EO-curry ranging from 15.93 to 55.80 mg L^{-1}; LC20 values ranged from 72.09 to 107.1 mg L^{-1} and from 13.41 to 35.19 mg L^{-1}, respectively.

Comparing the toxicity of the two EOs, the results clearly show that for all three species, either the LC50 or the LC20 values EO-thyme were significantly higher than the corresponding values for EO-curry ($p < 0.001$ for all comparisons within each species). In addition, the 95% CL of all compared values never overlapped, and all the respective ratios between EO-thyme and EO-curry were above a 2.5-fold factor, ranging from 2.7 to 5.3 for the LC50 values and from 3.0 and 5.4 for the LC20 values.

Table 5. Lethal concentrations (mg L^{-1}) of the essential oils (EOs) of *Thymus mastichina* (aerial parts; EO-thyme) and *Helichrysum italicum* (inflorescences; EO-curry) causing 50 (LC50) and 20% (LC20) mortality (and the respective 95% confidence limits (CLs) in parentheses) for the toxicity tests performed with standard aquatic invertebrates *Daphnia magna* (48 h exposure), *Thamnocephalus platyurus*, and *Artemia* sp. (24 h exposure).

Toxicity Test	Results (mg L^{-1})	Essential Oil		Essential Oil Ratio (Highest/Lowest)
		EO-Thyme	EO-Curry	
D. magna	LC50 [a]	87.84 (73.17–104.4) a	28.71 (26.28–31.68) b	3.06 *
T. platyurus		84.78 (78.03–93.60) a	15.93 (14.67–17.19) a	5.32 *
Artemia sp.		153.0 (136.8–171.0) b	55.80 (47.25–65.61) c	2.74 *
Species ratio (highest/lowest)		1.80	3.50	
D. magna	LC20 [a]	83.25 (69.21–99) ab	24.66 (21.51–26.82) b	3.38 *
T. platyurus		72.09 (61.56–78.30) a	13.41 (11.79–14.58) a	5.38 *
Artemia sp.		107.1 (90.00–121.5) b	35.19 (27.45–42.03) c	3.04 *
Species ratio (highest/lowest)		1.49	2.62	

Note: [a] LC50/LC20 values followed by a different lowercase letter differ significantly relative to one another ($p < 0.01$ for EO-curry comparing LC20 values between *D. magna* and *Artemia* sp., $p < 0.001$ in all remaining significant cases; $p = 0.068$ to $p = 0.72$ in the three non-significant comparisons for EO-thyme). * indicates a significant difference between EOs within each species (for all LC50/LC20 EO comparisons, $p < 0.001$).

Regarding the comparison of the sensitivity among the three invertebrate species, for EO-curry, all three showed significant differences between their LC50 values and between their LC20 values ($p < 0.01$ when comparing LC20 values between *D. magna* and *Artemia* sp.; $p < 0.001$ in all remaining cases). The species showing the lowest values (thus, the most sensitive) was *T. platyurus*, followed by *D. magna* and *Artemia* sp. The ratios between the highest and lowest LC50 or LC20 values among the three species for EO-curry were 3.5 and 2.6, respectively, and the 95% CL never overlapped. In the toxicity tests with EO-thyme, *T. platyurus* appeared to be the most sensitive, but no significant differences were found between their LC50 or LC20 values and those of *D. magna* (and their 95% CL overlapped). Such significant differences were only observed relative to *Artemia* sp., which showed significantly higher LC50/LC20 values than those of *T. platyurus*, with no overlapping of their 95% CL, although the observed ratios were low, ranging from 1.5 to 1.8. A significant difference between the sensitivity of *D. magna* and that of *Artemia* sp. to EO-thyme was found regarding the LC50 values but not the LC20 values, and an overlap of the 95% CL was observed in both comparisons.

