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Chemical profile and eco-safety evaluation of essential oils and hydrolates from *Cistus ladanifer*, *Helichrysum italicum*, *Ocimum basilicum* and *Thymbra capitata*

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ABSTRACT

The demand for natural-based products for industrial applications is increasing sharply and therefore the search for new alternatives to the plants traditionally used is growing. These alternative plants can be an important source of bioactive compounds under a circular economy approach. Considering the potential future use of new plant products by the industry, it is necessary to assess the risk associated with their introduction into the ecosystem. This work aims to provide an insight into the volatile profiles and evaluate the potential (eco)toxic effects of essential oils (EO's) and hydrolates of four plant species, namely rockrose (*Cistus ladanifer*), curry plant (*Helichrysum italicum*), conehead thyme (*Thymbra capitata*) and basil (*Ocimum basilicum*). Chemical analysis were performed by GC-MS and acute toxicity tests were performed using the model organism *Daphnia magna*. The essential oil and the hydrolate from *H. italicum*, as well as all the other hydrolates caused no immobilization up to the highest concentrations tested, suggesting that all hydrolates present low to no risk towards *D. magna*. Similarly, the essential oil of *H. italicum*, presented negligible risk towards *D. magna*. For *C. ladanifer* and *T. capitata* essential oils, the EC_{50} (the concentration estimated to immobilize 50 per cent of the *Daphnia*) at 48 h varied between 199.7 mg/L and 12.1 mg/L, respectively. The essential oil from *C. ladanifer* was mainly characterised by monoterpene hydrocarbons, while the *H. italicum* was richer in sesquiterpene hydrocarbons. Both essential oil and hydrolate from *T. capitata* contained exclusively monoterpene hydrocarbons with a particularly high content of carvacrol. The higher acute toxicity of *T. capitata* essential oil can be attributed to the high amount of carvacrol present in the distillate. Overall, of the essential oils and hydrolates tested, all can be classified as practically non-toxic, except for *T. capitata* essential oil that, according to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) of the United Nations, can be classified as moderately toxic.

1. Introduction

Plants have been used for centuries for their beneficial properties, and today they are an important source of bioactive organic compounds of high economic interest. Plant products and their purified active components have been used in several industrial applications such as the food processing, pharmaceuticals and in cosmetics (Rafinska et al., 2019). In the food industry, these compounds are mainly used for their antioxidant activity to increase the shelf life of food items as well as new functional foods that aim to promote health and decrease the probability

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of developing certain types of diseases (Granato et al., 2017). In the pharmaceutical industry, plant products are used, for example, in skincare products (Harhaun et al., 2020), in biomedicine in wound care, healing (Renu et al., 2019) and in a wide variety of products due to their antimicrobial properties. In fact, the use of plant metabolites with antimicrobial activity is gaining importance, as they could be used to replace antibiotics, an extremely important aspect considering the growing phenomena of antimicrobials' resistance (Aleksic Sabo and Knezevic, 2019). Different plant metabolites such as essential oils, and hydrolates can be obtained from plants. Essential oils are defined as the products obtained by distillation of a plant or any of its parts, or by a mechanical process (without the application of heat) from the epicarp of Citrus fruits. During the procedure of obtaining essential oils, hydrolates can also be attained as a by-product of the process. A hydrolate is the distilled water that remains after the distillation process and is usually rich in water-soluble components of the essential oil (Hamdi et al., 2017; ISO, 2013).

Due to the growing interest of industry and the general public in natural ingredients, alternative and still largely unexploited plants are being studied to be used by the cosmetics and pharma industries. The use of these alternative species is particularly important for the valorisation of endogenous resources that are still largely unexplored, including, for example, *Cistus ladanifer* (L.), *Helichrysum italicum* (Roth) G. Don fil., *Ocimum basilicum* (L.), and *Thymbra capitata* (L.).

