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Ecotoxicity of plant extracts

Celso Afonso Pestana Ferraz

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Orientador: Prof. Doutor Manuel Ramiro Dias Pastorinho
Co-orientadores: Prof. Doutora Ana Catarina Sousa e Prof. Doutora Ana Palmeira-de-Oliveira

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Dedicatória

A todos os que acreditaram em mim.

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Resumo

Desde a antiguidade, as plantas têm sido utilizadas pela Humanidade como uma importante fonte de compostos bioativos com diversas utilizações desde a medicina tradicional, passando pela alimentação, perfumaria e cosmética. Atualmente, a sua utilização distribui-se por quase todos os setores económicos e novas aplicações continuam a emergir. Algumas das mais importantes aplicações de compostos bioativos extraídos de plantas são realizadas recorrendo a óleos essenciais. Plantas que produzem óleos essenciais fazem-no naturalmente e esta produção está ligada a diversos fatores como resposta a stress, defesa contra ataques de agentes patogénicos e até como forma de atrair polinizadores que desempenham um papel fundamental na reprodução da planta, estando também ligada às condições ambientais e ecológicas em que a planta cresce. Estes óleos essenciais são obtidos industrialmente das plantas que os produzem por um processo de destilação ou em certos casos por um processo mecânico. Durante o processo de destilação para a obtenção de óleos essenciais, outro produto com interesse económico pode ser obtido: os hidrolatos, que são geralmente compostos por moléculas mais hidrossolúveis que permanecem na água de destilação. Outro produto que pode ser obtido das plantas são os extratos, que são obtidos através do tratamento de partes específicas de uma planta com um solvente.

A produção global de óleos essenciais tem crescido continuamente ao longo da última década e estima-se que esta tendência continue nos próximos anos. Esta crescente produção pode ser ligada à cada vez maior preocupação por parte dos consumidores na utilização de compostos naturais. Do ponto de vista académico, também podemos afirmar que a pesquisa por novos compostos e novas aplicações de produtos extraídos de plantas tem mostrado uma tendência crescente. Desta forma, o Projeto "InovEP – Inovação com Extratos de Plantas" tem como objetivo fazer a ligação entre a academia e a indústria, produzindo conhecimento científico que possa ser utilizado por empresas ligadas à produção de óleos essenciais e outros extratos de plantas, assim como empresas que queiram utilizar estes produtos para desenvolver novas aplicações baseando-se em evidências científicas. Entre os vários estudos efetuados, um deles tem como objetivo o estudo da segurança ambiental destes produtos.

O trabalho descrito na presente dissertação focou-se em avaliar os efeitos ecotoxicológicos de óleos essenciais, hidrolatos e extratos obtidos de várias plantas estudadas no projeto: *Cistus ladanifer*; *Cupressus lusitanica*, *Echinacea purpurea*,

Eucalyptus globulus, *Hamamelis virginiana*, *Helichrysum italicum*, *Humulus lupulus*, *Matricaria chamomilla*, *Ocimum basilicum*, *Thymbra capitata*, *Thymus citriodorus* e *Syzygium aromaticum*. Os testes realizados focaram-se na toxicidade aguda em organismos aquáticos utilizando como organismo modelo o cladócero *Daphnia magna*. Popularmente chamada de "pulga de água", é um dos organismos preferenciais para a realização de testes de toxicidade em ambiente aquático e é recomendada por várias organizações internacionais como a União Europeia (UE), a Organização para a Cooperação e Desenvolvimento Económico (OCDE), a Sociedade Americana para a Testagem e Materiais (ASTM) e a Organização Internacional para Padronização (ISO).

De uma forma geral, os resultados obtidos demostraram que os óleos essenciais são capazes de causar efeitos em concentrações mais baixas comparativamente aos extratos e aos hidrolatos estudados. O óleo essencial de *S. aromaticum* foi o que causou efeitos a concentrações mais baixas, seguido pelo óleo essencial de *T. capitata*, óleo essencial de *E. globulus* e óleo essencial de *C. ladanifer*. De todos os óleos essenciais apenas o de *H. italicum* não causou efeitos até à máxima concentração testada. De todos os extratos de *H. lupulus* testados, apenas se verificou imobilização dos organismos teste com o extrato clorofórmico, obtido das flores da planta, a altas concentrações. Todos os outros extratos não causaram imobilização até às máximas concentrações testadas, verificando-se a mesma tendência com todos os hidrolatos testados.

Em termos de classificação da toxicidade aguda dos óleos essenciais, extratos e hidrolatos testados foi seguido o sistema GHS (*Globally Harmonized System for Classification and Labelling of Chemicals*) proposto pelas Nações Unidas e utilizado também na União Europeia. Assim o óleo essencial de *S. aromaticum* pode ser classificado como tóxico para sistemas aquáticos na categoria "Agudo 2", e os óleos essenciais de *T. capitata* e *E. globulus* na categoria "Agudo 3". Os óleos essenciais de *C. ladanifer*, *H. italicum*, tal como todos os extratos e hidrolatos testados não podem ser classificados, sendo considerados não tóxicos, uma vez que os resultados obtidos são superiores aos limites de classificação propostos pela GHS.

Os óleos essenciais de *S. aromaticum*, *T. capitata* e *E globulus* podem causar efeitos agudos adversos em sistemas aquáticos, particularmente em organismos do mesmo nível trófico que *D. magna* e precauções devem ser tomadas de forma a evitar contaminações acidentais ou intencionais de sistemas aquáticos. Para os restantes óleos essenciais, extratos e hidrolatos, as mesmas precauções devem ser tomadas uma vez que, apesar de não serem classificados como tóxicos, os efeitos que podem causar em organismos de outros níveis tróficos são ainda desconhecidos.

Palavras-chave

Óleos essenciais; extratos; hidrolatos; toxicidade aguda; *Daphnia magna*

Abstract

Since ancient times, plants have been used by mankind as an important source of bioactive compounds with different uses ranging from traditional medicine, food, perfumery, and cosmetics. Currently, its use spreads to almost all economic sectors and new applications keep emerging. One of the great applications of compounds that can be extracted from plants is obtained in the form of essential oil. Plants that produce essential oils do it naturally and this production is linked to many factors such as response to stress, as a defence against pathogen attacks and even as a way to attract pollinators that play an important role in the reproduction of the plant, being also linked to the environmental and ecological conditions of the area in which the plant grows. These essential oils are obtainable industrially from the plants that produced them by a distillation process or in certain cases by a mechanical process. During the distillation process to obtain essential oils, another economically interesting product can be attained: hydrolates, which are generally composed of more hydrosoluble molecules that remain in the distillation water. Another product that can be obtained from plants are extracts, which are extracted by treating parts of a plant with a solvent.

Global essential oil production has been continuously increasing over the last decade and it is expected to continue rising over the next years. This growing production can be linked to the ever-bigger demand by consumers for natural products. From an academic point of view, it can also be said that research for new compounds and new applications of products extracted from plants has been increasing. In this way, the "InovEP Project – Innovation with Plant Extracts" has the goal to connect the university and the industry, providing scientific knowledge that can be used by companies linked to the production of essential oils and other plant extracts, as well as companies that want to use these products to develop new applications based in scientific evidence. Among the studies performed, one of the goals is to study the environmental safety of these products.

The work described in this dissertation focused on the ecotoxicological evaluation of essential oils, hydrolates and extracts obtained from several plants studied in the project: *Cistus ladanifer*; *Cupressus lusitanica*, *Echinacea purpurea*, *Eucalyptus globulus*, *Hamamelis virginiana*, *Helichrysum italicum*, *Humulus lupulus*, *Matricaria chamomilla*, *Ocimum basilicum*, *Thymbra capitata*, *Thymus citriodorus* and *Syzygium aromaticum*. The tests performed focused on the acute toxicity towards aquatic organisms using the cladoceran *Daphnia magna* as model organism. Commonly called

"water-flea", *D. magna* is one of the recommended organisms to perform toxicity tests in aquatic systems by several international organizations such as the European Union (EU), Organization for Co-operation and Economic Development (OECD), the American Society for Testing and Materials (ASTM), and the International Organization for Standardization (ISO).

The results, in general, show that essential oils can cause effects at lower concentrations when compared to the studied extracts and hydrolates. The *S. aromaticum* essential oil caused effects at lower concentrations, followed by the *T. capitata* essential oil, *E. globulus* essential oil and *C. ladanifer* essential oil. Of all the essential oils, only the one from *H. italicum* did not cause effects up to the highest concentration tested. Of all the *H. lupulus* extracts tested, immobilisation of the test organisms was only observed with high concentrations of the chloroform extract, obtained from the flowers. All the other extracts did not cause immobilisation up to the highest concentrations tested, and the same trend was observed with all the hydrolates tested.

In terms of classification of the acute toxicity of essential oils, extracts and hydrolates, the GHS (Globally Harmonized System for Classification and Labelling of Chemicals) proposed by the United Nations was followed, which is also used in the European Union. Thus, the *S. aromaticum* essential oil can be classified as toxic to aquatic systems under the "Acute 2" category, and the *T. capitata* and *E. globulus* essential oils under the "Acute 3" category. The essential oils from *C. ladanifer*, *H. italicum* and all the extracts and hydrolates tested can not be classified, being considered not toxic, as the obtained results are above the classification limits proposed in the GHS.

The *S. aromaticum*, *T. capitata* and *E. globulus* essential oils can cause acute adverse effects in aquatic systems, particularly in organisms in the same trophic level as *D. magna,* and so, precautions should be taken to avoid accidental or intentional contaminations of aquatic systems. For the other essential oils, extracts and hydrolates, the same precautions should be taken since, although they can not be classified as toxic, the effects that they can cause in organisms from different trophic levels remain unknown.

Keywords

Essential oils; extracts; hydrolates; acute toxicity; *Daphnia magna*

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Lista de Acrónimos

ISO	International Organization for Standardization
EU	European Union
AMR	Antimicrobial Resistance
GHS	Globally Harmonized System for Classification and Labelling of Chemicals
UN	United Nations
CLP	Classification, Labelling and Packaging of Substances
LC_{50}	Lethal Concentration 50
EC_{50}	Effect Concentration 50
ErC_{50}	Effect (growth) Concentration 50
NOEC	No Observed Effect Concentration
K_{ow}	Octanol-water partition coefficient
BCF	Bioconcentration Factor
ASTM	American Society for Testing and Chemicals
OECD	Organization for Economic Co-operation and Development
MIC	Minimum Inhibitory Concentration
NCI-H460	Human non-small-cell lung cancer cell line
MCF-7	Human breast cancer cell line
HepG2	Human liver cancer cell line
HeLa	Human cervical cancer cell line
MFC	Minimum Fungicidal Concentration
DPPH	2-2-diphenyl-1-picrylhydrazyl
U937	Human myeloid leukaemia cell line
MRSA	Methicillin-Resistant *Staphylococcus aureus*
HCT	Human collon carcinoma cells
IPCB	*Instituto Politécnico de Castelo Branco*
DMSO	Dimethyl Sulfoxide
ECHA	European Chemicals Agency
EC	European Community number
CAS	Chemical Abstract Service registry number
HSBD	Hazardous Substances Data Bank

Chapter 1 - Introduction

1.1 - Essential oils and plant extracts

Essential oils and plant extracts have been used for centuries in traditional medicine, as flavour enhancers in cooking, perfumery and cosmetics due to their unique properties (Ríos, 2016). Essential oils are volatile liquids obtained by distillation of any part of a plant or by a mechanical process when obtained from the epicarp of a citrus fruit at ambient temperature. During the process to extract essential oils, hydrolates can also be obtained as a by-product. An hydrolate is, according to the International Organization for Standardization (ISO), the distilled water that remains after the distillation process and is usually rich in water-soluble components of the essential oil (ISO, 2013) as depicted in figure 1. Conversely, an extract is "a product obtained by treating a natural raw material with a solvent then, after filtration, removal of the solvent by distillation, except in the case of use of a non-volatile solvent" (ISO, 1997).

Plants produce essential oils naturally as secondary metabolites in response to stress, as a defence against pathogen attacks and to attract pollinators that play a determinant role in the reproduction of the plant (Roohinejad et al., 2017). Variations in environmental conditions and ecological factors directly impact the ability of a plant to produce essential oils, affecting also the type of compounds produced, and the quality and quantity of the oils (Chrysargyris et al., 2020; Figueiredo et al., 2008b).

