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 extracts 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 was 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 48h varied from 199.7 mg/L and 12.05 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.

Keywords: Aromatic plants, bioactive compounds, ecotoxicity, invertebrates, acute toxicity, *Daphnia magna*

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 extracts 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 products as well as new functional foods that aim to promote health and decrease the probability of developing certain types of diseases (Granato et al., 2017). In the pharmaceutical industry, plant extracts are used, for example, in skincare products (Harhaun et al., 2020), in biomedicine for wound care, and healing (Renu et al., 2019) and in a wide variety of products for their antimicrobial properties. In fact, the use of plant extracts 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 extracts can be obtained from plants such as essential oils, and hydrolates. Essential oils are defined as the product 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 extract, 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) extracts present a wide variety of important properties and a large variety of extracts can be prepared from this plant, with resulting products differing 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 formations 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 and 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

115 organisms. There are a few reports on the toxicity of plant extract towards aquatic
116 invertebrates, particularly *Daphnia magna* Straus. This organism, commonly known as
117 water flea, is a primary consumer from the Cladocera order, with a short life cycle. When
118 maintained in a laboratory under ideal conditions of food, medium, temperature, pH,
119 and photoperiod its reproduction is parthenogenic, resulting in genetically identical
120 female offspring (Ebert, 2005; OECD, 2004). *D. magna* is one of the recommended
121 organisms to perform ecotoxicity tests according to the Organization for Economic Co-
122 operation and Development (OECD) and the American Society for Testing and Materials
123 (ASTM) (ASTM, 1997; OECD, 2004).

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

130

131 **2. Materials and Methods**

132 **2.1 Plants, essential oils and hydrolates preparation**

133 *Cistus ladanifer* aerial parts were collected from wild plants growing in the central-west
134 region of Portugal. *H. italicum* aerial parts were obtained from cultivated plants in the
135 central-north region of Portugal. *T. capitata* and *O. basilicum* were obtained from
136 cultivation fields respectively in southern Beira Interior and the Northwestern
137 Portuguese coast. 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. However, the details on the distillation method and apparatus used were not provided by the manufacturers. The essential oil and hydrolate from *T. capitata*, were obtained from the aerial parts of the plant (flower, stem and leaf), while for *O. basilicum* hydrolate only the leaves were used. The *T. capitata* essential oil was obtained according to the procedure described in the European Pharmacopoeia (Europe, 1997). The aerial parts of *T. capitata* were harvested at the flowering stage and subjected to hydrodistillation for 2h, using a Clevenger-type apparatus, in which the essential oil and hydrolate were obtained as the final product. The leaves of *O. basilicum* were also harvested at the flowering stage and used to obtain the hydrolate by using the same process described above. 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) equipped with a capillary column, HP-5MS (30m × 0.25mm × 0.25 µm). Helium was the carrier gas used with a flow rate of 1mL.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 minutes. The transfer line and the ion source were programmed at a temperature of 250°C and 220°C respectively. The extracts were analyzed from electron impact

ionization mass spectrometry (EI-MS) at 70eV, 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. Regarding the hydrolates of *H. italicum*, *O. basilicum* and *T. capitata*, they 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 analyzed 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 was 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) stock culture was maintained in ASTM hard water medium under continuous aeration at 20°C ± 1 and a photoperiod of 16/8h light/dark cycle. The daphnids were fed daily with a suspension of the green algae *Raphidocelis subcapitata*

185 (3.0×10^5 cells/mL) from a culture maintained in house, and the culture medium was
186 changed every other day. Before tests, adult female daphnids were isolated in 100 mL
187 glass beakers (1 per beaker) and maintained under the same standard conditions.
188 Parthenogenic daphnids (<24h old) descending from the isolated adults, between the
189 2nd and 5th brood were selected to perform the tests, while the 6th brood was used to
190 start a new culture.

191

192 **2.4 Range finding tests**

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

204

205 **2.5 Test solutions**

206 Due to low solubility in the culture medium (ASTM hard water), DMSO was used as a
207 solvent for the *C. ladanifer*, *H. italicum* and *T. capitata* essential oils. For the hydrolates

extracts, no solvent was necessary as they are water-based. 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 Dimethyl sulfoxide (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.

Table 1: Essential oils and extracts concentrations used in the acute toxicity tests.