Cistus ladanifer is an evergreen woody shrub part of the Cistaceae family (Papaefthimiou et al., 2014). It is known by the common name rockrose and it is very abundant in the wild areas of the western Mediterranean region (Spain, Portugal, south of France, and north of Morocco) (Frazão et al., 2018). It has been used in the perfumery industry due to a particular metabolite, the "labdanum", used as a fixative, and in the cosmetic industry in the form of essential oil (Barrajón-Catalán et al., 2016; Zidane et al., 2013). Recent studies have shown relevant properties of its essential oil and extracts (e.g., aqueous, hydroalcoholic, acetone:water) as antimicrobial, antioxidant, cytotoxic, anti-inflammatory, and anti-nociceptive, phytotoxic, and insecticidal activity (Raimundo et al., 2018). As with *C. ladanifer*, *Helichrysum italicum* (Asteraceae) products present a wide variety of important properties and a large variety of goods can be prepared from this plant, differing widely in their chemical composition. *H. italicum,* most commonly known as curry plant or immortelle, is also widely distributed in the Mediterranean region (Kladar et al., 2015). Extracts can be obtained from several parts of the plant (leaves and flowerheads, flowers, flowering tops, and aerial parts) using different solvents (acetone, diethyl ether, ethanol, methanol, or even by supercritical CO_2) while the essential oil is generally obtained from the flowers (Antunes Viegas et al., 2014). The essential oil from *H. italicum* is one of the most popular essentials oils being used in cosmetics, particularly in skin regeneration and anti-age treatments, and also in soaps and perfumes due to its characteristic scent (Sarkic and Stappen, 2018).

Thymbra capitata (Lamiaceae) known generally as conehead thyme, is an important aromatic plant and its essential oils are rich in the phenolic monoterpene carvacol. The essential oil can be used to inhibit *Pseudomonas aeruginosa* biofilm formation, which are often associated with multidrug-resistant infections (Qaralleh, 2019). A reduction in biofilm biomass and metabolic activity of *Candida albicans* has also been reported (Palmeira-de-Oliveira et al., 2012). This essential oil also exhibits strong antibacterial and antioxidant activity in vitro (Anastasiou et al., 2020) and it displays a high potential to be used as a natural preservative in emulsions (Neves et al., 2017).

Ocimum basilicum is another example of an aromatic plant from the Lamiaceae family used commercially in many countries, with particular importance in the food industry (Açıkgöz, 2020). It has been used in traditional medicine as an antispasmodic, aromatic, digestive, carminative, galactagogue, stomachic, and tonic agent (Marwat et al., 2011). The high content of polyphenols such as flavonoids (e.g. anthocyanins) are responsible for the high antioxidant properties of extracts and essential oil from this plant (Ragab and Saad-Allah, 2020).

Although plant-based products are generally perceived as safe, some compounds extracted from the plants may be very toxic and present toxicological effects on certain organisms. There are a few reports on the toxicity of plant metabolites towards aquatic invertebrates, particularly *Daphnia magna* Straus (Ferraz et al, 2022). This organism, commonly known as water flea, is a primary consumer from the Cladocera order, with a short life cycle. When maintained in a laboratory under ideal conditions of feeding, medium, temperature, pH, and photoperiod its reproduction is parthenogenic, resulting in genetically identical female offspring (Ebert, 2005; OECD, 2004). *D. magna* is one of the recommended organisms to perform ecotoxicity tests according to the Organization for Economic Co-operation and Development (OECD) and the American Society for Testing and Materials (ASTM) (ASTM, 1997; OECD, 2004).

The objective of this work is to characterise the volatile profiles and evaluate the toxicity profile towards *Daphnia magna* of essential oils and hydrolates obtained from Portuguese aromatic plants that can be used as cosmetic and pharmaceutical ingredients, specifically the gum rockrose (*Cistus ladanifer*), curry plant (*Helichrysum italicum*), conehead thyme (*Thymbra capitata*), and basil (*Ocimum basilicum*).

2. Materials and methods

2.1. Plants, essential oils and hydrolates preparation

The essential oils and the respective hydrolates of *C. ladanifer* and *H. italicum* were purchased from the companies "Aromas do Valado" and "Planalto dourado", respectively. They were obtained by steam distillation of the flowering aerial parts (including flower, stem and leaves) of wild *C. ladanifer* plants growing in the central-west region of Portugal, and the flowering aerial parts of *H. italicum* obtained from cultivation fields in the central-north region of Portugal. However, the details on the distillation method and apparatus were not provided by the manufacturers. *T. capitata* and *O. basilicum* were harvested at the flowering stage from cultivation fields in Southern Beira Interior and the Northwestern Portuguese coast, respectively. The essential oil and hydrolate from *T. capitata* aerial parts (flowers, stems and leaves) and the hydrolate from *O. basilicum* leaves were obtained according to the procedure described in The European Pharmacopoeia (Europe, 1997) using 100 g of dried plant material, completely submerged in distilled water and subjected to hydro distillation for 2 h, using a Clevenger-type apparatus, in which the essential oil and hydrolates were obtained as final products. All the essential oils and hydrolates obtained were stored in dark vials at 4 °C until further assays.