4. Discussion

In the present study, the toxicity of the EOs of *T. mastichina* (EO-thyme) and *H. italicum* (EO-curry) in standard aquatic species—both freshwater (*D. magna* and *T. platyurus*) and saltwater (*Artemia* sp.)—was assessed based on the estimated LC50 and LC20 values. The results demonstrate that both EOs exhibited lethal toxicity towards the tested aquatic species. Prior to the performance of the toxicity tests and due to the lipophilic nature of the EOs, DMSO was demonstrated to be an effective and safe solvent to be used in the toxicity tests with the selected invertebrate species. In order to more comprehensively discuss the potential toxicity of both EOs towards the aquatic organisms, their chemical compositions were characterized qualitatively and quantitatively based on the presence of monoterpenes and sesquiterpenes. To the best of our knowledge, the present study provides the first report on the toxicity of *T. mastichina* toward *D. magna* and *T. platyurus*, as well as on the toxicity of *H. italicum* toward *T. platyurus*.

4.1. Essential Oils Chemical Characterization

The chemical characterization of EO-thyme resulted in the identification of 36 compounds, corresponding to 74% oxygenated monoterpenes, 19% hydrocarbon monoterpenes, and 4.4% hydrocarbon sesquiterpenes. The major identified compounds were 1,8-cineole

(62%), β-pinene (5.1%), and α-terpineol (3.9%). The presence of these major compounds aligns with previous studies on Portuguese *T. mastichina* EO, where high levels of oxygenated monoterpenes and 1,8-cineole were consistently reported, often accompanied by α-terpineol or β-pinene as important constituents [42,62,63]. Nonetheless, some variability exists among Portuguese *T. mastichina* species, particularly regarding the second major compound found in EO-thyme (β-pinene), as other studies have reported different compounds, such as α-terpinyl acetate [64], α-pinene, camphene, and camphor [65]. Comparing the chemical composition of *T. mastichina* EOs obtained in the present study with those from other countries, a similar pattern emerges, with oxygenated monoterpenes being the major group and 1,8-cineole as the predominant compound; *T. mastichina* species from Spain [66,67] and Italy [68] have shown 1,8-cineole as the primary compound, with concentrations ranging from 38.8 to 74.0%. In contrast to our findings, linalool was reported as the second major compound in the latter studies, with percentages ranging between 2.2 and 42.7%.

In EO-curry, a total of 41 compounds were identified, corresponding to 45% hydrocarbon sesquiterpenes, 24% oxygenated monoterpenes, and 15% hydrocarbon monoterpenes. The major detected compounds were γ-curcumene (16%), α-pinene (13%), and neryl acetate (12%). These findings are consistent with the composition of Portuguese *H. italicum* EO reported by Ferraz et al. [43], which also exhibited a high percentage of sesquiterpenes (67%), followed by monoterpenes (33%), with γ-curcumene (16%), italicene (13%), and neryl acetate (12%) as the major constituents. The composition of *H. italicum* EO reported in the present study aligns with findings from other countries (Italy and Bosnia and Herzegovina), indicating a predominance of the sesquiterpene group and of the γ-curcumene compound [44,69]. However, it is worth noting that a significant variability in the composition of *H. italicum* EO is commonly observed, and at least 10 chemotypes have been identified, primarily based on variations in the main compounds; the chemotype rich in γ-curcumene (14 to 28%) corresponds to the profile found in the present study for EO-curry. Viegas et al. [5] also described different chemotypes of *H. italicum* EO, indicating significant intraspecific differences in response to environmental factors, particularly soil properties. The variability in the chemical composition of EOs can be attributed to various other factors, including the extraction method, sample homogeneity, the plant part used, plant physiological stage, chemotype and subspecies, seasonal influences, harvesting time, and location, among others [5,15,16,70–72]. This chemotype variability limits comparisons between studies, particularly when it comes to the toxicity of the EO.