Essential oils and plant extracts have been continuously used as a source of bioactive molecules. Due to the increasing interest from customers for natural and safer products, the demand for natural-based products has increased during the last few years. In fact, the applications of these plant-derived compounds are currently spread throughout almost all sectors of economic activities such as food, agriculture, pharmaceuticals, cosmetics and textile (Jugreet et al., 2020). Industries are keeping up with the consumer demand for natural products. In May 2020, a market size analysis on the global essential oils market by Grand View Research, estimated the global demand for these products to be around 247 kilotons in 2020 and it is expected to continue growing for the next years. The same report also estimates that the demand will almost double by 2027 to a whopping 473 kilotons (Grand_View_Research, 2020). By far, the most produced oils are orange, lemon and mint oils. These oils represent more than two-thirds of total essential crop production. In the EU (European Union), in 2016 the production of essential oils was about 41 kilotons, although the major producers around the world are Asian countries such as China, India and Indonesia (Barbieri and Borsotto, 2018). This increased interest from the industry is also accompanied by increasing interest from academia. This intensive research by the scientific community is responsible for the discovery of new compounds and new applications of compounds extracted from plants (Rana and Das, 2017; Zhang et al.,

2020). Amongst these new applications, some are being used to fight the most pressing health care problems of our society, for example, antimicrobial resistance (AMR), vector-borne diseases and cancer. Recently, natural products (such as essential oils) have been an important ally in developing products with antibacterial properties that can be used instead of antibiotics (Abdelli et al., 2018; Alonso-Esteban et al., 2019; Reid et al., 2020; Tavares et al., 2020a). Another growing concern is the resistance of insects to synthetic chemical pesticides and the environmental issues linked to the widespread usage of these chemicals that often result in long term contamination of soils and water bodies, with severe consequences to ecosystems and human health. Essential oils and extracts of some plants have been studied for their potential to be used as biopesticides which sometimes have similar effects as chemical pesticides with the advantage of – most of the time – having high degradability in the environment and being relatively safe to non-target organisms (Benelli et al., 2020a; Pavela et al., 2020; Pintong et al., 2020). Besides antimicrobial properties and the potential use as biopesticides, essential oils and plant extracts have also been intensively studied for their cytotoxic activity in tumour cells, which can lead to new cancer therapies or to enhance the effectiveness of existing cancer drugs (El-Garawani et al., 2019; Saleh et al., 2017; Salehi et al., 2020).

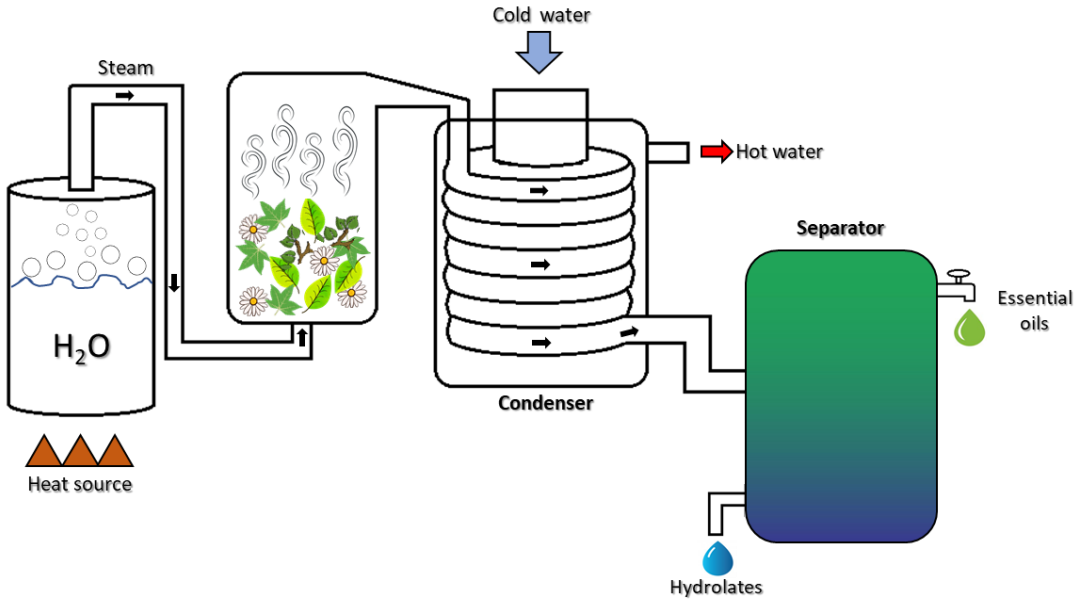

Figure 1: Schematic representation of a steam distillation process to obtain essential oils and hydrolates.

The intensive research regarding essential oils and other plant extracts is spread throughout several fields of study. Most of the studies published on plant extracts and essential oils focus on pharmaceuticals, agriculture, medicine, and biochemistry, which account for over 75% of all research published (Figure 2).

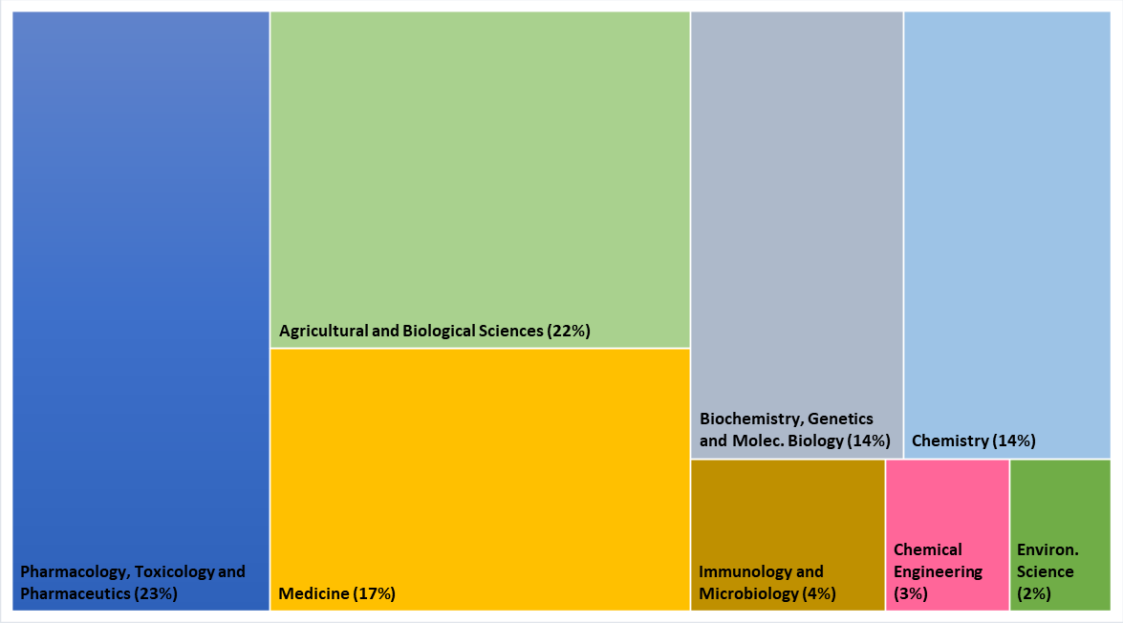

Figure 2: Relative distribution of the number of papers published between 2000 and 2020 across eight main categories. Data retrieved from Scopus Database on December 18th, 2020, using the keywords: Plant extract(s); Essential oil(s).

Overall, most of the research focuses on industrial applications of these products, including antibacterial and antifungal properties, potential development of new biopesticides, applications in new drugs and therapies or even applications in anti-corrosion chemicals. Remarkably, despite all the increasing interest in these resources, and the consequent expected increase in their production, possible environmental impacts of essential oils and plant extracts received far less attention from the scientific community. In fact, only 2% of the studies (n=320) were published in the environmental sciences category (c.f. Figure 2). The general idea that plants and their components are generally harmless and safe, could explain this lack of studies. However, some plants can produce metabolites that can be highly toxic (Falkowski et al., 2020; Zárybnický et al., 2018) and therefore the evaluation of their possible toxic effects on non-target organisms needs to be performed.

1.2 Toxicity of essential oils

Although generally perceived as safe and green products, essential oils and other plant extracts can have toxic properties (Falkowski et al., 2020; Gupta, 2016; Zárybnický et al., 2018). Some toxic properties of these products can be beneficial. As mentioned before, the toxic properties that some essential oils and extracts have, are helping in the development of new and "safer" biopesticides. Some of these products also have cytotoxic properties that can lead to the development and/or enhancement of cancer therapies and help us in the recent concern with growing resistance of microorganisms to antibiotics. Despite all these benefits, the industrial production of essential oils and plant extracts can also have an impact on the environment, and following the precautionary principle, it is important to assess this impact. Several regulations

and legislation are now in place throughout the world that aim to guarantee a safer use, transport and disposal of chemicals. The most ambitious is the Globally Harmonized System for Classification and Labelling of Chemicals (GHS) by the United Nations (UN). The objective is to have in place a harmonized system that can ensure that information regarding hazards and toxicity from chemicals is available to everyone in order to enhance human health and environmental protection. In the European Union (EU), the GHS has been implemented into EU law as the classification, labelling and packaging of substances and mixtures (CLP) regulation. In the GHS, a system of classification for environmental hazards of chemicals and mixtures of chemicals is set, based on the information of the 48h LC_{50}, EC_{50} or ErC_{50} (ErC_{50} - growth rate of algae or aquatic plants) values obtained when test organisms are exposed to these substances. Essential oils and other plant extracts are classified for their acute toxicity under the GHS. This system is divided into three categories for short-term (acute) aquatic toxicity on the basis of EC_{50} or LC_{50} values. Table 1 describes the classification system for acute toxicity to aquatic organisms. Substances that are classified under one of these categories are considered "hazardous to the aquatic environment" (UN, 2019).

Table 1: Categories for short-term (acute) toxicity of substances to aquatic environment as proposed by the GHS. Adapted from UN (2019).

Acute toxicity		
Category	**Endpoint**	**Range**
Acute 1	96h LC_{50} (fish) 48h EC_{50} (crustacea) 72 or 96h ErC_{50} (algae and aquatic plants)	≤ 1 $mg.L^{-1}$ and/or ≤ 1 $mg.L^{-1}$ and/or ≤ 1 $mg.L^{-1}$
Acute 2	96h LC_{50} (fish) 48h EC_{50} (crustacea) 72 or 96h ErC_{50} (algae and aquatic plants)	>1 - ≤ 10 $mg.L^{-1}$ and/or >1 - ≤ 10 $mg.L^{-1}$ and/or >1 - ≤ 10 $mg.L^{-1}$
Acute 3	96h LC_{50} (fish) 48h EC_{50} (crustacea) 72 or 96h ErC_{50} (algae and aquatic plants)	>10 - ≤ 100 $mg.L^{-1}$ and/or >10 - ≤ 100 $mg.L^{-1}$ and/or >10 - ≤ 100 $mg.L^{-1}$ and/or

Besides the acute toxicity classification, which is based only on the LC_{50} or EC_{50} data, the GHS also proposes a system for chronic toxicity classification. This system is divided into four categories based on the acute toxicity data and environmental fate data (degradability and bioaccumulation) as well as No Observed Effect Concentration (NOEC) data. An overview of this classification system is presented in table 2.

Table 2: Categories for long term (chronic) toxicity of substances to aquatic environment as proposed by the GHS. K_{ow} – octanol-water partition coefficient; BCF – Bioconcentration factor. Adapted from UN (2019).

Chronic toxicity		
Category	**Endpoint**	**Range**
Chronic 1	96h LC_{50} (fish) 48h EC_{50} (crustacea)	≤ 1 $mg.L^{-1}$ and/or ≤ 1 $mg.L^{-1}$ and/or

	72 or 96h ErC_{50} (alage and aquatic plants)	≤ 1 $mg.L^{-1}$
	And the substance is not rapidly degradable and/or the log K_{ow} ≥ 4 (unless the experimentally determined Bioconcentration factor (BCF) <500).	
Chronic 2	96h LC_{50} (fish) 48h EC_{50} (crustacea) 72 or 96h ErC_{50} (alage and aquatic plants)	>1 - ≤ 10 $mg.L^{-1}$ and/or >1 - ≤ 10 $mg.L^{-1}$ and/or >1 - ≤ 10 $mg.L^{-1}$
	And the substance is not rapidly degradable and/or the log K_{ow} ≥ 4 (unless the experimentally determined Bioconcentration factor (BCF) <500) unless the chronic toxicity NOECs are > 1 $mg.L^{-1}$.	
Chronic 3	96h LC_{50} (fish) 48h EC_{50} (crustacea) 72 or 96h ErC_{50} (alage and aquatic plants)	>10 - ≤ 100 $mg.L^{-1}$ and/or >10 - ≤ 100 $mg.L^{-1}$ and/or >10 - ≤ 100 $mg.L^{-1}$ and/or
	And the substance is not rapidly degradable and/or the log K_{ow} ≥ 4 (unless the experimentally determined Bioconcentration factor (BCF) <500) unless the chronic toxicity NOECs are > 1 $mg.L^{-1}$.	
Chronic 4	Poorly soluble substances for which no acute toxicity is recorded at levels up to the water solubility, and which are not rapidly degradable and have a log Kow ≥ 4, indicating a potential to bioaccumulate, will be classified in this category unless other scientific evidence exists showing classification to be unnecessary. Such evidence would include an experimentally determined BCF < 500, or a chronic toxicity NOECs > 1 mg/l, or evidence of rapid degradation in the environment.	