2.2. Essential oils and hydrolates analysis by GC-MS

The volatile profile of the essential oils of *C. ladanifer*, *H. italicum* and *T. capitata* were analyzed, in triplicate, by gas chromatography coupled with mass spectrometry (GC/MS SCION-SQ 456 GC, Bruker) using a capillary column, HP-5MS (30 m × 0.25 mm × 0.25 μm). Helium was the carrier gas used with a flow rate of 1 mL min^{-1}. The initial oven temperature was programmed to 45 °C, gradually increasing 3 °C/min to 175 °C, finally increasing to 300 °C with a heating rate of 15 °C/min, maintaining this final temperature for 10 min. The transfer line and the ion source were programmed at a temperature of 250 °C and 220 °C respectively. The products were analyzed from electron impact ionization mass spectrometry (EI-MS) at 70 eV, and the compounds were identified in scan mode with positive polarity of ions 20–300 *m/z* with a time of 250.0 ms. All the essential oils were injected with a volume of 1 μL, using a split ratio of 1:350 for *C. ladanifer* and *T. capitata*, and 1:100 for *H. italicum*, which was previously diluted with an organic solvent. The hydrolates of *H. italicum*, *O. basilicum* and *T. capitata* were subjected to a liquid-liquid extraction (LLE) with an organic solvent (hexane) (Collin and Gagnon, 2016; Riani et al., 2017). The aqueous phase was

separated from the organic phase, and the organic phase was injected and analysed using the same chromatographic method as essential oils. All hydrolate samples after undergoing this process were injected with a volume of 1 μL, in triplicate, except the hydrolate of *C. ladanifer* which was injected with a volume of 0.5 μL. The split ratio was 1:50 for *H. italicum* and *O. basilicum*, 1:20 for *T. capitata* and 1:10 for *C. ladanifer*. The identification of the compounds was based on the retention index (RI) comparing with the RI given by the MS library (NIST 17 version 2.3) and with RI calculated from the n-alkane series standards (C7-C18 and C19-C30) that were injected under the same chromatographic conditions and with the same column as the samples of essential oils and hydrolates. The relative amount of each compound was expressed as a percentage of the relative peak area of the compound, relative to the total area of the peaks identified in the samples.

2.3. Daphnia magna culture

Daphnia magna (clone K6) long-standing stock culture (originally provided by the Environmental Changes, Hazards & Conservation (ECHC) Research Group from CESAM and Biology Department, University of Aveiro, Portugal) was maintained in ASTM hard water medium under continuous aeration at 20 °C ± 1 and a photoperiod of 16/8 h light/dark cycle. The daphnids were fed daily with a suspension of the green algae *Raphidocelis subcapitata* (3.0 × 105 cells/mL) from a culture maintained in house, and the culture medium was changed every other day. Before tests, adult female daphnids were isolated in 100 mL glass beakers (1 per beaker) and maintained under the same standard conditions. Parthenogenic daphnids (<24 h old) descending from the isolated adults, between the 2nd and 5th brood were selected to perform the tests, while the 6th brood was used to start a new culture.

2.4. Range finding tests

Cistus ladanifer and *Helichrysum italicum* essential oils chemical composition is highly heterogeneous, and therefore, a range-finding test was performed to obtain information on the appropriate concentrations to be used in the acute toxicity test (OECD, 2004). Since there is no available data on *H. italicum* essential oil toxicity towards *D. magna*, neonates (<24 h old) were also exposed to widely spaced concentrations of the test substance (1, 10, 100, 500 and 1000 mg/L). The range of concentrations used for the *T. capitata* essential oil was set according to available toxicity information of the major compound present to *D. magna*. In the range-finding tests, five organisms were used by concentration in one replicate and immobilisation was observed and recorded at the end of the test (48 h). This process was continuously repeated narrowing the concentrations until appropriate test concentrations were obtained.

2.5. Test solutions

Due to low solubility in the culture medium (ASTM hard water), the essential oils were previously diluted in Dimethyl sulfoxide (DMSO). The test substances were weighed and dissolved in the solvent at the concentration of 1% in ASTM hard water. When no solvent was used, the test substance was directly dissolved in ASTM hard water. The obtained solutions were subjected to serial dilution in culture medium until reaching the working concentrations (Table 1). When DMSO was used as a solvent, the highest amount of solvent in the work solutions was below 0.1% to minimize any possible effects of the solvent on the results as recommended by the followed guideline (OECD, 2019). An overview of the different essential oils and hydrolates tested and the concentrations used are presented in Table 1. The essential oil from *O. basilicum* was not included as only the hydrolate was obtained after distillation.