4.2. Toxicity of the Dimethyl Sulfoxide Solvent

Based on the results of the present study including three aquatic species, DMSO is an effective and safe solvent of EOs for lethal ecotoxicity estimations with these invertebrate species when used at concentrations up to 1% (*v*/*v*; 10 mL L^{-1}). The present results agree with previous studies reporting the safe use of DMSO as a carrier solvent for diverse chemicals in sublethal and even lethal toxicity tests with different aquatic species at concentrations ranging from 0.01 to (exceptionally) 2% (*v*/*v*). Barbosa et al. [73] previously evaluated the lethal and sublethal toxicity of DMSO to *D. magna* at various concentrations ranging from 16.5 to 34.1 g L^{-1} (1.5 to 3.1%, *v*/*v*). The authors found that the 48 h LC50 was 24.6 g L^{-1} (2.24%) and recommended the use of ranges between 0.00062 and 0.001% (*v*/*v*) when testing effects on *D. magna* reproduction and growth. Huang et al. [54] compared the lethal and behavioral effects of DMSO on model species *D. magna*, *A. franciscana*, *Allorchestes compressa*, and *D. rerio* at concentrations between 0.1 and 10% (*v*/*v*). *Daphnia magna* was found to be the most sensitive, with a 48 h LC50 value of 0.5% (*v*/*v*), whereas concentrations up to 1% (*v*/*v*) did not present significant lethality for any other organisms. However, DMSO induced behavioral abnormalities in response to sublethal concentrations in all test species. As stated in the Results section, we also observed behavioral swimming changes in all tested species exposed to the highest tested DMSO concentration of 1% (*v*/*v*). Recently, Ferraz et al. [43] reported the safe use of 0.1% (*v*/*v*) DMSO as a solvent for several EOs and

hydrolates to evaluate the lethal toxicity in *D. magna* exposed to maximum concentrations of the test substance ranging from 400 to 800 mg L^{-1} for EOs and 400 to 8000 mg L^{-1} for hydrolates. Similarly, Hallare et al. [74] evaluated the effect of DMSO on *D. rerio* (zebrafish) embryos exposed to concentrations ranging from 0.0001 to 2.0% (*v*/*v*), reporting no lethal effects, although, based on the evaluated sublethal endpoints, they suggested the use of concentrations below 1.5% (*v*/*v*). Recently, Akermi et al. [56] confirmed that the use of DMSO at 0.1% (*v*/*v*) to evaluate the toxicity of *Cupressus sempervirens* EO to zebrafish embryos did not result in adverse effects on the embryos.

Notwithstanding the sensitivity of the various aquatic species to DMSO as a single substance, when DMSO is used as a solvent, its potential interference as a vehicle for chemicals (with different molecular structures and polarities) cannot be disregarded. Kais et al. [75] studied the influence of DMSO (0.1 to 10%, *v*/*v*) on the function of *D. rerio* chorion and found that up to a concentration of 1%, DMSO decreased the barrier function of the chorion, increasing the 48 h uptake of 2,7-dichlorofluorescein. Conversely, antagonist effects were reported between DMSO and anti-inflammatory drug diclofenac in lethal tests with *D. magna* [53] and *D. rerio* embryos [76]. For *D. magna*, DMSO at a concentration of 0.6 mL L^{-1} (0.06%, *v*/*v*) influenced the bioactivity of diclofenac, resulting in a decrease in its 48 h LC50 value [53]. In turn, diclofenac had a modulating effect on DMSO by inducing a concentration-dependent increase in stress protein hsp70 of zebrafish [76], although no differences in fish mortality or malformation incidences were observed up to the highest tested concentration of DMSO (0.04%, *v*/*v*). In general, the results of the present study demonstrate that concentrations of DMSO up to 1% (*v*/*v*) are not lethal to different aquatic species (*D. magna*, *T. platyurus*, and *Artemia* sp.) and can therefore be used as solvent to test the lethal effects of EOs and other lipophilic substances, even though the complexity of the interactions in the compound mixtures should not be ignored.