1.3 Ecotoxicity

Michael C. Newman defines ecotoxicology as "the science of contaminants in the biosphere and their effects on constituents of the biosphere, including humans" (Newman, 2009).

Despite essential oils and plant extracts being produced in large quantities, which are expected to continue rising, the available data concerning the ecotoxicity of these products is very scarce. Some studies have addressed the potential toxicity of essential oils and plant extracts towards non-target organisms, and some oils and extracts have been shown to have toxic effects. By far, the most studied non-target species is *Daphnia magna*. The available data in the literature on the toxicity of plant extracts and essential oils towards this organism is presented in table 3. Other crustaceans have also been used for acute toxicity tests, such as *Daphnia pulex*, *Scapholeberis kingi* and *Artemia salina*, and are also presented in table 3. In this table is also included the classification regarding the toxicity reported based on 48h EC_{50} values as proposed in the Globally Harmonised System for Classification and Labelling of Chemicals (GHS) by the UN. Organisms from the *Daphnia* genus, particularly *Daphnia magna*, are recommended for the performance of ecotoxicity tests of chemicals and other substances by international organizations like ASTM (American Society for Testing and Materials) and OECD (Organisation for Economic Co-operation and Development) (ASTM, 1997; OECD, 2004) to assess the risk of materials to aquatic organisms.

Table 3: Essential oils and plant extracts tested towards crustaceans (from 2000 – 2020) and categorisation according to the GHS (UN, 2019). (The reported values that do not follow the recommendations were considered not classifiable).

Plant				**Toxicity evaluation**				**Reference**
Family	**Species**	**Part(s) used**	**Type**	**Test species**	**Test/Endpoint**	**48h EC_{50}/LC_{50} ($mg.L^{-1}$)**	**GHS Classification**	
Amaranthaceae	*Amaranthus retroflexus*	Leaves	Ethanolic	*D. magna*	Acute toxicity	1053	Not toxic	(Dinu et al., 2017)
Apiaceae	*Anthriscus sylvestris*	Aerial parts	Aqueous			483.7	Not toxic	(Olaru et al., 2016)
			Hydroethanolic			102.4		
			Ethanolic			106.9		
	Ferula assa-foetida	Oleo-gum-resin	Essential oil			N. D.	N. C.	(Pavela et al., 2020)
	Ferula gummosa	Oleo-gum-resin	Essential oil			N. D.	N. C.	
Asteraceae	*Achillea millefolium*	Aerial parts	Essential oil			13.6	Acute 3	(Zanfirescu et al., 2020)
	Artemisia absinthium	N. S.	Ethanolic			0.093	Acute 1	(Pino-Otín et al., 2019a)
			Hexane			0.103	Acute 1	
			Methanolic			0.236%	N. C.	
	Petasites hybridus	Roots	Methanolic			178.6 (72h)	N. C.	

Plant				Toxicity evaluation				Reference
Family	**Species**	**Part(s) used**	**Type**	**Test species**	**Test/Endpoint**	**48h EC_{50}/LC_{50} ($mg.L^{-1}$)**	**GHS Classification**	
	Senecio vernalis	Aerial parts	Methanolic			83.31 (72h)	N. C.	(Seremet et al., 2018)
	Solidago canadensis	N. S.	Ethanolic			> 1000	Not toxic	(Huang et al., 2014)
	Tussilago farfara	Leaves	Methanolic			189.97 (72h)	N. C.	(Seremet et al., 2018)
Berberidaceae	*Berberis vulgaris*	Bark	Ethanolic			201.3 (24h)	N. C.	(Gîrd et al., 2017)
Boraginaceae	*Symphytum officinale*	Roots	Methanolic			801.0 (72h)	N. C.	(Seremet et al., 2018)
Cupressaceae	*Chamaecyparis lawsoniana*	Heartwood	Essential oil			1.9	Acute 2	(Duringer et al., 2010) (Duringer et al., 2010)
	Juniperus occidentalis	Leaves	Essential oil			> 5	N. C.	
	Taxodium distichum	Female cones	Essential oil			10.9	Acute 3	(Zanfirescu et al., 2020)
		Wood	Aqueous			6.49	Acute 2	

Plant				Toxicity evaluation				Reference
Family	**Species**	**Part(s) used**	**Type**	**Test species**	**Test/Endpoint**	**48h EC_{50}/LC_{50} ($mg.L^{-1}$)**	**GHS Classification**	
	Tetraclinis articulata				Acute and chronic toxicity (reproduction)	EC50 8.17 NOEC 0.49 LOEC 0.83	Acute 2	(Montassir et al., 2017)
Ericaceae	*Ledum palustre*	Aerial parts	Essential oil			N. D.	N. C.	(Benelli et al., 2020a)
Euphorbiaceae	*Hura crepitans*	Bark	Aqueous			0.036	Acute 1	(Iannacone et al., 2014)
Equisetaceae	*Equisetum arvense*	Bark+Leaves	Ethanolic			50-100	Acute 3	(Andreu et al., 2018)
Fabaceae	*Medicago sativa*	Aerial parts	Ethanolic			1008 (24h)	N. C.	(Gîrd et al., 2017)
	Robinia pseudoacacia	Leaves	Aqueous		Acute toxicity	4290 (96h)	N. C.	(Alonso et al., 2020)

Plant				Toxicity evaluation				Reference
Family	**Species**	**Part(s) used**	**Type**	**Test species**	**Test/Endpoint**	**48h EC_{50}/LC_{50} ($mg.L^{-1}$)**	**GHS Classification**	
	Tephrosia vogelii	Leaves	Aqueous			0.00047 (24h)	N. C.	(Li et al., 2015)
Geraniaceae	*Pelargonium gravolens*	Flowering aerial parts	Dry extract			203.3	Not toxic	(Neagu et al., 2018)
Humiriaceae	*Humiria balsamifera*	Bark	Ethyl acetate			N. D	N. C.	(Falkowski et al., 2020)
Lamiaceae	*Origanum vulgare*	Aerial parts	Ethanolic			364.4	Not toxic	(Gîrd et al., 2016)
Lythraceae	*Trapa japonica*	Leaves	Methanolic			4.7-22.0	Acute 2/3	(Ishimota et al., 2019)
Monimiaceae	*Peumus boldus*	Leaves	Essential oil			N. D.	N. C.	(Pavela et al., 2019)

Plant				Toxicity evaluation				Reference
Family	**Species**	**Part(s) used**	**Type**	**Test species**	**Test/Endpoint**	**48h EC_{50}/LC_{50} ($mg.L^{-1}$)**	**GHS Classification**	
Oleaceae	*Fraxinus angustifolia*	Leaves	Aqueous			9500 (96h)	N. C.	(Alonso et al., 2020)
Papaveraceae	*Chelidonium majus*	Aerial parts	Ethanolic			258.1	Not toxic	(Jancula et al., 2007)
	Dicranostigma lactucoides	Roots	Aqueous			31.25	Acute 3	
	Macleaya microcarpa	Roots	Aqueous			> 1000	Not toxic	
	Sanguinaria canadensis	Roots	Aqueous			62.0	Acute 3	
	Stylophorum lasiocarpum	Roots	Aqueous			> 400	Not toxic	

Plant				Toxicity evaluation				Reference
Family	**Species**	**Part(s) used**	**Type**	**Test species**	**Test/Endpoint**	**48h EC_{50}/LC_{50} ($mg.L^{-1}$)**	**GHS Classification**	
Plantaginaceae	*Plantago lanceolata*	Leaves	Ethanolic			375.0	Not toxic	(Zanfirescu et al., 2020)
Polygonaceae	*Fallopia aubertii*	Flowers	Aqueous			3019.95 (24h)	N. C.	(Olaru et al., 2015)
			Hidroethanolic			2398.83 (24h)		
			Ethanolic			2951.20 (24h)		
	Fallopia convulvulus	Stems, Leaves, Flowers and fruits	Hydroethanolic			N. D.	N. C.	
	Fallopia dumetorum		Hydroethanolic			4073.8 (24h)	N. C.	(Olaru et al., 2015)
Salicaceae	*Populus alba*	Leaves	Aqueous			9500 (96h)	N. C.	(Alonso et al., 2020)
Sapindaceae	*Aesculus hippocastanum*	Seeds	Ethanolic			7.5	Acute 2	(Zanfirescu et al., 2020)

Plant				Toxicity evaluation				Reference
Family	**Species**	**Part(s) used**	**Type**	**Test species**	**Test/Endpoint**	**48h EC_{50}/LC_{50} ($mg.L^{-1}$)**	**GHS Classification**	
	Mataya arborescens	Fruits	Ethyl acetate			N. D.	N. C.	(Falkowski et al., 2020)
Simaroubaceae	*Ailanthus altissima*	Leaves	Aqueous			10100 (96h)	N. C.	(Alonso et al., 2020)
Solanaceae	*Solanum nigrum*	Leaves	Ethyl acetate			N. D.	N. C.	(Rawani et al., 2014b)a
			Petroleum ether			N. D.	N. C.	(Rawani et al., 2014a)b
			Benzene			N. D.	N. C.	(Rawani et al., 2014a)b
			Chloroform/Methanol			N. D.	N. C.	(Rawani et al., 2017)
			Ethanolic			N. D.	N. C.	(Rawani et al., 2014a)b
Quillajaceae	*Quillaja saponaria*	Bark	Commercial extract			27.3	Acute 3	(Jiang et al., 2018)

Plant				Toxicity evaluation				Reference
Family	**Species**	**Part(s) used**	**Type**	**Test species**	**Test/Endpoint**	**48h EC_{50}/LC_{50} ($mg.L^{-1}$)**	**GHS Classification**	
Asteraceae	*Artemisia absinthium*	Aerial parts	Ethanolic	*D. pulex*		150-200	N. C.	(Andreu et al., 2018)
	Artemisia vulgaris	Aerial parts	Ethanolic			50-100	N. C.	
Equisetaceae	*Equisetum arvense*	Bark + leaves	Ethanolic			50-100	N. C.	
Salicaceae	*Salix alba*	Bark + leaves	Ethanolic			150-200	N. C.	
Lythraceae	*Trapa japonica*	Leaves	Methanolic	*S. kingi*		1.2-6.9	Acute 2	(Ishimota et al., 2019)
Asteraceae	*Petasites hybridus*	Roots	Methanolic	*A. salina*		296.48 (24h)	N. C.	(Seremet et al., 2018)

Plant				Toxicity evaluation				Reference
Family	**Species**	**Part(s) used**	**Type**	**Test species**	**Test/Endpoint**	**48h EC_{50}/LC_{50} ($mg.L^{-1}$)**	**GHS Classification**	
	Senecio vernalis	Aerial parts	Methanolic			131.22 (24h)	N. C.	
	Tussilago farfara	Leaves	Methanolic			222.33 (24h)	N. C.	
Boraginaceae	*Symphytum officinale*	Roots	Methanolic			707.95 (24h)	N. C.	
Eleagnaceae	*Eleagnus angustifolia*	Flowers	Essential oil			2.25	Acute 2	(Torbati et al., 2016)
		Leaves				11.0	Acute 3	
Polygonaceae	*Fallopia aubertii*	Flowers	Aqueous			2239.55	Not toxic	(Olaru et al., 2015)
		Stems+leaves	Hydroethanolic			2576.36		
		Flowers	Ethanolic			1872.16		
	Fallopia convulvulus	Stems, Leaves, Flowers and fruits	Hydroethanolic			N. D.	N. C.	

Plant				Toxicity evaluation				Reference
Family	**Species**	**Part(s) used**	**Type**	**Test species**	**Test/Endpoint**	**48h EC_{50}/LC_{50} ($mg.L^{-1}$)**	**GHS Classification**	
	Fallopia dumetorum	Stems, Leaves, Flowers and fruits	Hydroethanolic			2689.09	Not toxic	

N.S. – Not specified; N.D. – Not determined; N. C. – Not classifiable.

Most of the essential oils and plant extracts tested for the acute toxicity to *Daphnia magna* have shown low toxicity, i.e., EC_{50}> 100 mg.L^{-1}, as shown in table 2. Nevertheless, some extracts have shown high acute toxicity. The best example of that is the aqueous extract of *Tephrosia vogelii* that was reported to be remarkably toxic to *D. magna* with a 24h LC_{50} value of 0.47 µg.L^{-1} (Li et al., 2015). This plant, belonging to the Fabaceae family, has been used in India and other tropical regions as a fish-poison, insecticide or for soil enrichment, and various studies have shown the potential of the essential oils of *T. vogelii* to be used as bioinsecticides and larvicides (Bravim dos Santos et al., 2021; Touqeer et al., 2013). An aqueous extract from the bark of *Hura crepitans* (Euphorbiaceae) also showed to be acutely toxic towards *D. magna*, although to a lesser extent with 48h LC_{50}= 0.036 mg.L^{-1} (Iannacone et al., 2014). Like *T. vogelii*, *H. crepitans* is a tree known for its toxicity, especially the latex it produces which is rich in huratoxin (Trinel et al., 2018; Vassallo et al., 2020b). A comparison of reported 48h EC_{50} values of essential oils and other plant extracts to *D. magna* is presented in figure 3 as well as the respective classification according to the GHS.