Table 1
Concentrations of essential oils and hydrolates used in the acute toxicity tests.

Plant		Product	Concentrations tested (mg/L)
C. ladanifer	Aerial parts	Essential oil	50; 100; 150; 200; 400
		Hydrolate	125; 250; 500; 1000; 2000
H. italicum	Aerial parts	Essential oil	50; 100; 200; 400; 800
		Hydrolate	125; 250; 500; 1000; 2000
O. basilicum	Leaves	Hydrolate	500; 1000; 2000; 4000; 8000
T. capitata	Aerial parts	Essential oil	5; 7.5; 10; 25; 50
		Hydrolate	50; 100; 150; 200; 300; 400

2.6. Acute toxicity tests

Toxicity tests were performed according to the OECD Test No. 202: *Daphnia* sp. Acute Immobilisation Test (OECD, 2004). Neonates (<24 h) from the 2nd to 5th brood were selected for the tests. For each test, five groups with increasing concentrations of the essential oils or hydrolates, plus the control group (ASTM medium or DMSO 0.1% - solvent control) were tested. For each concentration, five replicates were used with five animals per replicate. The tests were performed in multi-well plates containing 10 mL of test medium/well and the animals were not fed during the test. Immobilization of the daphnids was observed and recorded at 24 and 48 h. An organism was considered immobilized if no movement was observed within 15 s after gentle agitation of the test vessels. During the experiments, daphnids were kept under a constant temperature of 20°C ± 1 °C and a photoperiod of 16/8 light/dark cycle and were not fed until the end of the test.

2.7. Test validation

To validate the tests, the reference toxic potassium dichromate ($K_2Cr_2O_7$) was used as recommended in the OECD guideline. Immobilization at 24 h was registered and the EC_{50} value was calculated. The EC_{50} was $1.714 \pm 1.205 \times 10^{-5}$ mg/L which is within the 0.6 – 2.1 mg/L range proposed by the International Organization for Standardization (ISO) (ISO, 2012) and recommended by the OECD guideline.

2.8. Statistical analysis

Based on the immobilisation results obtained, the EC_{50} (concentration estimated to immobilise 50% of the daphnids) value was calculated using a non-linear regression model (variable slope) using GraphPad Prism 8 software.

3. Results and discussion

3.1. Phytochemical profile

The analysis of essential oils from the selected species show that *C. ladanifer* essential oil is mainly composed of monoterpene compounds, being α-pinene the most abundant (35.8%) (Table 2). Similar results were obtained for *T. capitata*, for which all major compounds are monoterpenes with a high percentage of carvacrol (79.9%) (Table 2). In contrast, *H. italicum* essential oil has a higher percentage of sesquiterpene compounds such as γ-Curcumene (16%), (-)-Italicene (12.5%), neryl acetate (11.5%) and α-Curcumene (10.1%) and monoterpenes (Table 2). Comparing the composition of the essential oils and hydrolates, only the products obtained from *T. capitata* showed similar composition with carvacrol being also the main compound (98.1%) in the hydrolate. The other hydrolates displayed different compositions from their essential oils' counterparts. The hydrolate from *H. italicum* was mainly composed of α-terpineol (30.5%) and interestingly, the hydrolate from *C. ladanifer* had lower percentages of monoterpene hydrocarbons than the essential oil, being the major compounds identified 4-hydroxy-3-methylacetophenone (21.6%), (-)-Myrtenol (11.2%) and p-cymen-8-ol (10.7%), which were not identified in the essential oil

Table 2
Chemical composition of *C. ladanifer*, *H. italicum*, *T. capitata* and *O. basilicum* and essential oils (EO) and respective hydrolates (except for *O. basilicum* for which no essential oil was obtained).