4.3. Essential Oils Ecotoxicity towards Aquatic Organisms

The main objective of our study was to contribute to the knowledge of the potential adverse environmental effects of two widely used EOs and, ultimately, to develop a more informed approach to implement appropriate management strategies. Although we believe that our results make an important contribution to—while suggesting the need for—the construction of toxicity databases, we also highlight that this issue has been overlooked. Unfortunately, due to the small number of toxicity studies of these two EOs in aquatic organisms, together with the lack of EO characterization (plant part, composition, etc.), our discussion of results was forcedly limited to a few non-target species.

Corroborating our hypothesis, both *T. mastichina* and *H. italicum* EOs exhibited lethal effects in all tested aquatic species at relatively low concentrations, which is plausible to be found in natural aquatic systems. Our results with respect to the 48 h survival of *D. magna* exposed to EO-thyme showed an LC50 value of 87.84 mg L^{-1}. Limited information is available on the toxicity of the EOs of *Thymus* species to invertebrates. Arslan et al. [77] found toxic effects of thyme oil on *D. magna*, with a 48 h LC50 of 11.79 mg L^{-1}, and You et al. [78] reported toxicity of white thyme EO to *D. magna*, with a 48 h LC50 of 2.5 mg L^{-1}. In both cases, the toxicity thresholds were lower than those observed in the present study. However, the abovementioned studies do not provide the scientific name of the plant or the EO characteristics (e.g., plant part, chemical composition, density, etc.), making it difficult to compare results among studies. The results of the *T. platyurus* lethal test with EO-thyme showed a 24 h LC50 of 84.78 mg L^{-1}. Few studies have used *T. platyurus* to assess the toxicity of plant species in the aquatic environment, and to the best of our knowledge, this is the first time the toxicity of a *Thymus* species EO has been evaluated in *T. platyurus*. Mayorga et al. [35] assessed the toxicity of extracts from ten Guatemalan plant species using *T. platyurus*, and all plant extracts, except one, exhibited lethal effects on *T. platyurus*, with 24 h LC50 values ranging from 12 to 492 mg L^{-1}. In accordance, the lethal toxicity obtained in our study for *T. platyurus* falls within the reported range. For *Artemia* sp., the lethal toxicity of EO-thyme observed in the present study was equal to

a 24 h LC50 of 153.0 mg L^{-1}. Arantes et al. [42] also found *T. mastichina* EO to be toxic to *A. salina*, reporting a 24 h LC50 of 74.1 mg L^{-1}, which is consistent with our findings. This could be explained by the similar chemical composition of the EOs, with a predominance of oxygenated monoterpenes (73.7 to 85.9%) and 1,8-cineole as the major compound (61.5 to 71.2%).

For EO-curry, *D. magna* lethal tests showed a 48 h LC50 of 28.71 mg L^{-1}. In contrast to our findings, Ferraz et al. [43] did not observe lethal effects of *H. italicum* EO on *D. magna*, even at the highest tested concentration of 800 mg L^{-1} and after 48 h of exposure. The substantial divergence between these results is surprising, especially considering the similar characteristics reported for both EOs, which are of the same commercial origin (Planalto Dourado, Portugal), i.e., the same plant part, extraction method, and composition, with a high content of sesquiterpenes and a predominance of γ-curcumene (15.7 to 16.0%). This fact strongly suggests that the major compound alone may not explain EO differences in toxicity. Variations in toxicity may also be attributed to other compounds and/or interactions among them [37,40,79]. The results of *T. platyurus* exposed to EO-curry showed a 24 h LC50 of 15.93 mg L^{-1}. Other studies reporting the toxicity of EO from *Helichrysum* species to *T. platyurus* were not found. However, Mayorga et al. [35], reported the 24 h lethal toxicity toward *T. platyurus* of an extract from *Neurolaena lobata*, a plant from the same family (Asteraceae), to be equal to an LC50 of 251 mg L^{-1}. The results of *Artemia* sp. exposed for 24 h to EO-curry revealed an LC50 of 55.80 mg L^{-1}. Judzentiene et al. [44] also observed mortality in *A. salina* exposed to *H. italicum* EO, reporting a 24 h LC50 of 15.99 mg L^{-1}, which is consistent with our results, though pointing to a somewhat higher level of toxicity. The authors reported a slightly higher γ-curcumene content (21.5%) in *H. italicum* EO than what we found (15.7%), suggesting that differences in toxicity may be explained, to some extent, by this chief compound. However, it is important to note that γ-curcumene is highly unstable and easily transforms into italicene, isoitalicene, and α-curcumene when exposed to light [15], which may contribute to variations in the observed toxicological effects.