Plant		Type	Concentrations tested (mg/L)
Cistus ladanifer	Aerial parts	Essential oil	50; 100; 150; 200; 400
		Hydrolate	125; 250; 500; 1000; 2000
Helichrysum italicum	Aerial parts	Essential oil	50; 100; 200; 400; 800
		Hydrolate	125; 250; 500; 1000; 2000
Ocimum basilicum	Leaves	Hydrolate	500; 1000; 2000; 4000; 8000

Thymbra capitata	Aerial parts	Essential oil	5; 7.5; 10; 25; 50
		Hydrolate	50; 100; 150; 200; 300; 400

219

220 **2.6 Acute toxicity tests**

221 Toxicity tests were performed according to the OECD Test No. 202: *Daphnia* sp. Acute
222 Immobilisation Test (OECD, 2004). Neonates (<24h) from the 2nd to 5th brood were
223 selected for the tests. For each test, five groups with increasing concentrations of the
224 essential oils or hydrolates, plus the control group (ASTM medium or DMSO 0.1% -
225 solvent control) were tested. For each concentration, five replicates were used with five
226 animals per replicate. The tests were performed in multi-well plates containing 10 ml of
227 test medium in each well and the animals were not fed during the test. Immobilization
228 of the daphnids was observed and recorded at 24 and 48h. An organism was considered
229 immobilized if no movement was observed within 15 seconds after gentle agitation of
230 the test vessels. During the experiments, daphnids were kept under a constant
231 temperature of 20°C ± 1°C and photoperiod of 16/8 light/dark cycle and were not fed
232 until the end of the test. Based on the immobilisation results obtained, the EC_{50}
233 (concentration estimated to immobilise 50% of the daphnids) value was calculated using
234 GraphPad Prism 8 software.

235

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 24h 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.

3. Results and Discussion

3.1 Phytochemical profile

The analysis of essential oils from the four selected species shows that *C. ladanifer* essential oil is mainly composed of monoterpene compounds. Similar results were obtained for the *T. capitata*, for which all major compounds belong to the class of monoterpene compounds. In contrast, *H. italicum* essential oil has a higher percentage of sesquiterpene compounds and some monoterpenes. The hydrolates of *H. italicum* and *T. capitata* display different compositions in relation to their essential oils. For *H. italicum*, the hydrolate sample main compound was α-terpineol (30.5%), while for the essential oil γ-curcumene (16.1%) was the major compound. Concerning *T. capitata*, the major compound, carvacrol, was the same in both essential oil and hydrolate with an amount of 79.9% and 98.1% respectively. It is also legitimate to state that all compounds belong to the monoterpene class. The *O. basilicum* hydrolate was characterized mainly by a compound widely used in perfumes and flavours, eugenol, and it is the one that has the lowest percentage of monoterpene compounds. Very low yields were obtained for *O. basilicum* essential oil hydrodistillation process and thus only a hydrolate was

acquired and analysed in this work. On the other hand, the essential oil and the hydrolate of *C. ladanifer* showed some differences regarding the quantity and quality of the two extracts. In relation to essential oil, it has a large percentage of monoterpenes, in which the major compound is α-pinene and in the case of hydrolate, the major compound is 4-Hydroxy-3-methylacetophenone and α-pinene is not among the five compounds majority. This shows the importance of analyzing the chemical profile of the two types of extracts since they may have different compounds that could possibly have different bioactivities. All the data is shown in Table 2.

Table 2: Chemical composition of *C. ladanifer*, *H. italicum*, *T. capitata* and *O. basilicum* and essential oils 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	
	RI[a]	Peak Area (%)	RI	Peak Area (%)	RI	Peak Area (%)	RI	Peak Area (%)	RI	Peak Area (%)	RI	Peak Area (%)	RI	Peak Area (%)
Compounds														
α-pinene	920	35.8			917	7.4								
Camphene	930	6.7												
β-myrcene									968	2.1				
o-cymene	1004	4.6												
ρ-cymene									1000	5.5	995	0.4		
α-terpinene									991	1.8				
1,8-cineole							1011	15.4			1002	0.4	1007	3.4
2,2,6-trimethylcyclohexanone	1014	6.7												
γ-terpinene									1037	6.1				
Linalool													1084	38.3
Camphor													1129	1.1
Endo-borneol			1156	8.4			1160	3.0						
Terpinen-4-ol							1173	2.0			1166	0.7		