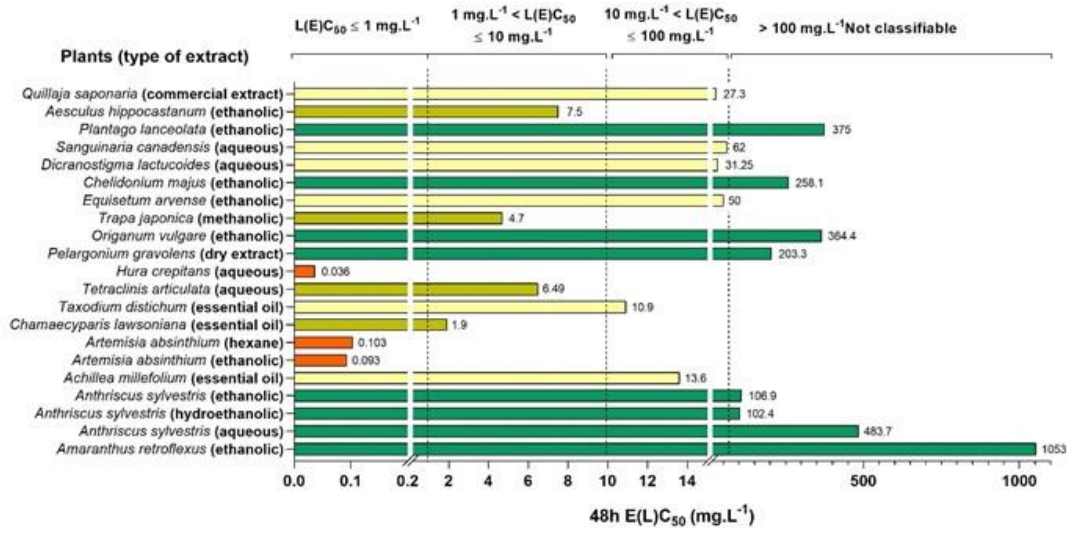

Figure 3: Classification of the 48h acute toxicity of essential oils and extracts based on L(E)C_{50} values towards *D. magna* reported in the literature according to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) by the United Nations.

As shown in Table 3 and Figure 3, essential oils and other extracts obtained from plants, although generally perceived as "green" and safe products, can have adverse effects on aquatic organisms on the same trophic level as *D. magna*. Considering the potentially toxic effects that these plant products can have in the environment, and in the framework of the InovEP Project – "Inovação com extratos de plantas" which aims to provide scientific data regarding applications and safety of essential oils and extracts from plants that are present in the Portuguese territory, toxicity tests were performed to evaluate the potentially toxic effects that the industrial exploitation of these products can have in the environment, particularly aquatic systems, using the model organism *Daphnia magna*.

1.4 Animal models for ecotoxicity tests

Several organisms are recommended by international organisations to assess the effects on different biotic systems, for example the microalgae *Raphidocelis subcapitata* (OECD, 2011), the zebrafish *Danio rerio* (OECD, 2013, 2019), the crustacean *Daphnia magna* (OECD, 2004), the earthworm *Eisenia fetida* (OECD, 2016), the aquatic plant *Lemna minor* (OECD, 2006a) and several terrestrial plants such as the common onion *Allium cepa* (OECD, 2006b). In the OECD Guidelines for the Testing of Chemicals, Section 2, there are 49 guidelines used by governments, industry, and independent laboratories to identify and characterise potential hazards of chemicals. Tables 4, 5, 6 and 7 summarise different essential oils, hydrolates and extracts that have been studied regarding their toxic effects on microalgae, fish, plants and earthworms, respectively.

Table 4: Essential oils, hydrolates and plant extracts tested towards microalgae (from 2000 – 2020) and categorization according to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) by the United Nations. (The reported values that do not follow the recommendations were considered not classifiable).

Plant				**Toxicity evaluation**				**Reference**
Family	**Species**	**Part(s) used**	**Type of Extract**	**Test species**	**Endpoint**	**EC_{50} ($mg.L^{-1}$)**	**GHS Classification**	
Asteraceae	*Artemisia absinthium*	N. S.	Hydrolate	*C. reinhardtii*	Photosynthetic activity	16.49% (1h)	N. C.	(Pino-Otín et al., 2019a)
Cupressaceae	*Juniperus occidentalis*	Leaves	Essential oil	*R. subcapitata*	Algal cell density	1.7 (96h)	Acute 2	(Duringer et al., 2010)
					biomass	1.7 (96h)		
					growth	2.0 (96h)		
	Chamaecyparis lawsoniana	Heartwood	Essential oil		Algal cell density	> 5	N. C.	
					biomass	> 5		
					growth	> 5		
Fabaceae	*Pterodon emarginatus*	Fruits	Oleoresin (in nanoemulsion)	*C. vulgaris*	Algal density, growth	N. D.	N. C.	(Oliveira et al., 2016)
Malvaceae	*Alcea rosea*	Leaves	Aqueous extract + nanoparticles		biomass	0.0336	Acute 1*	(Khoshnamvand et al., 2020)
					Chlorophyll a	N. D.		
Papaveraceae	*Chelidonium majus*	Roots	Aqueous	*R. subcapitata* (1); *S. quadricauda* (2)	Growth	(1) 96h: 60.87	(2) 96h: 78.01	Acute 3	(Jancula et al., 2007)
	Dicranostigma lactucoides					21.27	20.61	Acute 3	

Plant				Toxicity evaluation				Reference
Family	**Species**	**Part(s) used**	**Type of Extract**	**Test species**	**Endpoint**	**EC_{50} ($mg.L^{-1}$)**	**GHS Classification**	
	Macleaya microcarpa					> 600 > 600	N. C. (not toxic)	
	Sanguinaria canadensis					23.90 29.05	Acute 3	
	Stylophorum lasiocarpum					114.10 117.48	N. C. (not toxic)	
Pinaceae	*Pinus radiata*	Bark	Methanolic (polyflavonoids extracts)	*R. subcapitata*	Growth	N. D.	N. C.	(García et al., 2017)

N.S. – Not specified; N.D. – Not determined; N. C. – Not classifiable.

Table 5: Essential oils and plant extracts tested towards fish (from 2000 – 2020).

Plant				Toxicity evaluation			Reference
Family	**Species**	**Part(s) used**	**Type of extract**	**Test species**	**Endpoint(s)**	**LC_{50} (mortality)**	
Apocynaceae	*Cascabela thevetia*	Fruits	Methanolic	*D. rerio*	Mortality; Developmental abnormalities; Coagulation; Embryonic movements and heart rate; Length; Failure to straighten; Edema	(72h) 1000 $mg.L^{-1}$	(Haldar et al., 2015)

Plant				Toxicity evaluation			Reference
Family	**Species**	**Part(s) used**	**Type of extract**	**Test species**	**Endpoint(s)**	**LC_{50} (mortality)**	
Asteraceae	*Solidago canadensis*	N. S.	Ethanolic		Mortality	(72h) 320 $mg.L^{-1}$	(Huang et al., 2014)
Lamiaceae	*Leonurus japonicus*	Aerial parts	Essential oil		Mortality; Developmental abnormalities; Embryo hatching rate; Embryo heartbeat	(24 hpf) ~10 $mg.L^{-1}$; (48 hpf) ~60 $mg.L^{-1}$	(He et al., 2018)
Piperaceae	*Piper turbeculatum*	Roots	Piplartine isolated from the methanolic extract		Mortality; Swimming activity; Developmental abnormalities	1.69 $mg.L^{-1}$	(Rapado et al., 2013)
Zingiberaceae	*Zingiber cassamunar*	Rhizomes	Essential oil		Mortality; Developmental abnormalities	N. D.	(Mektrirat et al., 2020)
Apiaceae	*Heracleum sprengelianum*	Leaves	Essential oil	*G. affinis*	Mortality; Swimming activity	4219 $mg.L^{-1}$	(Govindarajan and Benelli, 2016)
Zingiberaceae	*Zingiber nimmonii*	Leaves	Essential oil		Mortality	(48h) 9250.12 $mg.L^{-1}$	(Govindarajan et al., 2016)
Cupr	*Chamaecyparis lawsoniana*	Leaves	Essential oil	*O. mykiss*	Mortality	(96h) >5 $mg.L^{-1}$	(Duringer et al., 2010)

Plant				Toxicity evaluation			Reference
Family	**Species**	**Part(s) used**	**Type of extract**	**Test species**	**Endpoint(s)**	**LC_{50} (mortality)**	
	Juniperus occidentalis	Heartwood	Essential oil		Mortality	(96h) >5 $mg.L^{-1}$	(Duringer et al., 2010)
Fabaceae	*Tephrosia vogelii*	Leaves	Aqueous	*O. niloticus*	Mortality	5.31 $\mu g.L^{-1}$	(Li et al., 2015)

N.S. – Not specified; N.D. – Not determined; N. C. – Not classifiable.

Table 6: Essential oils and plant extracts tested towards plants (from 2000 – 2020).

Plant				Toxicity evaluation				Reference
Family	**Species**	**Part(s) used**	**Type of extract**	**Test species**	**Family**	**Endpoint**	**EC_{50} ($mg.L^{-1}$)**	
Amaranthaceae	*Amaranthus retroflexus*	Leaves	Hydroalcoholic	*T. aestivum*	Poaceae	Cytotoxicity and genotoxicity	N. D.	(Dinu et al., 2017)
Asteraceae	*Achillea biebersteinii*	Flowering aerial parts	Essential oil and extracts (n-hexane, Acetone, methanolic)	*A. retroflexus*	Amaranthaceae	Germination, root growth and seedling growth	N. D.	(Çakır et al., 2015)
				C. album	Chenopodiaceae			
	Achillea biserrate			*C. juncea*	Asteraceae			
	Achillea coarctata			*L. serriola*				
				T. officinale				
	Achillea wilhelmsii			*R. crispus*	Polygonaceae			
	Artemisia absinthium	N. S.	Hydrolate	*A. cepa*	Amaryllidaceae	Root growth	3.87% (v/v)	(Pino-Otín et al., 2019b)

Plant				Toxicity evaluation				Reference
Family	**Species**	**Part(s) used**	**Type of extract**	**Test species**	**Family**	**Endpoint**	**EC_{50} ($mg.L^{-1}$)**	
Papaveraceae	*Chelidonium majus*	Roots	Aqueous	*L. minor*	Araceae	Growth	484.69	(Jancula et al., 2007)
	Dicranostigma lactucoides	Roots	Aqueous				N. D.	
	Sanguinaria canadensis	Roots	Aqueous				N. D.	
	Stylophorum lasiocarpum	Roots	Aqueous				> 500	
	Macleaya microcarpa	Roots	Aqueous				N. D.	
Pinaceae	*Pinus radiata*	Bark	Extract of polyflavonoids	*L. sativa*	Asteraceae	Percentage of germination and radicle length	N. D.	(García et al., 2017)

N.S. – Not specified; N.D. – Not determined.

Table 7: Essential oils and plant extracts tested towards earthworms (from 2000 – 2020).

Plant				Toxicity evaluation			Reference
Family	**Species**	**Part(s) used**	**Type of extract**	**Test species**	**Endpoint**	**EC_{50}**	
Asteraceae	*Artemisia absinthium*	N. S.	Hydrolate	*E. fetida*	Mortality	0.07 mL.g-1	(Pino-Otín et al., 2019b)
	Stevia rebaudiana	Flowering branches	Essential oil			N. D.	(Benelli et al., 2020b)
Apiaceae	*Cuminum cyminum*	Seeds	Essential oil			N. D.	(Benelli et al., 2018)
	Ferula assa-foetida	Oleoresin	Essential oil			N. D.	(Pavela et al., 2020)
	Ferula gummosa	Oleoresin	Essential oil			N. D.	
	Foeniculum vulgare	N. S.	Commercial essential oil			N. D.	(Pavela, 2018)
	Pimpinella anisum	Seeds	Essential oil			N. D.	(Benelli et al., 2018)

Meliaceae	*Swietenia mahagoni*	Leaves	Essential oil	*E. eugeniae*		N. D.	(Dinesh-Kumar et al., 2018)
Pieraceae	*Piper betle*	Leaves	Essential oil			N. D.	(Vasantha-Srinivasan et al., 2016)

N.D. – Not determined.

1.5 Test organism – *Daphnia magna*

Daphnia magna is part of the Cladocera order. Popularly called "water-fleas", these organisms are recommended by main organizations like the OECD (OECD 201, OECD 202) (OECD, 2004, 2011), or ISO (ISO 6341:2012) (ISO, 2012) to perform acute and chronic aquatic ecotoxicity tests although other species can be used (e.g., *Daphnia pulex*).