Overall, each species' sensitivity ratios of either LC50 or LC20 values within each studied EO revealed *T. platyurus* to be the most sensitive species in the present study, followed by *D. magna*, although the latter was more sensitive than *Artemia* sp. only when exposed to EO-curry and not when exposed to EO-thyme. *Artemia* sp. was the least sensitive organism—always significantly less sensitive than both freshwater species. This higher sensitivity of *T. platyurus* to plant-based products in relation to *A. salina* is supported by a previous study conducted by Mayorga et al. [35], who demonstrated that *T. platyurus* was more sensitive than *A. salina* in detecting the toxicity of plant extracts. Regarding *D. magna*, crustaceans are among the most sensitive aquatic organisms to several chemicals, including metals, pesticides, and solvents [80–82]. Limited information is available on the toxicity of chemicals to *T. platyurus*, but comparable toxicity to metal compounds was reported between *D. magna* and *T. platyurus* by Blinova et al. [83]. *Artemia* sp., on the other hand, is generally considered to be less sensitive than other aquatic invertebrates [30]. *Daphnia* species and *A. salina* are among the aquatic organisms that have been most studied regarding the toxic effects of plant extracts and EOs [21], but limited information is available regarding the sensitivity of aquatic species to the EOs evaluated in the present study. Nevertheless, our study contributes by demonstrating the potential of freshwater (*D. magna* and *T. platyurus*), as well as saltwater (*Artemia* sp.) crustaceans, in assessing the toxicity of EOs.

Despite the aforementioned scarcity of studies on the toxicity of *T. mastichina* and *H. italicum* EOs, the cytotoxicity of *H. italicum* EO of exactly the same commercial origin, as well as using DMSO as a solvent, was previously determined using Hep G2 cell lines in tandem resazurin tests to assess the cells' metabolic activity [84]. The abovementioned study showed that EO-curry was not only highly toxic to cell lines (24 h LC50 of 0.5781 μg L^{-1}) but more than five orders of magnitude more toxic than for the aquatic organisms tested in the present study (24 h LC50 values ranging from 15.93 to 55.80 mg L^{-1}). However, other studies on the cytotoxicity of *H. italicum* toward several cell lines, including Hep G2, suggest

low levels of toxicity of EOs and extracts from this plant [14,17] but very likely with different characteristics (e.g., plant part and detailed composition). Gismondi et al. [17] evaluated the cytotoxicity of *H. Italicum* EO to B16F10 murine melanoma cells and found that at 1:50 and 1:10 dilutions of DMSO the EO increased the percentage of cell death by a maximum of 8.8% and 9.9%, respectively. Kramberger et al. [14] reviewed the data supporting the internal use of *H. Italicum* in humans, indicating that the plant extract showed low cytotoxicity to cell lines, including the Hep G2, N9, and S17 cells, after 72 h of exposure at tested concentrations up to 100 μg mL^{-1}. Despite the reported differences in cell line sensitivity, it is a fact that cell-based toxicity tests are an emerging alternative to conventional tests with aquatic organisms, which are costly and time-demanding and raise ethical issues; however, it is necessary to evaluate cell lines that show sensitivities comparable to those of aquatic species [85,86] or even similar sensitivity rankings to preliminarily categorize the toxicity of diverse chemicals. Therefore, more research including tests performed under similar experimental conditions is required to assess the suitability of cell-based assays as an alternative to conventional tests with aquatic organisms.