δ-Terpineol							1162	6.6						
p-cymen-8-ol			1179	10.7										
α-Terpineol							1188	30.5					1182	3.1
(-)-Myrtenol			1191	11.2										
D-verbenone			1205	9.8										
Bornyl acetate	1289	4.9								-				
Carvacrol							1311	29.6	1316	79.9	1311	98.1		
4-Hydroxy-3-methylacetophenone			1310	21.6										
Eugenol													1364	52.5
Nerol acetate					1379	11.5								
(-)-Italicene					1388	12.5								
γ-Curcumene					1493	16.0								
α-Curcumene					1496	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]*RI: Retention index calculated by the standard n-alkane series.*

271

272 **3.2 Acute toxicity**

273 The essential oils tested exhibited different levels of toxicity to *D. magna*. Generally,
274 when detected, the acute toxicity of the essential oils and extracts was dose and time-
275 dependent. The essential oil from the aerial parts of *C. ladanifer* caused immobilisation
276 of the daphnids at high concentrations (48h EC_{50} ~200 mg/L). The essential oil obtained
277 from the aerial parts of *H. italicum* showed no observable toxic effects up to the highest
278 concentration tested of 800 mg/L. The essential oil obtained from the aerial parts of *T.*
279 *capitata* caused the highest observable toxic effects, presenting the lowest EC_{50} value
280 of 10.81 mg/L (95% CI 9.55 – 12.60) after 48h of exposure (Table 3).

281

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

Plant	Type	Parts used	EC_{50} mg/L (24h)	95% (CI) mg/L	EC_{50} mg/L (48h)	95% (CI) mg/L
Cistus ladanifer	Essential oil	Aerial parts	201.10	*	199.70	*
Thymbra capitata	Essential oil	Aerial parts	12.05	11.03 – 13.31	10.81	9.55 – 12.60

284 * Not possible to calculate

285

All the hydrolates tested caused no observable acute effects to *D. magna* after 48h of exposure up to the highest concentrations tested. The hydrolates and the essential oil that caused no immobilisation to *D. magna* are presented in Table 4 alongside the highest concentrations tested. The maximum concentrations for which no observable effects were observed were always above the limit to be considered toxic by international regulations. The toxicity was ranked according to the Globally Harmonized System for Classification and Labelling of Chemicals (GHS) proposed by the United Nations (Table 5).

Table 4: Maximum concentrations tested of *H. italicum* essential oil and hydrolates that caused no observable effects in *D. magna* after 48h of exposure.

Plant	Type of extract	Part(s) used	Maximum concentration tested (mg/L)
Cistus ladanifer	Hydrolate	Aerial parts	2000
Helichrysum italicum	Essential oil	Aerial parts	800
	Hydrolate		2000
Ocimum basilicum	Hydrolate	Leaves	8000
Thymbra capitata	Hydrolate	Aerial parts	400

3.2.1 *Cistus ladanifer* essential oil and hydrolate

The EC_{50} for the *C. ladanifer* ranged between 201.1 and 199.7 mg/L at 24 and 48h, respectively. The chemical analysis of the essential oil used in this study identified 37 compounds present 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 that evaluated the composition of essential oils obtained from *C. ladanifer*. 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 amphene 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 - 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 24h of exposure and 63.2 mg/L after 48h of exposure. It was classified as long term hazardous to the aquatic environment under the Chronic 3 category ($EC_{50\,(48h)}$ within 10 and 100 mg/L and degradability of the test substance in the environment 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 48h EC_{50} value above 100 mg/L, which cannot be considered hazardous to the environment (Table 5). 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 cause no observable effects to *D. magna* after 48h of exposure up to 2000 mg/L. As mentioned above, *C. ladanifer* essential oil and hydrolate differ slightly 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 monoterpenes 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 in the present work, the samples were acquired commercially

while in the study by 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.

Table 5: Classification of essential oils and hydrolates tested according to the Globally Harmonized System for classification and labelling of chemicals (GHS) proposed by the United Nations. The proposed values are based on 48 hours EC_{50} values for crustaceans. Acute 1: ≤ 1 mg/L; 1 < Acute 2 ≤ 10 mg/L; 10 < Acute 2 ≤ 100 mg/L; Non-toxic: >100 mg/L.