		C. ladanifer		*H. italicum*		*T. capitata*		*O. basilicum*
		EO	Hydrolate	EO	Hydrolate	EO	Hydrolate	Hydrolate
Compounds	RI[a]	Peak Area[b] (%)						
α-pinene	920a	35.8		7.4				
	917c							
Camphene	930a	6.7						
β-myrcene	968e					2.1		
o-cymene	1004a	4.6						
ρ-cymene	1000e					5.5	0.4	
	995 f							
α-terpinene	991e					1.8		
1,8-cineole	1011d				15.4		0.4	3.4
	1002 f							
	1007 g							
2,2,6-trimethylcyclohexanone	1014a	6.7						
γ-terpinene	1037e					6.1		
Linalool	1084 g							38.3
Camphor	1129 g							1.1
Endo-borneol	1156b		8.4		3.0			
	1160d							
Terpinen-4-ol	1173d				2.0		0.7	
	1166 f							
δ-Terpineol	1162d				6.6			
p-cymen-8-ol	1179b		10.7					
α-Terpineol	1188d				30.5			3.1
	1182 g							
(-)-Myrtenol	1191b		11.2					
D-verbenone	1205b		9.8					
Bornyl acetate	1289a	4.9						
Carvacrol	1311d				29.6	79.9	98.1	
	1316e							
	1311 f							
4-Hydroxy-3-methylacetophenone	1310b		21.6					
Eugenol	1364 g							52.5
Neryl acetate	1379c			11.5				
(-)-Italicene	1388c			12.5				
γ-Curcumene	1493c			16.0				
α-Curcumene	1496c			10.1				
Class composition								
Monoterpene hydrocarbons		80.2	34.0	32.8	96.5	100	100	46.7
Oxygenated monoterpenes		8.4						
Sesquiterpene hydrocarbons				67.2				
Others		11.5	66.0		3.5			53.4

a,b,c,d,e,f and g correspond respectively to *C. ladanifer* EO, *C. ladanifer* hydrolate, *H. italicum* EO, *H. italicum* hydrolate, *T. capitata* EO, *T. capitata* hydrolate and *O. basilicum* hydrolate;

[a] RI: retention index calculated by the standard n-alkane;

[b] Peak area results represent compounds with percentages > 1.5% for essential oils and > 0.3% for hydrolates

(Table 2). These results demonstrate the importance of analysing the chemical profile of the two types of products (essential oils and hydrolates) that can be obtained from the same plant since they may have different compounds that could possibly have different bioactivities. The *O. basilicum* hydrolate was characterized mainly by the phenylpropanoid eugenol, a compound widely used in perfumes and flavours. It also exhibited the lowest percentage of monoterpene compounds. Very low yields were obtained for *O. basilicum* essential oil hydrodistillation process and thus only the hydrolate was obtained and analysed in this work.

3.2. Acute toxicity

The essential oils tested exhibited different levels of toxicity to *D. magna*. Generally, when detected, the acute toxicity of the essential oils was dose and time dependent, and the results are presented in Table 3. All the hydrolates tested caused no observable acute effects on *D. magna* after 48 h of exposure up to the highest concentrations tested. The toxicity classification according to the Globally Harmonized System for Classification and Labelling of Chemicals (GHS) proposed by the United Nations of the tested hydrolates and essential oils is summed up in Table 4.

Table 3
Daphnia magna acute toxicity of tested essential oils after 24 and 48 h of exposure with the indication of the respective EC_{50} and 95% Confidence Interval (CI).

Plant	**EC_{50} mg/L (95% CI)**	
	24 h	48 h
C. ladanifer	201.10 ([a])	199.70([a])
T. capitata	12.05 (11.03 – 13.31)	10.81 (9.55 – 12.60)
H. italicum	[a]	[a]

[a] Not possible to calculate

We will now present the acute toxicity results for the essential oils/hydrolates of each of the plants tested.

3.2.1. Cistus ladanifer essential oil and hydrolate

The EC_{50} for the *C. ladanifer* essential oil ranged between 201.1 and 199.7 mg/L at 24 and 48 h, respectively. The chemical analysis of this essential oil identified 37 compounds, making up 92.6% of the total composition. The most abundant compound was α-pinene (35.8%) followed by camphene (6.7%), which is in accordance with other studies.

Table 4
Maximum concentrations tested for the plants products ([Max]tested) and the 48 h EC_{50} values obtained for each product alongside with the Globally Harmonized System for classification and labelling of chemicals (GHS) proposed by the United Nations.

Plant	Type of product	[Max] tested	48 h EC_{50} (mg/L)	GHS Classification
C. ladanifer	Essential oil	400	> 100	Non-toxic
	Hydrolate	2000	> 100	Non-toxic
H. italicum	Essential oil	800	> 100	Non-toxic
	Hydrolate	2000	> 100	Non-toxic
O. basilicum	Hydrolate	8000	> 100	Non-toxic
T. capitata	Essential oil	400	10.8	Acute 3
	Hydrolate	400	> 100	Non-toxic

The proposed values for GHS are based on 48 h EC_{50} values for crustaceans. Acute 1: ≤ 1 mg/L; 1 < Acute 2 ≤ 10 mg/L; 10 < Acute 3 ≤ 100 mg/L; Non-toxic: >100 mg/L.