4.4. Toxicity and Chemical Composition of Essential Oils

The specific mode(s) of action behind the toxic effects found in the present study for the EOs of *T. mastichina* and *H. italicum* on aquatic invertebrates and for the latter EO on Hep G2 cell lines [84] is not known. However, it is likely that it may be largely explained by the chemical composition of the EO, considering both the individual compounds and their potential interactions [37,40,79]. Regarding EO-thyme, the major compounds identified in this study were 1,8-cinelole (61.5%) and β-pinene (5.1%). Bullangpoti et al. [36] reported that 1,8-cineole exhibits moderate toxicity to the non-target fish *P. reticulata* (24 h LC50 of 1701.93 mg L^{-1} for male fish and 3997.07 mg L^{-1} for female fish), inhibiting acetylcholinesterase (AChE) and carboxyl esterase (CarE) enzymes in both genders. In another study conducted by Astani et al. [87], the maximum non-cytotoxic concentration of 1,8-cineole against RC-37 cell lines was 1.25 mg mL^{-1}. Few data report the toxic effects of β-pinene on aquatic organisms, indicating markedly higher toxicity to *Onchorhynchus mykiss* fish (60 d LC50 of 1.2 mg L^{-1}) than to crustacean *A. salina* (24 h LD50 of 491 mg L^{-1}) [88]. Cytotoxic effects of β-pinene (1 h IC50 of 67 μg mL^{-1}) were reported by Machado et al. [89] in oral squamous carcinoma cell lines, demonstrating its ability to induce apoptotic cell death and selectivity towards oral cancer cells. Hence, 1,8-cineole and β-pinene may have contributed to the toxicity found for EO-thyme in the aquatic species evaluated in the present study, although other factors need to be considered to explain the observed degree of toxicity of the EOs. Regarding EO-curry, the most representative compounds found in the present study were γ-curcumene (15.7%), α-pinene (13.2%), and neryl acetate (11.6%). Limited scientific data exist regarding the toxicity of these compounds to aquatic organisms, with a few studies conducted on α-pinene toxicity to crustaceans *D. magna* and *A. salina* [88] and fish *Gambusia affinis* [90] and *D. rerio* [91]. According to the reported toxicity end points, α-pinene was particularly toxic to *D. rerio* (48 h LC50 of 14.45 μL mL^{-1}) and *D. magna* (24 h LC50 of 68 mg L^{-1}), with *A. salina* showing less susceptibility (24 h LC50 of 494 mg L^{-1}) [88,91]. Regarding the mode of action, information was only provided in relation to α-pinene, which is known to inhibit AChE activity [87]. In contrast to our finding, Ferraz et al. [43] did not find the EO of *H. italicum* to be toxic to *D. magna*, and their study revealed similar concentrations of major compounds γ-curcumene (16%) and neryl acetate (11.5%). Similarly, Gismondi et al. [17] demonstrated that *H. italicum* EO induced only low levels of cytotoxicity in B16F10 tumor cells. The major compounds found in our study were similar to those reported in the abovementioned studies, suggesting that other compounds could have influenced the divergencies in the outcomes.

The mode of action of EO compounds is also related to their polarity, which influences their ability to penetrate the cuticle and cell membranes, as observed in insects [41,92]. Hydrophobic hydrocarbon compounds like *p*-cymene may face challenges in penetrating the hydrophilic chitin layer due to the lack of necessary oxygenation for hydrogen bonding [41].