D. magna is a freshwater crustacean commonly found in lakes and ponds, playing an important role as a primary consumer of phytoplankton and being an important food source for predator fish (Bownik, 2017; Lampert and Sommer, 2007; Mergeay et al., 2006).

Its life cycle is characterized by sexual and asexual reproduction. In ideal conditions, like laboratory conditions, a female will produce genetically identical offspring from 7 to 10 days after its birth. After the first brood, the female will continue producing genetically identical females every 3 days. The life cycle of *D. magna* is illustrated in figure 4. This and other characteristics of these organisms, such as their relatively easy maintenance in the laboratory, small size, short life cycle, high fecundity, possessing transparent bodies, and sensitivity in relation to chemicals makes *Daphnia magna* a very suitable animal for scientific testing (Ebert, 2005; Tkaczyk et al., 2020). For all these reasons, *Daphnia magna* has been, for many years, used as "the" test species for studies in aquatic toxicology (Martins et al., 2007).

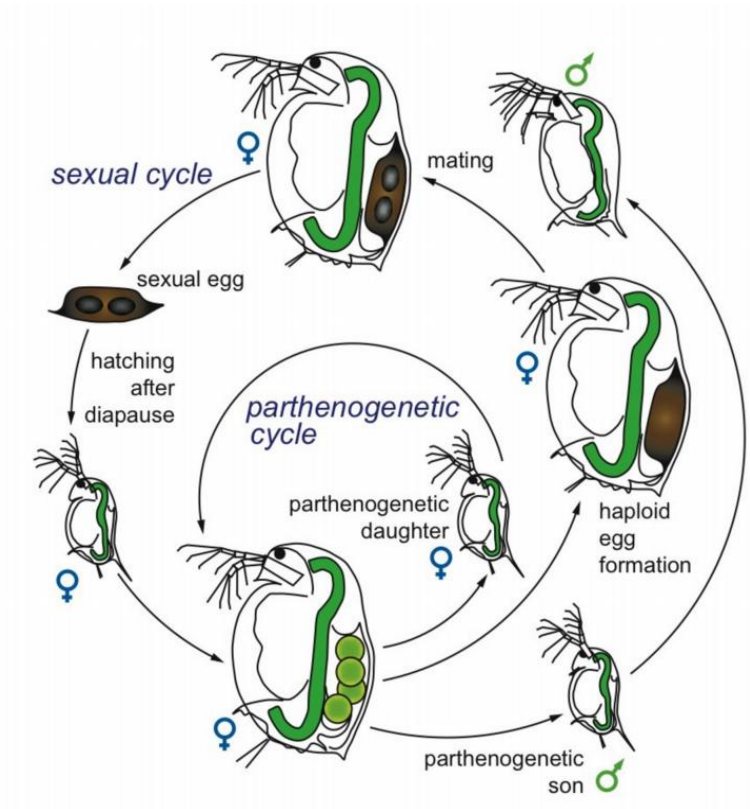

Figure 4: *Daphnia magna* sexual and asexual (parthenogenic) life cycle. Image adapted from Ecology, Epidemiology, and Evolution of Parasitism in *Daphnia* (Ebert, 2005).

Daphnia magna was the organism used to perform aquatic acute toxicity tests of the essential oils and plant extracts studied in this dissertation as it is a recommended organism by the OECD guideline (OECD, 2004) followed in this study.

1.6 Plants studied

1.6.1 *Cistus ladanifer*

Cistus ladanifer is an evergreen woody shrub part of the Cistaceae family (Papaefthimiou et al., 2014). It is also known as rockrose (or "esteva" in Portuguese) and it is very abundant in the wild areas of the western Mediterranean region (Portugal, Spain, south of France, north of Morocco) (Frazão et al., 2018). Specifically, in Portugal, it outspreads throughout the country, predominantly in the centre and south regions (Alves-Ferreira et al., 2019). It has high importance in the perfumery industry due to a particular extract, the "labdanum", that is used as a fixative (Zidane et al., 2013). The "labdanum" is a sticky exudate covering leaves and stems of the plant, rich in monoterpernes and other lipophilic compounds labdane- and clerodane-type diterpenes. It is also used in the cosmetic industry in the form of essential oil (Barrajón-Catalán et al., 2016; Rauwald et al., 2019). *C. ladanifer* has been used for centuries as a natural incense, and in traditional and folk medicine as anti-inflammatory, anti-ulcerogenic, anti-tumour and anti-spasmodic (Deforce, 2005; Demetzos et al., 2001; Dimas et al., 1998; Papaefthimiou et al., 2014).

Figure 5: *Cistus ladanifer* flowering aerial parts (left) and detail of a branch (right) from Vale de Prazeres, Beira Interior, Portugal.

Recently, *C. ladanifer* essential oil and extracts have been studied in terms of their bioactive properties, such as a preservative of stored products. Upadhyay et al (2018) have reported that an essential oil obtained from *C. ladanifer* was effective in reducing contamination of common fungi contaminants of the seeds of the brown mustard (*Brassica juncea*), peanuts (*Arachis hypogaea*), sesame (*Sesamum indicum*), sunflower (*Helianthus annuus*) and mustard (*Brassica campestris*), being especially effective against the most aflatoxigenic isolate from these seeds, *Aspergillus flavus* AF-M-KS, completely inhibiting fungal growth (MIC (minimum inhibitory concentration)= 0.6 $\mu L.L^{-1}$) and completely inhibited the production of aflatoxin B1 by this microorganism at 0.5 $\mu L.L^{-1}$. In the same study, it is also reported that α-asarene to be the most abundant compound of the essential oil, followed by camphene and α-pinene (Upadhyay et al., 2018). In another study, the antibacterial and antifungal potential of the *C. ladanifer* essential oil was also shown. It was effective against both Gram-positive bacteria (*Staphylococcus aureus* and *S. epidermis*) and Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) as well as the fungi *Candida albicans*, *Trichophyton rebrum* and *Aspergillus niger*. The fungicidal activity was the most noticeable with MICs from 10 $mg.L^{-1}$ for *T. rubrum* and 0.001 $mg.mL^{-1}$ for *A. niger* and *C. albicans*, emphasizing the antifungal properties of this essential oil, which possessed a different chemical composition than the essential oil studied by Upadhyay, S., et al, with verticiol and camphene as the major compounds present (Mohammed et al., 2018). These bioactive properties of the essential oil obtained from *C. ladanifer* could lead to the development of new treatments for *Candida albicans* infections, as a resistance of this organism to commonly used antifungals is well documented (Whaley et al., 2017). The chemical composition of essential oils obtained from plants in different regions usually differ in the most abundant compounds. A recent study analysed C. *ladanifer* essential oils obtained from the Beira Baixa region, Portugal, from plants collected at different times of the year. The authors reported that the major compound found was α-pinene (figure 6) in all studied essential oils, but the amount of the second most abundant component, camphene, varied according to the time when the plant was collected and the extraction process utilized (Tavares et al., 2020a).

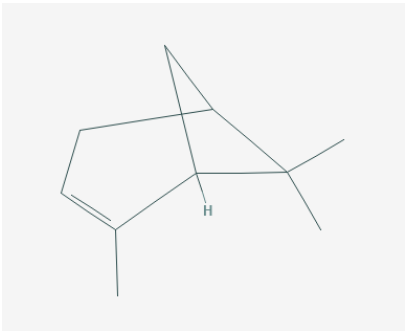

Figure 6: Chemical structure of α-pinene (PubChem).

1.6.2 *Cupressus lusitanica*

Commonly known as Cedar-of-Goa or Mexican cypress, *Cupressus lusitanica* (Cupressaceae) or "Cedro-do-Buçaco" in Portuguese, is a conifer tree native from central America and first described by Miller in 1768 from specimens identified in the region of Coimbra, Portugal (Farjon, 1993; Watt et al., 2009). It is very common throughout the Portuguese territory, in the wild and also as an ornamental plant, especially in gardens (Carmo and Frazao, 1989). It is also used as a source of timber for multiple purposes. Throughout the world, it has been used in traditional medicine, especially its leaves used to treat skin conditions, particularly conditions caused by dermatophytes, alleviate coughs and cold symptoms, and also as a protector of stored grains from insects (Kamatenesi-Mugisha et al., 2013; Kuiate et al., 2006).

Figure 7: *Cupressus lusitanica* branch (Jardim Botânico UTAD, Flora digital de Portugal).

This tree has been studied in the past years due to important bioactive compounds that can be extracted from its aerial parts, especially essential oils and other extracts that can contribute to the development of new industrial products. Tavares C. S. et al have shown that the essential oils, hydrolates and other extracts (ethanolic and acetone) can have important antioxidant and anti-inflammatory activity (Tavares et al., 2020a; Tavares et al., 2020b). The most abundant compounds that can be extracted from *C. lusitanica* have been reported to be α-pinene (figure 6), β-pinene, umbellulone, limonene and camphor (figure 8) (Bett et al., 2016; Hassanzadeh et al., 2010; Santos Filho et al., 2011; Tavares et al., 2020a) although some chemical variation can occur according to the region the plant was grown in.

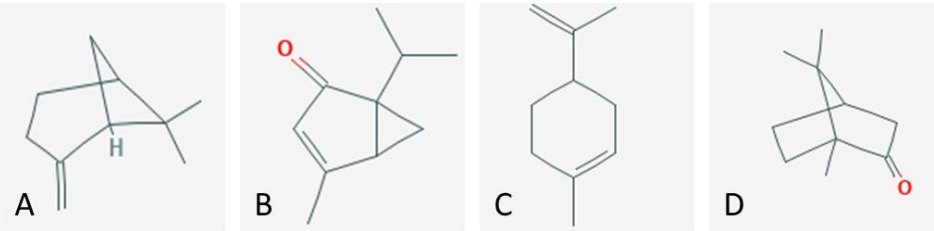

Figure 8: Chemical structure of β-pinene (A); umbellulone (B); limonene (C); camphor (D) (PubChem).

1.6.3 *Echinacea purpurea*

The purple coneflower *Echinacea purpurea* (Asteraceae) is an important medicinal plant native from North America but now grown in many parts of the world (Bałan et al., 2012). Extracts from *E. purpurea* are amongst the most used in traditional medicine, mainly as a treatment for respiratory and urinary diseases (Sharifi-Rad et al., 2018). Its immunomodulatory, anti-inflammatory, antioxidant, and antiviral properties are well documented in the literature (Barnes et al., 2005; Barrett, 2003; Kumar and Ramaiah, 2011) along with other studies that also show antifungal and antibacterial activity, especially against bacteria that often cause respiratory infections such as *Streptococcus pyogenes*, *Hemophilus influenzae* and *Legionella pneumophila* (Sharma et al., 2010).

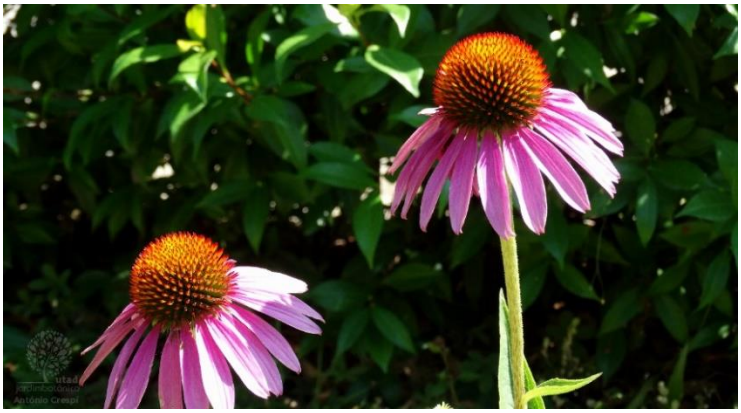

Figure 9: Detail of *Echinacea purpurea* flowers (Jardim Botânico UTAD, Flora digital de Portugal).

Different extracts can be obtained from this plant with different bioactive properties. Coelho, J. et al have recently reported that dichlorometane, ethyl acetate and acetone extracts show the highest antimicrobial activity especially against *Enterococcus faecalis* and *Listeria monocytogenes*, and cytotoxicity was observed with the dichloromethane and *n*-hexane extracts in the human tumour cell lines NCI-H460, MCF-7, HepG2 and HeLa (Coelho et al., 2020). Many bioactive compounds are reported to be obtained from *E. purpurea*, such as carbohydrates, fatty

acids, derivates of caffeic tartaric acids and phenolic compounds, mainly chicoric and caftaric acids (Bałan et al., 2012). Essential oils are reported to be rich in germacrene D and other compounds such as β-caryophyllene (figure 10) and α-pinene (figure 6) can also be found in smaller quantities (Hudaib et al., 2002; Thappa et al., 2004).

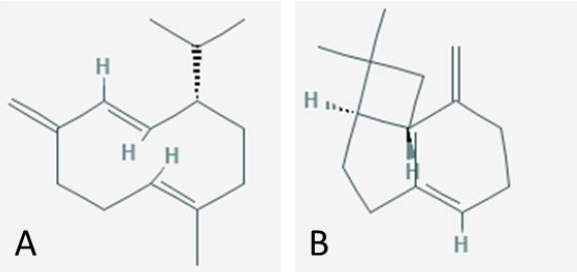

Figure 10: Chemical structure of germacrene D (A) and β-caryophyllene (B) (PubChem).