Other important compounds present were 2,2,6-trimethylcyclohexanone (6.7%), bornyl acetate (4.9%), o-cymene (4.6%), D-limonene (3.3%), viridiflorol (3.5%) and pinocarveol (3.1%). Twenty-eight other compounds were present at concentrations below 3% showing the high heterogeneity of compounds present in the *C. ladanifer* essential oil. The chemical composition of essential oils obtained from this plant has been intensively studied. Most of the studies report α-pinene as the major compound present (Costa et al., 2007; Greche et al., 2009; Gülz et al., 1984; Mariotti et al., 1997; Robles et al., 2003; Rossi et al., 2007; Tavares et al., 2020). Camphene is usually the second most abundant compound present, although some authors reported camphene as the major compound (Zidane et al., 2013). Other compounds including *trans*-Pinocarveol and Viridiflorol (Gomes et al., 2005; Verdeguer et al., 2012) or 1,8-Cineole (Viuda-Martos et al., 2011) have also been reported. The acute toxicity of α-pinene towards *D. magna* was reported to be in the range between 0.22 and 1.44 mg/L, whereas the acute toxicity of a mixture containing α-pinene, camphene, β-pinene and δ-3-carene was reported to be 4.29 mg/L in the US EPA (United States Environmental Protection Agency) database. Although α-pinene is reported to be toxic to *D. magna* at low concentrations, in this study, the percentage of this compound is relatively low (~36%) and several other compounds were detected which can reduce the toxicity of the essential oil. In the European Chemicals Agency (ECHA) database, an essential oil obtained from the stems and leaves of *C. ladanifer* by distillation is registered as having toxic effects to *D. magna* with EC_{50} values ranging from 94.2 mg/L after 24 h of exposure and 63.2 mg/L after 48 h of exposure. It was classified as long term hazardous to the aquatic environment under the Chronic 3 category ($EC_{50\ (48\ h)}$ within 10 and 100 mg/L) and the degradability of the test substance in the environment was considered to be low (ECHA, 2016). However, no other information about the chemical composition of the essential oil or the origin of the plant material is provided by ECHA. The *C. ladanifer* essential oil used in this work presented a 48 h EC_{50} value above 100 mg/L, which cannot be considered hazardous to the environment (Table 4). These differences in the observed toxicity can be explained by the chemical variations that are common in essential oils. The chemical variability of Portuguese *C. ladanifer* essential oils was studied recently, showing differences in the chemical composition of the essential oils according to the time of the year the plant was harvested and the extraction process (steam distillation vs hydrodistillation) (Tavares et al., 2020). These normal variabilities of the chemical composition of essential oils from *C. ladanifer* can be the explanation of the different toxicity observed with the essential oil in this study and the one reported in the ECHA database. The hydrolate caused no observable effects to *D. magna* after 48 h of exposure up to 2000 mg/L. As mentioned above, *C. ladanifer* essential oil and hydrolate differ in their chemical profile. According to a previous study (Tavares et al., 2020) the hydrolate major compounds were trans-pinocarveol, borneol and terpinen-4-ol, which in comparison with this study only verbenone fits in the identified major compounds. On the order hand, the percentage of monoterpene hydrocarbons was very low in relation to the other classes of compounds, which was also verified in the present work. It is important to note that for this study, the samples were acquired commercially while in Tavares et al. the aerial parts of the plant were collected in Beira Baixa in 2017 and 2018 and subjected to steam distillation. The extraction method as well as the time of harvesting the plant are variables that influence the chemical characterization and consequently the bioactivity analyses.