On the other hand, certain monoterpenes such as 1,8-cineole exhibit both hydrophilic and hydrophobic properties, enabling them to cross both layers in insect cell membranes, potentially leading to DNA damage [92]. Similar to insects, crustaceans possess a protective cuticle with a lipophilic waxy outer layer and a hydrophilic exocuticle containing chitin, creating a physical barrier [93]. In this context, previous studies evaluating the sensitivity of aquatic species to different pesticides found that physiological characteristics such as the degree of cuticle of sclerotization and lipid content determine sensitive responses across species [94,95]. In the present study, both 1,8-cineole and *p*-cymene were found in the evaluated EOs, and their modes of action in aquatic species may be similar to those observed in insects exposed to these compounds. Furthermore, all reported findings suggest that differences in sensitivity between studies may be explained by differences in the dissolution of EOs caused by their polarities and low solubility, making comparisons difficult. However, this assumption needs to be investigated.

The relationship between EO composition and toxicity might not solely or predominantly depend on the major compounds. EOs often exhibit greater toxicity against insects than their most abundant terpenes, and one possible explanation may be the synergistic effect occurring between EO compounds [40]. Studies conducted with the components of *T. vulgaris* EOs against insect pests demonstrated synergistic effects between major and minor compounds, leading to increased toxicity to insect larvae [41,79]. For instance, the combination of major monoterpenes thymol and *p*-cymene resulted in *p*-cymene enhancing the penetration of thymol through the integument of larvae, thereby increasing the overall toxicity of thymol [41]. In our study, *p*-cymene was found to be a minor compound in both *T. mastichina* and *H. italicum* EOs and therefore may have enhanced the toxicity of other compounds. The extent of the synergistic effects between compounds can be influenced by several factors, including the molecular structure of the compounds, the type and position of functional groups, the ratios in the mixtures, and the potential antagonistic relationships [40,41]. An alternative explanation to synergistic effects was proposed by Scalerandi et al. [37] based on the influence of insect metabolic detoxification on enhanced insecticide activity of EOs relative to their major compounds. They suggested that insects preferentially oxidize the major component in the mixture, while the minor terpene acts as a toxicant, exhibiting higher toxicity than when tested alone. Similarly to insects, aquatic organisms have also developed specific mechanisms to cope with several chemical toxicants, including metabolic detoxification mechanisms [24,96]. However, more research is needed to clarify the specific pathways used by aquatic invertebrates to deal with the toxicity of EO compounds. On the other hand, the adverse effects of EO compounds on aquatic organisms are influenced by their persistent and accumulative patterns in the water environment, which is determined by their physicochemical properties and lipophilic nature [26,27]. The limited data available on aromatic organic compounds indicate their effective removal through wastewater treatment processes [25,26], although additional data are required for further discussion. Overall, the available data seem to indicate that major compounds of the EO of *T. mastichina* present lower toxicity than the compounds less prevalent in the EO of *H. italicum* and that factors such as synergisms and metabolic detoxification mechanisms may influence the extent of toxicity. These results seem to be consistent with the findings of our study, which clearly demonstrate that EO-curry was more toxic compared than EO-thyme for all tested species and in sensitivity comparisons based on either LC50 or LC20 values; all differences in toxicity were statistically significant ($p < 0.001$) and without an overlap of the 95% CL. The toxicity end points (24 h LC50) reported in the literature for both EOs in *A. salina* also indicate that *H. italicum* [44] is more toxic than *T. mastichina* [42]. Regarding the other aquatic species, the existing data do not allow for direct comparisons of the toxicity of the two EOs, either due to a lack of data, as in the case of *T. platyurus*, or because the studies did not report toxicity end points or provide the scientific name of the plant, as in the case of *D. magna*.