1.6.4 *Eucalyptus globulus*

The Tasmanian blue gum *Eucalyptus globulus* (Myrtaceae) or just eucalyptus, as it is more commonly referred to, is an evergreen tree native to south-eastern Australia, introduced in the Iberian Peninsula mainly for industrial purposes (source of timber and pulp). In Portugal, it can be found throughout the entire continental territory, and it is currently the most abundant species of tree (Alegria et al., 2020; Cerasoli et al., 2016). Recently, attention has been drawn to ways of valorisation of *E. globulus* residues left by the industry in the context of a circular economy. For example, it has been shown that bark leftovers, after industrial usage of the tree, can be used to obtain extracts rich in a wide variety of compounds such as triterpenoids, fatty acids, phenolic compounds, condensed tannins and flavonoids, with antibacterial and antifungal activities (Gominho et al., 2020; Neiva et al., 2020).

Figure 11: *Eucalyptus globulus* trees (left) and detail of the leaves and flowers (right) (Jardim Botânico UTAD, Flora digital de Portugal).

The essential oils obtained from *E. globulus* are used in many industries. In the food industry it is approved to be used as a flavouring agent in the European Union, and in the United States of America and China commercial insect repellents containing these oils are in the market (Batish et al., 2008; Harkat-Madouri et al., 2015). Moreover, it is a common active ingredient of oral products such as mouthwash and toothpaste (Dhakad et al., 2018). The wide range of bioactivities and applications are mainly attributed to the most abundant compounds found in the essential oils and extracts such as 1,8-cineole (commonly referred to as eucalyptol)(figure 12), α-pinene and limonene (figures 6 and 8C). Variability of the abundance of compounds, and chemical composition are common, although the main component of the essential oils is usually eucalyptol (Dhakad et al., 2018; Gominho et al., 2020; Jerbi et al., 2017).

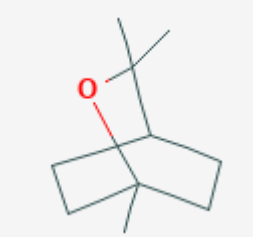

Figure 12: Chemical structure of eucalyptol (PubChem).

1.6.5 *Hamamelis virginiana*

Popularly called witch hazel, *Hamamelis virginiana* is a shrub native from North and South America known for its interesting reproductive biology. Unlike many other plants its flowering stage occurs in late autumn and fertilisation only occurs in the following spring (Anderson and Hill, 2002).

Extracts from this plant have been used in skin care, especially in the treatment of small wounds, skin irritations and its anti-inflammatory properties are known as well (Deters et al., 2001; Korting et al., 1993; Wolff and Kieser, 2007). The usage of extracts and distillates such as essential oils in cosmetic products (skin lotions, hair tonics, nutrition creams) is well documented (Deters et al., 2001; Korting et al., 1995). These bioproperties have been attributed to the complex mixture of compounds, mainly polyphenols, that can be extracted from the bark and leaves of this plant (Engel et al., 1998; Kreidel and Jhaveri, 2021). An extensive study reported about 175 compounds in the extract from the leaves and around 160 from the bark. The most abundant compounds present were heptacosane, pentacosane and tricosane (figure 14) in small relative percentages (<20%) (Engel et al., 1998) emphasising the incredibly complex composition of compounds that can be extracted from *H. virginiana*.

Figure 13: *Hamamelis virginiana* flowers and leaves.

Figure 14: Chemical structure of heptacosane (A); pentacosane (B) and tricosane (C) (PubChem).

1.6.6 *Helichrysum italicum*

Helichrysum italicum, commonly known as immortelle, sandy everlasting, curry plant or "perpétua das areias" in Portuguese, is a member of the Asteraceae family and it is the most studied species of the genus *Helichrysum*. This plant, widely distributed in the Mediterranean region (Kladar et al., 2015), has been used for decades in traditional medicine, mainly in respiratory, digestive and skin conditions and today is mostly exploited to produce essential oils and extracts used in perfumery, cosmetics and aromatherapy, and in recent years the demand for *H. italicum* essential oil has increased substantially (Andreani et al., 2019; Antunes Viegas et al., 2014). Several studies confirm some of these applications of compounds extracted from *H. italicum*, and others show that more interesting activities can be exploited (Djihane et al., 2017; Gismondi et al., 2020; Kladar et al., 2015). For example, it was showed that the *H. italicum* essential oil is effective against *Candida albicans* (Minimum fungicidal concentration - MFC)= 4.00 – 24.17 $mg.mL^{-1}$ and inhibition of growth after 24h exposure to 5% of *H. italicum* essential oil (Dzamic et al., 2019; Oliva et al., 2020). The essential oil also displays insecticidal properties against larvae of *Aedes albopictus* mosquitoes, which was able to cause 100% mortality of the

larvae at a concentration of 300 mg.L^{-1} after 24h of exposure (Conti et al., 2010). Another interesting property of the essential oil from *H. italicum* is related to collagen regeneration which has led to the development of anti-ageing creams (Millou et al., 2010).

Figure 15: *Helichrysum italicum* - detail of the aerial parts of the plant (Jardim Botânico UTAD, Flora digital de Portugal).

The most important compounds present in the extracts from *H. italicum* are terpenes, especially mono- and sesquiterpenes, such as neryl-acetate, β-eudesmene and α-pinene (figures 16 and 6) which are reported to be responsible for most of the biological activities of the isolates (Maksimovic et al., 2017; Oliva et al., 2020), although chemical composition may vary depending on the origin of the plant, properties of the soil where it grows and even the stage which the plant is collected, as stated in some studies (Bianchini et al., 2001; Leonardi et al., 2013).

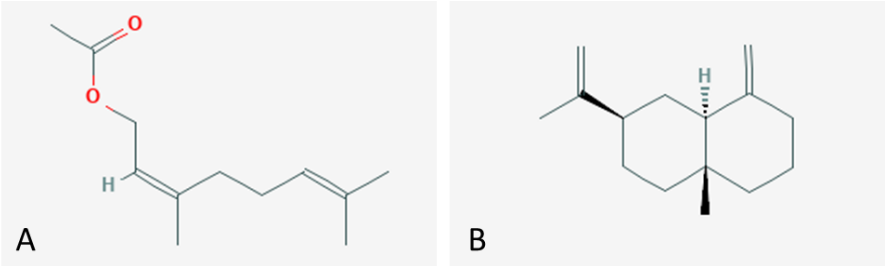

Figure 16: Chemical structure of neryl acetate (A) and β-eudesmene (B) (PubChem).

1.6.7 *Humulus lupulus*

The hop plant, *Humulus lupulus*, is widely known for being one of the main ingredients of beer, where only female cones are used for beer brewing due to the characteristic aroma and antimicrobial properties (Alonso-Esteban et al., 2019; Murakami et al., 2006). *H. lupulus* belongs to the Cannabaceae family and is widely distributed throughout Europe and in Portugal, wild hop is common in the north and centre parts of the country (Rocha, 2005).

Figure 17: Aerial parts of *H. lupulus* with its characteristic cone flowers (Jardim Botânico UTAD, Flora digital de Portugal).

Apart from its usage by the beer brewing industry, hops have been used for centuries in traditional and folk medicine to control anxiety, spasms, cough, fever and inflammation, just to name a few (Alonso-Esteban et al., 2019). Due to the richness of compounds that can be extracted from this plant, it has become an important plant in the pharmaceutical industry. The sedative effects of extracts from *H. lupulus* are well documented (Franco et al., 2012; Schiller et al., 2006; Smyly, 2020) and in recent years, many biological activities, such as antioxidant, antimicrobial, anti-metastatic, apoptosis-inducing, among others have been described. For example, it was shown that methanol extracts from the seeds of *H. lupulus* have DPPH free radical scavenging activity and reducing power and also showed good antimicrobial activity against Gram-positive and Gram-negative bacteria. The authors attribute these properties to the presence of the phenolic compounds (+)-catechin and (-)-epicatechin present in the extract (Alonso-Esteban et al., 2019; Hrnčič et al., 2019)

The main components reported to be found in hop extracts are monoterpenes like α-pinene and myrcene (figures 6 and 18B), and sesquiterpenes such as α-humulene (figure 18A) and β-caryophyllene (figure 10B), although many other compounds are present (Hrnčič et al., 2019) and can vary according to where the plant is grown. For example, an essential oil from the cones of *H. lupulus* cultivated in Brazil showed *trans*-β-farnesene, β-selinene, myrcene, α-selinene, β-caryophyllene and 2-undecanone as major components, while another from *H. lupulus* grown in

North America was richer in α-humulene, myrcene, β-caryophyllene and *trans*-β-farnesene (da Rosa Almeida et al., 2020).

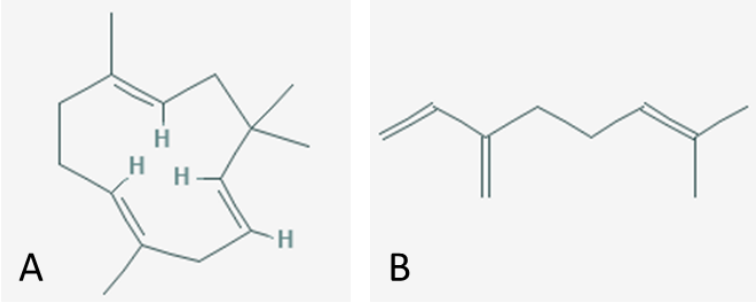

Figure 18: Chemical structure of α-humulene (A) and myrcene (B) (PubChem).

1.6.8 *Matricaria chamomilla*

Matricaria chamomilla or just chamomile as it is commonly called is an important medicinal plant part of the Asteraceae family. In Europe it is used in a wide variety of products, being used in natural medicine, especially as herbal tea, used to relieve inflammations of the gastrointestinal tract as well as a gentle sedative. It is widely used in the industry, mostly in the form of infusions and essential oil to produce soaps, perfumes, detergents, lotions, hair products, baked goods, beverages, and herbal teas (McKay and Blumberg, 2006; Singh et al., 2011).

Figure 19: *Matricaria chamomilla* aerial parts (left) and flowers (right) (Jardim Botânico UTAD, Flora digital de Portugal).

The pharmacological activities of *M. chamomilla* are associated with its essential oil and the content of flavonols. According to the European Pharmacopoeia, the flowers used for medicinal purposes "*Matricariae flos*" should contain at least 4 ml.Kg^{-1} of essential oil and a minimum of 0.25% of apigenin 7-glucoside (Commission et al., 2010). The most abundant compounds reported to be found in essential oils from *M. chamomilla* are usually α-bisabolol and its oxides (α-bisabolol oxide A and B), farnesene and β-farnesene (figure 20) (Singh et al., 2011). Methanolic extracts have been shown to contain important bioactive phenolic compounds such as quercetin, ferulic acid, caffeic acid or apigenin (Roby et al., 2013). A hydrolate obtained from

M. chamomilla was also reported to contain the important bioactive compounds thymol, α-bisabolol oxide A (figure 21) and carvacrol (figure 25) (Hamedi et al., 2017).

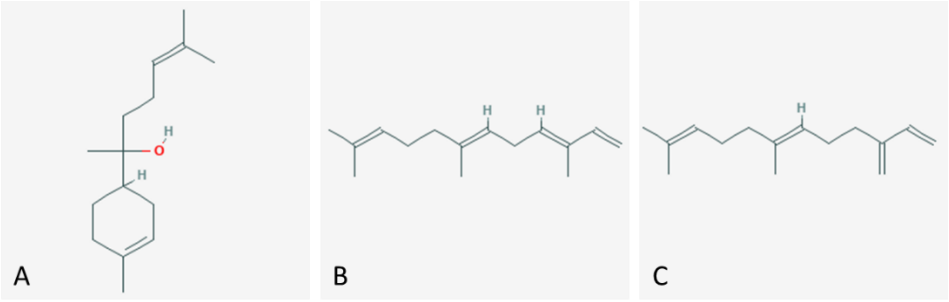

Figure 20: Chemical structure of α-bisabolol (A); farnesene (B) and β-farnesene (C) (PubChem).

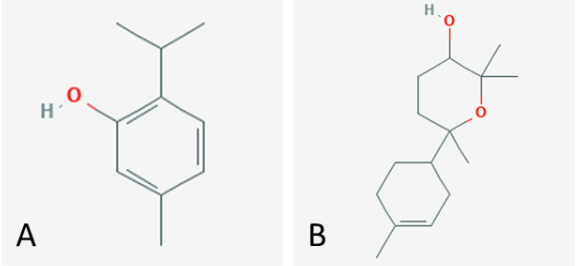

Figure 21: Chemical structure of thymol (A) and α-bisabolol oxide A (B) (PubChem).