3.2.2. *Helichrysum italicum essential oil and hydrolate*

Helichrysum italicum essential oil showed no acute toxic effects to *D. magna* up to 800 mg/L suggesting very low toxicity towards this organism. The chemical analysis identified 27 compounds present, which were responsible for 93.6% of the total chemical composition of the essential oil. The three most abundant compounds were γ-curcumene (16.1%), followed by (-)-italicene (12.6%) and neryl acetate (11.5%). It is noticeable that the compounds present in the essential oil are all in relatively low percentages. Regarding the effects of these three major compounds, there is no available data on toxicological effects towards *D. magna* or other aquatic invertebrates. The hydrolate studied also did not cause any observable toxic effect to *D. magna* up to high concentrations (2 $g.L^{-1}$). In total, 17 compounds were identified in the hydrolate, being the most abundant L-α-terpineol (30.6%), followed by carvacrol (29.6%) and 1,8-cineole (15.4%). There are significant chemical differences between the essential oil and the hydrolate obtained from *H. italicum*. To the authors' best knowledge, there is no available data on the literature about the chemical composition of *H. italicum* hydrolates, as this type of product has attracted far less attention when compared with the essential oil. The high heterogeneity of compounds present in similar percentages in the essential oil and hydrolate might explain the lack of toxic effects observed to *D. magna* up to relatively high concentrations. To the authors' best knowledge, this is the first time that the toxicity towards *D. magna* of an essential oil and hydrolate from *H. italicum* are tested. The obtained results are also an important contribution for the characterization of the hydrolate which is obtained simultaneously with the essential oil, and that can be regarded as a source of bioactive compounds instead of "waste" of the distillation process.

The chemical composition of essential oils obtained from *Helichrysum italicum* can vary depending on the region the plant is grown, and it can present very distinct chemotypes depending on subspecies and environmental factors (e.g. soil properties). An essential oil obtained by hydrodistillation of the aerial parts of *H. italicum* from Croatia was predominantly constituted of neryl acetate (20.5%), γ-curcumene (14.1%) and trans-α-bergamotene (7.0%), and other 59 compounds were identified in lower percentages (Dzamic et al., 2019). Another essential oil obtained from the aerial parts of *H. italicum* in Montenegro showed similar chemical composition with neryl acetate and γ-curcumene as the two most abundant compounds (29.2% and 18.8%, respectively), but neryl propanoate was the third most abundant with 10.1% (Kladar et al., 2015). Interestingly, another study reported β-eudesmene, β-bisabolene and α-pinene as the three most abundant compounds (21.6%, 19.9% and 16.9%, respectively) of an essential oil from *H. italicum* collected in Montenegro (Oliva et al., 2020). On the other hand, an essential oil from aerial parts of *H. italicum* collected in the North of Algeria showed a high diversity of compounds in small percentages, with α-cedrene (13.6%), α-curcumene (11.4%) and geranyl acetate (10.1%) the three most abundant (Djihane et al., 2017). Finally, an essential oil obtained by hydrodistillation of the aerial parts of *H. italicum* subsp. picardii collected in the south of Portugal was rich in α-pinene (53.5%) and γ-curcumene (27.4%) (Costa et al., 2015).

3.2.3. *Thymbra capitata essential oil and hydrolate*

The *Thymbra capitata* essential oil was the most toxic towards *D. magna*. The EC_{50} values obtained varied from 12.1 mg/L after 24 h of

exposure to 10.8 mg/L after 48 h of exposure. This essential oil can be classified as acutely hazardous to the aquatic environment under the acute 3 category of the GHS (Table 4) as the 48 h EC_{50} obtained is within 10 and 100 mg/L. The toxicity observed at relatively low concentrations can be explained by the high amount of carvacrol present in the essential oil (80%). This phenolic monoterpenoid is registered in ECHA as being toxic to aquatic life, with a reported 48 h EC_{50} value to *D. magna* of 6.06 mg/L (95% CI 5.10–7.28 mg/L), 96 h EC_{50} for the zebrafish *Danio rerio* of 6.17 mg/L (95% CI 4.83 – 9.92 mg/L) and 72 h EC_{50} to the green microalgae *Raphidocelis subcapitata* of 4.05 mg/L (ECHA). This shows that carvacrol is toxic towards different aquatic organisms from microalgae to fish, and contamination of water bodies by this substance or solutions of the substance should be avoided. The observed EC_{50} value to *D. magna* in this study follows closely the EC_{50} value reported of carvacrol in the ECHA database (ECHA, 2021a). Moreover, the low biodegradability of carvacrol in the environment has led to the chronic 2 category classification, as toxic to aquatic life with long-lasting effects by the ECHA. The chemical composition of essential oils obtained from *T. capitata* are usually very similar in composition and carvacrol is consistently reported as the major compound. An essential oil obtained from the aerial parts of *T. capitata* collected in north Morocco, by hydrodistillation, presented 75.5% of carvacrol (Charfi et al., 2019). In another study, different essential oils from the aerial parts of *T. capitata* collected from 2002 to 2004 in both flowering and fruiting stages in the Badajoz area, Spain, consistently showed high amounts of carvacrol (>74%) (Salas et al., 2010) and another essential oil from the aerial parts of *T. capitata* collected in the south of Portugal showed the same trend with carvacrol accounting up to 75% (Palmeira-de-Oliveira et al., 2012). The potential toxicity of the *T. capitata* hydrolate towards *D. magna* was also assessed. Despite carvacrol being the most abundant compound (98.1%), no toxic effects were observed up to 400 mg/L. Hydrolates are a by-product of the process of obtaining an essential oil (hydro- or steam-distillation) they usually contain lower concentrations of compounds than essential oils, and these are generally water-soluble compounds that end up as a residue of the process to obtain essential oils. This leads to usually softer scents and lower biological activity of these products when compared with the essential oils (Catty, 2001). The hydrolate was mainly composed of carvacrol. This composition is consistent with another study that compared the chemical composition of an essential oil and a hydrolate obtained from *T. capitata* that links this high abundance to the hydrophilic character of carvacrol (Moukhles et al., 2020). It has been shown that the yield of compounds present in an essential oil and the corresponding hydrolate from *T. capitata* is much lower in the hydrolate form (1.99% for the essential oil vs 0.45% for the hydrolate) (Moukhles et al., 2019). Although hydrolates are usually linked to lower bioactive activities, a recent study showed acute toxic effects of a hydrolate obtained from *Artemisia absinthium* towards *D. magna* at relatively low concentrations (EC_{50} = 0.24% of hydrolate dilution), and in this way, it is also important to evaluate potential toxic effects of these industry by-products to non-target organisms (Pino-Otín et al., 2019).