Research has demonstrated the adverse effects of EO sand their compounds on cellular lipid profiles and cell membrane permeability in microorganisms [39]. EOs, due to their

lipophilic nature, easily cross the cell wall and cytoplasmic membrane, disrupting their structure and permeabilizing them, resulting in a variety of other damage [97]. Such damage can vary greatly depending on the mode of action of the EO compounds, as demonstrated by Melkina et al. [38] for α-pinene and limonene, with the former only causing heat shock and the latter causing irreversible degradation processes in cells. Other studies have reported the cytotoxicity of isolated compounds identified in our *H. italicum* EO. For instance, 1,8-cineole was found to induce oxidative DNA damage in mammalian cell lines [98], while *p*-cymene, γ-terpinene, and myrcene from *Lippia gracilis* exhibited cytotoxicity to cell lines Hep G2, K562, and B16-F10 [99]. Regarding the higher toxicity of *H. italicum* EO to Hep G2 cell lines reported by Nogueira [84] when compared to the three aquatic crustaceans employed in the present study, this result may be attributed to the absence of a physical barrier such as the crustacean cuticle. Some compounds encounter difficulties in crossing this barrier and exert their biological activity, as previously discussed. However, this does not always occur, and the findings of previous studies demonstrate that differences in sensitivity between cell lines and aquatic organisms depend on the type of toxicant. For instance, Rodrigues et al. [100] found that cell line assays were less sensitive than fish lethal assays to pesticides. Conversely, in the case of pharmaceuticals, tests with several cell lines showed higher sensitivity than fish lethal tests in predicting their toxicity [86]. Regarding EOs, due to their complex mixture of compounds, they appear to not have specific cell targets [97]. This highlights the need for future research to explore the sensitivity of different cell lines in comparison to aquatic organism tests relative to the toxicity that each EO and its compounds may impart.

5. Conclusions

In the present, we study investigated the lethal ecotoxicity of two commercialized and commonly used EOs (from *T. mastichina* and *H. italicum*) to three standard aquatic invertebrates, including freshwater (*D. magna* and *T. platyurus*) and saltwater (*Artemia* sp.) species. Our study first demonstrates the safety of using DMSO (at concentrations up to 1% (*v*/*v*)) as a solvent for lethal ecotoxicity testing of these EOs and of organic compounds in general. This 1% concentration is higher than those usually employed in lethal studies with invertebrates, demonstrating that the effectiveness of DMSO as a solvent for organic compounds can be considerably increased, at least when using these three invertebrates. Our study also revealed that both EOs can be lethally toxic to the three aquatic species, with EO-curry being significantly more toxic than EO-thyme for all species, providing valuable insights into the relationship between the chemical composition and toxicity of the two EOs. The second major compound of EO-thyme, β-pinene, appears more toxic to aquatic life than major compound 1,8-cineole when tested alone. However, 1,8-cineole facilitates penetration into the epidermis of crustaceans due to its hydrophilic and hydrophobic properties, which may increase the toxicity of other compounds in the EO. Regarding the main compounds of EO-curry, data were only found for α-pinene, which shows toxicity to various aquatic organisms. Furthermore, it is possible that minor compounds contribute to the overall toxicity through synergistic effects, as in the case of *p*-cymene, which is present in both EOs and is known to enhance the penetration of other EO compounds. When major compounds are preferentially oxidized by the organism's metabolic detoxification pathways, trace compounds may also act as toxic substances, and further research on this issue should be considered. Regarding species sensitivity, *T. platyurus* was found to be the most sensitive, followed by *D. magna* (particularly regarding EO-curry), and *Artemia* sp. However, because limited information is available in the scientific literature regarding the toxicity of the two EOs toward aquatic organisms, it is difficult to provide a robust and wide-ranging appraisal of species sensitivity differences before further research on non-target aquatic species is conducted. Overall, the observed differences in sensitivity among aquatic species, as well as the probable relationship between toxicity and EO compound constituents, need to be better comprehended and underscore the need for further research in this domain. It should also be noted that given the highest sensitivity of *T. platyurus*, this species might

have the potential to be used for preliminary screenings, at least for EOs obtained from plants belonging to the same families as those evaluated in this study (Lamiaceae and Asteraceae), although this needs to be confirmed by additional research.

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