1.6.9 *Ocimum basilicum*

Ocimum basilicum is a member of the Lamiaceae family and an important aromatic plant used in culinary. This plant is indigenous to India, but it is also present in the Mediterranean region and it is grown for industrial purposes, including food supplements. It has been used in traditional and folk medicine as a tonic, vermifuge and even as a treatment for snake bites (Ch et al., 2015; Mostafavi et al., 2019). The essential oils and extracts that can be obtained from this plant are known to have important bioactive properties such as anti-inflammatory, antibacterial, antioxidant, wound-healing, antidiabetic, antihypertensive and cardioprotective as well as sedative, and anxiolytic effects (Sestili et al., 2018).

Figure 22: *Ocimum basilicum* flowering aerial parts (Jardim Botânico UTAD, Flora digital de Portugal).

The major compounds that can be extracted from *O. basilicum* are linalool, geraniol, eugenol and 1,8-cineole (figures 23B,C and 12) (Ćavar Zeljković et al., 2020) even though phytochemical variability is can be found in extracts and essential oils obtained from this plant. Dev, N. et al have reported an essential oil from the leaves of *O. basilicum* grown in Bangladesh to be rich in eugenol (61.76% of the total composition). Other compounds such as isopropyl palpitate, 2,3-dihydroxy propyl elaidate and α-cubene were also present in smaller amounts (11,36%, 5.10%and 3.85%, respectively). The same authors also characterized different extracts obtained from the leaves by applying n-hexane, ethyl acetate and chloroform. They have shown that the ethyl acetate extracts are rich in 1, 2-dimethoxy-4-(2-propynel)-benzene (53.06%) and the chloroformic extract contained mostly eugenol (88.18%). The hexane extract contained more 2-pentanone (27.06%) and caryophyllene oxide (4.64%) (Dev et al., 2011). More recently, Rezzoug, M. et al have reported that an essential oil from the leaves of *O. basilicum* grown in Algeria to be rich in linalool (52.1%) (figure 23A) and linalyl acetate (19.1%) (figure 23D) (Rezzoug et al., 2019).

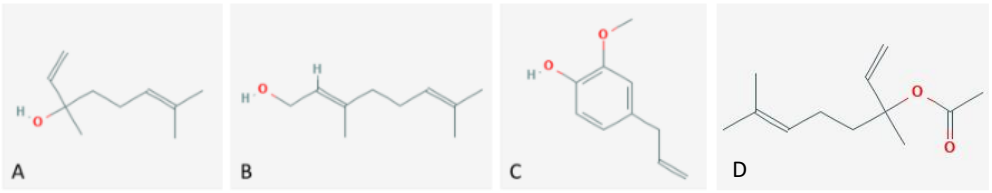

Figure 23: Chemical structure of linalool (A); geraniol (B), eugenol (C) and linalyl acetate (D) (PubChem).

1.6.10 *Thymbra capitata*

Thymbra capitata, is an endemic species in the southwest Iberian Peninsula (Rodrigues et al., 2006). Part of the Lamiaceae family, it is commonly called thyme or more specifically conehead thyme, with other similar species from the *Thymus* genus such as *Thymus vulgaris*. It is an important aromatic plant used in culinary, as well as ornamental or in traditional medicine (Figueiredo et al., 2008a).

Figure 24: *Thymbra capitata* flowering aerial parts (Jardim Botânico UTAD, Flora digital de Portugal).

The essential oils that can be obtained from this aromatic plant are usually very similar in their chemical composition, despite the area in which the plant grows. Several studies that assessed the chemical composition of the oils *from T. capitata* have reported carvacrol (figure 25) as the most abundant component (Charfi et al., 2019; Moukhles et al., 2020; Salas et al., 2010) although other components such as α-pinene and γ-terpinene have been found (Aazza et al., 2016). Many studies have reported important bioproperties of the essential oils obtained from *T. capitata* such as antifungal, being effective in reducing the biomass of *C. albicans* and other species of *Candida* biofilms as well as metabolic activity at the concentration of 0.64 $\mu L.L^{-1}$ of essential oil; antibacterial activity against *Listeria innocua*, *Bacillus cereus* and *Escherichia coli*; antioxidant and cytotoxic activity towards HeLa (adherent) and U937 (free-floating) cells at the highest concentration tested (0.1% v/v) (Charfi et al., 2019; Delgado-Adámez et al., 2017; Moukhles et al., 2020; Palmeira-de-Oliveira et al., 2012), showing a wide range of possible applications of this essential oil.

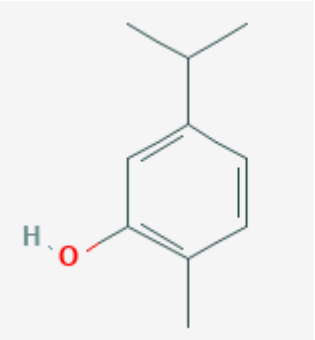

Figure 25: Chemical structure of carvacrol (PubChem).

1.6.11 *Thymus citriodorus*

The lemon thyme, *Thymus citriodorus*, is another member of the Lamiaceae family. It is widely distributed throughout the Mediterranean region and is grown primarily for culinary purposes due to the characteristic lemon scent of its leaves. Essential oils and other extracts are used in perfumes and cosmetics, food products as well as pharmaceuticals (Jurevičiūtė et al., 2019; Rita et al., 2018). Important bioactive activities have been attributed to this plant such as anti-bacterial, antioxidant and cytotoxic properties (Bayala et al., 2014; Sacchetti et al., 2005).

Figure 26: Characteristic leaves of *Thymus citriodorus*.

Essential oils obtained from *T. citriodorus* are usually characterized by high contents of geraniol (figure 23B). Other compounds are also present in significant amounts such as Z-citral which gives this plant its characteristic lemon scent, nerol and geranial (Duman and Özcan, 2017; Jurevičiūtė et al., 2019; Lisi et al., 2011; Stahl-Biskup and Holthuijzen, 1995). A hydrolate has also been reported to be rich in geraniol (Ntalli et al., 2020). Important biochemical and pharmacological properties have been attributed to geraniol: antioxidant, antibacterial, anti-fungal, anti-inflammatory and can also act as an insect repellent (Hadian et al., 2020). Solvent extraction is reported to be a good method to extract important phenolic compounds such as rosmarinic acid. For example, one study reported a hydroethanolic extract to contain 86.23% of rosmarinic acid and an aqueous decoction also showed a high content of this phenolic compound (51.80%) (Pereira et al., 2013; Taghouti et al., 2020). All these characteristics make *T. citriodorus* a good natural source of different chemicals that can be exploited in the development of new products.

1.6.12 *Syzygium aromaticum*

Clove (*Syzygium aromaticum*) is an important aromatic plant that has been exploited for centuries. It is one of the most valuable spices used in food and its uses in traditional medicine is well documented as well. This plant, part of the Myrtaceae family, is indigenous to Indonesia and it is cultivated in many parts of the world for commercial exploitation. The commercially exploited part of the plant is its flower buds that are rich in phytochemicals, and these are also used to produce essential oils. Other parts such as leaves and stems are also reported to be used for the production of this product. Up to 18% of essential oil can be present in the clove buds, and these are generally very rich in eugenol (figure 23C), eugenol acetate (figure 28) and β-caryophyllene (figure 10B)(Batiha et al., 2020; Bhowmik et al., 2012; Cortés-Rojas et al., 2014).

Figure 27: Aerial parts of the *S. aromaticum* tree (left) and detail of the commercially exploited flower buds (right) (Jardim Botânico UTAD, Flora digital de Portugal).

The chemical composition of essential oils from *S. aromaticum* has been intensively studied. Srivastava, A. K. and collaborators studied essential oils obtained from buds of *S. aromaticum* grown in India and Madagascar. Both essential oils were rich in eugenol. The oil obtained from plants grown in India had 70% of eugenol and also 19.5% of β-caryophyllene, while the oil obtained from plants grown in Madagascar contained 82.6% of eugenol, 7.2% of β-caryophyllene and 6% of eugenyl acetate (Srivastava et al., 2005). In an extensive study, 45 essential oils obtained from the buds, 32 essential oils obtained from the leaves and 44 essential oils obtained from the stems of *S. aromaticum* were compared for their chemical composition. In all the essential oils, eugenol was the most abundant compound ranging in concentrations from 72.08% to 96.65% of the total composition. The second most abundant compounds varied between eugenyl acetate and β-caryophyllene (Razafimamonjison et al., 2014).

Many biological activities of essential oils and extracts obtained from *S. aromaticum* have been reported: antioxidant, anti-inflammatory, antimicrobial, antiprotozoal, antidiabetic, antinociceptive and antidepressant (Batiha et al., 2020). Different extracts have also shown important bioactivities, for example, a dichloromethane extract from clove buds showed

antibacterial activity against Gram-positive (MRSA) and Gram-negative (*Escherichia coli* and *Salmonella typhi*) bacteria, while an ethanolic extract showed cytotoxic activity against human colon carcinoma cells (HCT) (Taha Yassin et al., 2020).

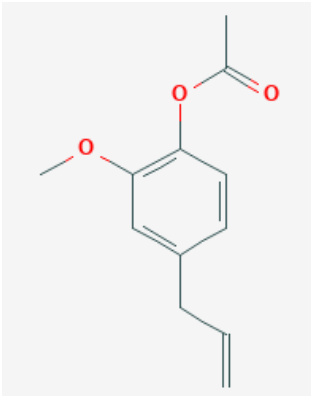

Figure 28: Chemical structure of eugenol acetate (PubChem).

The plants used in this study were selected due to their potential to be exploited industrially as a source of compounds that can be used in the development of new products. All of them are used industrially as a source of phytochemicals of great industrial interest due to their important properties and are being studied in the framework of the InovEP Project.

Chapter 2 - Objective

In the framework of the InovEP project, “Inovação com extratos de plantas”, essential oils and extracts obtained from different plants are studied for their bioactive properties and new potential applications. Following the precautionary principle, particularly what is consigned in the REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) regulation of the European Chemicals Agency (ECHA), it becomes necessary to evaluate the potential effects that the introduction of these products can have in the ecosystem.

This dissertation aims to study the potential (eco)toxic effects that essential oils, hydrolates and extracts obtained from twelve different plants: *Cistus ladanifer*, *Cupressus lusitanica*, *Echinacea purpurea*, *Eucalyptus globulus*, *Hamamelis virginiana*, *Helichrysum italicum*, *Humulus lupulus*, *Matricaria chamomilla*, *Ocimum basilicum*, *Thymbra capitata*, *Thymus citriodorus* and *Syzygium aromaticum* can have in aquatic ecosystems. To achieve this objective, the acute toxicity was evaluated using the model organism *Daphnia magna*.

Chapter 3 - Materials and methods

3.1 Essential oils and plant extracts

Twelve plants were studied in a total of twenty-two essential oils, hydrolates and extracts. The test substances were obtained from different sources and suppliers. Table 8 summarises the plants studied, the part(s) of the plant that were used, the source and the different products obtained and that were used in the acute toxicity tests.

3.2 *Daphnia magna* culture

The maintenance of a *Daphnia magna* culture in a laboratory is essential to have the organisms stipulated by the OECD 202 guideline to perform the tests (neonates with less than 24h after being spawn). Daphnids for the stock culture were maintained, in accordance with the guideline, in 800 mL flasks in ASTM hard water medium (stock culture - 15 organisms per flask), at a controlled temperature of 20° C ± 1, pH of 7.80 ± 0.1, and photoperiod of 16/8 light/dark cycle. The daphnids were fed every day with a cell suspension of the microalgae *Raphidocelis subcapitata* ($3{,}05\times10^5$ cells/mL/*daphnia*) also maintained in our laboratory, and the medium was changed every other day. The number of neonates was also assessed to evaluate the fitness of the cultures (the criteria being >30 neonates per daphnid from N2 onwards). Before the beginning of the test, organisms were maintained isolated in 100 mL flasks under the same conditions.

3.3 Fitness condition test

In order to evaluate the health and fitness conditions of the test organisms, an acute toxicity test with a reference toxic is recommended. This test should be done preferably every month or at least twice a year as recommended by OECD (OECD, 2004). The reference substance to be used is potassium dichromate ($K_2Cr_2O_7$, AnalaR NORMAPUR®, purity ≥ 99,8%).

The test was performed in six multiwell plates with five *D. magna* neonates (<24h) per well, and 5 replicates per concentration. After 24h, immobilisation was recorded and the EC_{50} value was calculated. For the test to be considered valid, and therefore for the organisms to be considered fit, the 24h EC_{50} value should be in the range of 0.6 – 2.1 $mg.L^{-1}$ (ISO, 2012).

Table 8: Essential oils and plant extracts studied. IPCB – *Instituto Politécnico de Castelo Branco*.