3.2.4. *Ocimum basilicum hydrolate*

The *Ocimum basilicum* hydrolate also showed no acute toxic effects to *D. magna* in this study up to very high concentrations (8000 mg/L). The hydrolate composition revealed the presence of seven compounds. Eugenol (52.5%) and linalool (38.3%) were the major compounds identified, but lower amounts of α-pinene, eucalyptol (or 1,8-cineole), camphor, α-terpineol and geraniol were also present. Although eugenol is reported to be acutely toxic to *D. magna* at very low concentrations, with one study reporting a 48 h EC_{50} of 0.70 mg/L (Gueretz et al., 2017), it has very low solubility in water (Baker and Grant, 2018) meaning it is not present at high concentrations in hydrolates. For linalool, an 48 h EC_{50} of 20 mg/L has been reported (Api et al., 2016), and in the ECHA database, a 48 h EC_{50} of 59 mg/L to *D. magna* is reported (ECHA, 2021b). Similarly to eugenol, linalool is also poorly soluble in water, and in this way, the amount of the compound present in the hydrolate is not expected to be very high. These facts can explain of the lack of toxicity of the hydrolate from *O. basilicum* tested towards *D. magna* up to very high concentrations.

Table 4 presents the classification of toxicity of the tested hydrolates and essential oils according to the Globally Harmonized System for Classification and Labelling of Chemicals (GHS) proposed by the United Nations, alongside the highest concentrations tested towards *D. magna*. The maximum concentrations for which no observable effects were observed were always above the limit to be considered toxic by international regulations, with the exception of the essential oil of *T. capitata*, classified as acute 3.

4. Conclusions

Essential oils are important raw materials used in many industries for decades. Recently, hydrolates, which were commonly disregarded and categorized as a waste from the distillation process, have been increasingly considered, as they possess interesting bioactivities and add extra value under a circular economy approach. Despite being generally regarded as green and safe products, essential oils, hydrolates and other extracts obtained from plants may pose environmental risks to certain organisms. Considering the possible industrial applications of essential oils and hydrolates from *C. ladanifer*, *H. italicum*, *O. basilicum* and *T. capitata* and following the Precautionary Principle, the acute toxicity of these products was tested for their eco-safety using the model organism *D. magna*. All the hydrolates tested presented no risk to this organism. The essential oils from *H. italicum* and *C. ladanifer* present low to no risk to *D. magna*. The essential oil from *T. capitata* showed moderate toxicity to *D. magna* and therefore precautions should be taken to avoid the contamination of water bodies when producing, handling, and using this product especially due to the high carvacrol content of the oil.

CRediT authorship contribution statement

Celso Afonso Ferraz: Investigation, Visualization, Writing – original draft. **Ana Catarina Sousa:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Débora Caramelo:** Investigation, Visualization, Writing - original draft. **Fernanda Delgado:** Resources, Supervision, Writing – review & editing. **Ana Palmeira de Oliveira:** Funding acquisition, Writing – review & editing. **M. Ramiro Pastorinho:** Conceptualization, Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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