Plant	Type of extract	48h EC_{50} mg/L	Classification
Cistus ladanifer	Essential oil	> 100	Non-toxic
	Hydrolate	> 100	Non-toxic
Helichrysum italicum	Essential oil	> 100	Non-toxic
	Hydrolate	> 100	Non-toxic
Ocimum basilicum	Hydrolate	> 100	Non-toxic
Thymbra capitata	Essential oil	10.8	Acute 3
	Hydrolate	> 100	Non-toxic

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 the toxicological effects of these compounds on *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 extract 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.

382 The chemical composition of essential oils obtained from *Helichrysum italicum* can vary
383 depending on the region the plant is grown, and it can present very distinct chemotypes
384 depending on subspecies and environmental factors (e.g. soil properties). An essential
385 oil obtained by hydrodistillation of the aerial parts of *H. italicum* from Croatia was
386 predominantly constituted of neryl acetate (20.5%), γ-curcumene (14.1%) and trans-α-
387 bergamotene (7.0%), and other 59 compounds were identified in lower percentages
388 (Dzamic et al., 2019). Another essential oil obtained from the aerial parts of *H. italicum*
389 in Montenegro showed similar chemical composition with neryl acetate and γ-
390 curcumene as the two most abundant compounds (29.2% and 18.8%, respectively), but
391 neryl propanoate was the third most abundant with 10.1% (Kladar et al., 2015).
392 Interestingly, another study reported β-eudesmene, β-bisabolene and α-pinene as the
393 three most abundant compounds (21.6%, 19.9% and 16.9%, respectively) of an essential
394 oil from *H. italicum* collected in Montenegro (Oliva et al., 2020). On the other hand, an
395 essential oil from aerial parts of *H. italicum* collected in the North of Algeria showed a
396 high diversity of compounds in small percentages, with α-cedrene (13.6%), α-curcumene
397 (11.4%) and geranyl acetate (10.1%) the three most abundant (Djihane et al., 2017). In
398 Portugal, for example, an essential oil obtained by hydrodistillation of the aerial parts of
399 *H. italicum* subsp. picardii collected in the south of Portugal was rich in α-pinene (53.5%)
400 and γ-curcumene (27.4%) (Costa et al., 2015).

401

402 **3.2.3 *Thymbra capitata* essential oil and hydrolate**

403 The *Thymbra capitata* essential oil was the most toxic towads *D. magna.* The EC_{50} values
404 obtained varied from 12.1 mg/L after 24h of exposure and 10.8 mg/L after 48h of

exposure. This essential oil can be classified as acutely hazardous to the aquatic environment under the acute 3 category of the GHS (Table 5) as the 48h 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 48h EC_{50} value to *D. magna* of 6.06 mg/L (95% CI 5.10 - 7.28 mg/L), 96h EC_{50} for the zebrafish *Danio rerio* of 6.17 mg/L (95% CI 4.83 – 9.92 mg/L) and 72h 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. 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 48h 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 48h EC_{50} of 20 mg/L has been reported (Api et al., 2016), and in the ECHA database, a 48h EC_{50} of 59 mg/L to *D. magna* is reported (ECHA). 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.

4. Conclusion

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 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.

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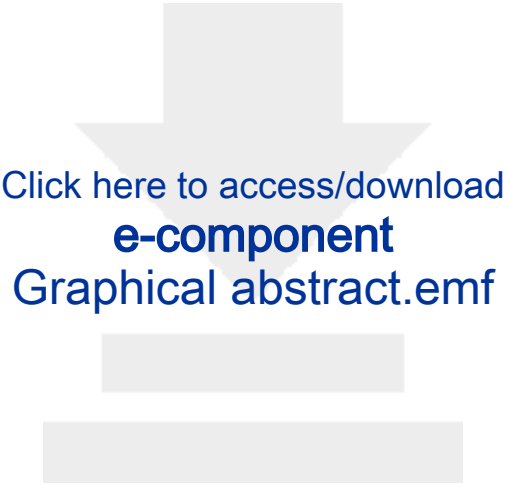

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Declaration of interests

☒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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