Family	Plant	Part used	Type	Source
Cistaceae	*Cistus ladanifer*	Aerial parts	Essential oil	Ervitas Catitas
			Hydrolate	IPCB
Cupressaceae	*Cupressus lusitanica*		Hydrolate	IPCB
Asteraceae	*Echinacea purpurea*		Hydrolate	IPCB
Myrtaceae	*Eucalyptus globulus*		Essential oil	Ervitas Catitas
Hamamelidaceae	*Hamamelis virginiana*		Hydrolate	IPCB
Asteraceae	*Helichrysum italicum*	Aerial parts	Essential oil	Planalto Dourado
			Hydrolate	IPCB
Cannabaceae	*Humulus lupulus*	Flowers + aerial parts (mix)	Hydrolate	IPCB
		Flowers	Hydrolate	IPCB
		Flowers + aerial parts (mix)	Chloroformic	IPCB
		Flowers	Chloroformic	IPCB
		Flowers + aerial parts (mix)	Ethanolic	IPCB
		Aerial parts	Hydro-ethanolic	IPCB
		Flowers + aerial parts (mix)	Methanolic	IPCB
		Flowers	Methanolic	IPCB
Asteraceae	*Matricaria chamomilla*		Hydrolate	IPCB
Lamiaceae	*Ocimum basilicum*	Leaves	Hydrolate	IPCB
Lamiaceae	*Thymbra capitata*	Aerial parts	Essential oil	IPCB
			Hydrolate	IPCB
Lamiaceae	*Thymus citriodorus*		Hydrolate	IPCB

Myrtaceae	*Syzygium aromaticum*	Essential oil	BonEscient aromatherapie

3.4 Test solutions

Different approaches were used to select the concentrations to be tested. For *H. italicum* and *C. ladanifer*, the chemical analysis of the essential oils showed high heterogeneity in the compounds present, and so, a range-finding test was performed before the final acute toxicity test to obtain an adequate concentrations' interval.

The range-finding tests were performed with *C. ladanifer* and *H. italicum* essential oils during 48h. Neonates (<24h) were exposed to widely spaced concentrations (1, 10, 100, 500 and 1000 $mg.L^{-1}$) and immobilisation was recorded. This procedure was repeated until a good range of concentrations was found to perform the actual acute toxicity tests. For the *H. italicum* essential oil, as no immobilisation was observed in the preliminary tests, an arbitrary maximum concentration of 800 $mg.L^{-1}$ was used due to the limited amount of test substance. For all the other extracts the acute toxicity tests were performed up to the highest possible concentration that the amount of test substance permitted. Maximum concentrations tested are presented in table 9.

The test concentrations for the *E. globulus*, *T. capitata* and *S. aromaticum* essential oils were set according to the *D. magna* toxicity data available for the major compound present in each essential oil. For all other extracts, the concentrations were set according to the available amount of test substance.

Due to low solubility in the dilution medium (ASTM hard water), DMSO 1% was used as a solvent for the *C. ladanifer*, *E. globulus*, *H. italicum*, *T. capitata* and *S. aromaticum* essential oils and for both *H. lupulus* chloroformic extracts. For *H. lupulus* methanolic extracts, methanol at a concentration of 1% was used, and for the ethanolic extracts, ethanol was used at the same concentration. For all other extracts, no solvent was used since they were water-based.

To obtain the test solutions, the extracts and essential oils were weighted in 10 mL volumetric flasks. The remaining volume was completed with a solution of ASTM hard water or the corresponding solvent at a concentration of 1% (v/v) in ASTM hard water or just ASTM hard water when no solvent was necessary. This first solution was continuously diluted in ASTM hard water until reaching the desired working concentrations, and when a solvent was used, the dilutions were performed in order to ensure that the maximum final concentration of solvent was never higher than 0.1%, to minimize the effect of the solvent on the results, as recommended by the OECD guideline.

3.5 Acute toxicity tests

The acute toxicity tests were performed according to the OECD guideline 202 (OECD, 2004).

Daphnia magna parthenogenic neonates (less than 24h old) from the 2nd to the 6th brood were selected to perform the acute toxicity tests. The tests were performed in multiwell plates (6 wells). 10 mL of each test concentration was pipetted into the corresponding well, with 2 mL of test substance per organism as recommended. Five concentrations (table 9) were used for each essential oil/ extract/ hydrolate, plus a control of ASTM hard water and when solvents were used a second solvent control was also included. For all the essential oils and the chloroformic extracts, DMSO at 0.1% was used as control and for the ethanolic and methanolic extracts, ethanol, and methanol at 0.1% were used, respectively.

After preparing all the test concentrations in the multiwell plates, the *D. magna* neonates were transferred to the test wells. Thirty-five neonates were used for each test divided into 7 wells, making 5 neonates per well, when a solvent control was included. For the water-based extracts, no solvent was used. After transferring the neonates to the test plates, these were placed inside a controlled temperature chamber (20°C ±1) as previously described (Fernandes et al., 2020). In all tests, 5 replicates were performed.

After 24h and 48h, the immobilisation of the daphnids was registered and the EC_{50} (concentration estimated to immobilise 50 per cent of the organisms in a given time) value was calculated. Organisms were considered immobilised if they were not able to swim for more than 15 seconds after gentle agitation of the test vessels (OECD, 2004). Figure 29 exemplifies the procedure of the acute toxicity tests performed.

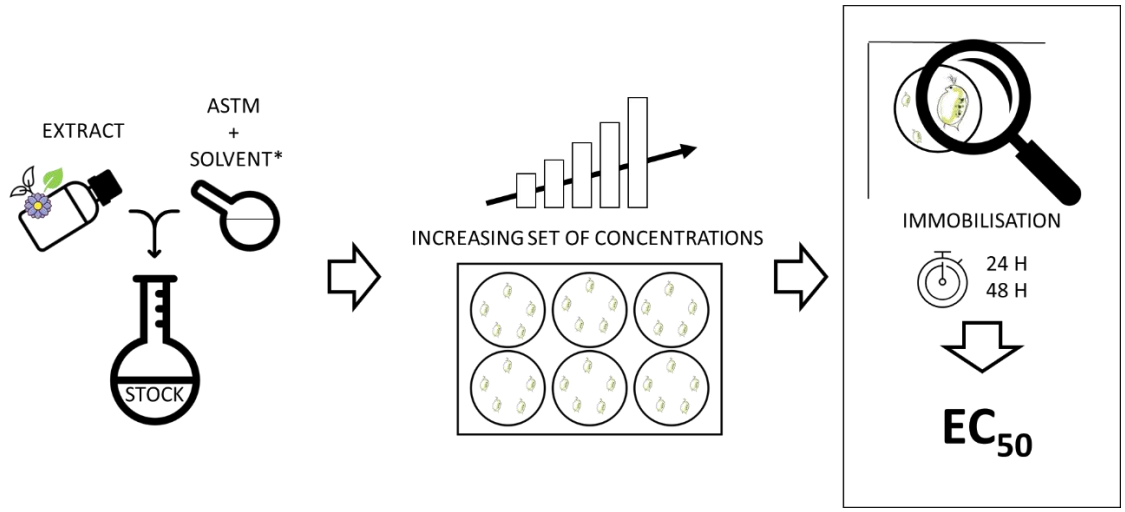

Figure 29: Schematic representation of the procedure performed in the acute toxicity tests. *Solvents were used when necessary and if so, a solvent control was included in the test.

Table 9: Concentrations used in the acute toxicity tests of the essential oils and extracts studied.

Plant	Type	Concentrations tested (mg.L^{-1})
C. ladanifer	Essential oil	50; 100; 150; 200; 400
	Hydrolate	125; 250; 500; 1000; 2000
C. lusitanica	Hydrolate	125; 250; 500; 1000; 2000
E. purpurea	Hydrolate	125; 250; 500; 1000; 2000
E. globulus	Essential oil	50; 100; 200; 400; 800
H. virginiana	Hydrolate	125; 250; 500; 1000; 2000
H. italicum	Essential oil	50; 100; 200; 400; 800
	Hydrolate	125; 250; 500; 1000; 2000
H. lupulus	Hydrolate (mix)	100; 200; 300; 400; 500
	Hydrolate (flowers)	100; 200; 300; 400; 500
	Chloroformic (mix)	125; 250; 500; 1000; 2000
	Chloroformic (flowers)	125; 250; 500; 1000; 2000
	Ethanolic (mix)	50; 100; 200; 400; 700
	Hydro-ethanolic (mix)	50; 100; 200; 400; 800
	Methanolic (mix)	50; 100; 200; 400; 800
	Methanolic (flowers)	50; 100; 200; 300; 400
M. chamomilla	Hydrolate	125; 250; 500; 1000; 2000
O. basilicum	Hydrolate	500; 1000; 2000; 4000; 8000
T. capitata	Essential oil	5; 7.5; 10; 25; 50
	Hydrolate	50; 100; 150; 200; 400
T. citriodorus	Hydrolate	125; 250; 500; 1000; 2000
S. aromaticum	Essential oil	0.8; 1.6; 3.2; 10; 25

3.6 Statistical analysis

Statistical analysis was performed using GraphPad Prism 8 software (GraphPad Software, California). For each concentration tested, the recorded number of immobilised organisms observed in the acute toxicity tests was introduced. The data was then normalized to percentage of immobilisation and analysed using a non-liner regression (curve fit). The equation used to obtain the EC_{50} values was [Inhibitor] vs normalised response -- variable slope (equation 1). The EC_{50} values as well as the 95% confidence interval was obtained, and dose-response curves were established.

$$Y = \frac{100}{(1 + \left(\frac{IC50}{x}\right)^{HillSlope})}$$

Equation 1: [Inhibitor] vs normalised response – variable slope.

Chapter 4 - Results

4.1 Fitness condition test

The fitness condition test was performed several times during this work whenever possible. The OECD guideline followed recommends performing a fitness condition test at least once a year to evaluate the response of the test organisms. The 24h EC_{50} values obtained were 1.71 $mg.L^{-1}$, 1.66 $mg.L^{-1}$, 2.01 $mg.L^{-1}$ and 1.68 $mg.L^{-1}$. The 24h EC_{50} values were in the range of 0.6 – 2.1 $mg.L^{-1}$ as proposed by ISO (Standardization, 2018) and the OECD guideline 202 being the test considered valid and therefore allowing the conclusion that the organisms are in good condition.

4.2 Acute toxicity

The toxicity observed was dose and time-dependant for the *C. ladanifer*, *E. globulus*, *T. capitata* and *S. aromaticum* essential oil as well as for the chloroform extract from the flowers of *H. lupulus*. Overall, the essential oils caused the highest immobilisation of the *D. magna* neonates, except for the *H. italicum* essential oil which caused no observable immobilisation up to the highest concentration tested (800 $mg.L^{-1}$). The different extracts from *H. lupulus* caused no immobilisation up to the highest concentrations tested, except the chloroformic extract obtained from the flowers (FC) of this plant that caused immobilisation of the daphnids although at very high concentrations. All the hydrolates tested caused no observable immobilisation up to the highest concentrations tested. General results are presented in table 10 and detailed results of the immobilisation observed are presented in the following sections. For *H. italicum* essential oil and hydrolate, *H. lupulus* hydrolates and extracts (mix chloroform - MC, mix ethanol - ME, mix hydroethanol - MHE, mix methanol - MM and flower methanol - FM) and all the other hydrolates (*C. ladanifer*, *C. lusitanica*, *E. purpurea*, *M. chamomilla*, *O. basilicum* and *T. citriodorus*) no further results will be provided as no immobilisation was observed.

Table 10: 24 and 48h EC_{50} results ($mg.L^{-1}$) and 95% confidence intervals (CI) of the studied essential oils and the extract that caused observable effects to *D. magna*. N. I. – No immobilisation observed.

		24h EC_{50}	95% CI	48h EC_{50}	95% CI
Essential oils	*C. ladanifer*	201.10	*	199.70	*
	E. globulus	222.80	(*) – 240.50	82.90	76.20 – (*)
	H. italicum	N. I.		N. I.	
	T. capitata	12.05	11.03 – 13.31	10.81	9.55 – 12.60
	S. aromaticum	11.63	8.50 – 16.40	2.42	2.25 – 2.62
Extracts	*H. lupulus* (FC)	1086	862.50 – 1366	905.7	695.80 - 1190
	H. lupulus (MC)	N. I.		N. I.	
	H. lupulus (ME)	N. I.		N. I.	
	H. lupulus (MHE)	N. I.		N. I.	
	H. lupulus (MM)	N. I.		N. I.	
	H. lupulus (FM)	N. I.		N. I.	
Hydrolates	*C. ladanifer*	N. I.		N. I.	
	C. lusitanica	N. I.		N. I.	
	E. purpurea	N. I.		N. I.	
	H. italicum	N. I.		N. I.	
	H. lupulus (mix)	N. I.		N. I.	
	H. lupulus (flowers)	N. I.		N. I.	
	M. chamomilla	N. I.		N. I.	
	O. basilicum	N. I.		N. I.	
	T. citriodorus	N. I.		N. I.	

*Not possible to calculate.

4.2.1 - *Cistus ladanifer* essential oil

The essential oil obtained from the aerial parts of *C. ladanifer* was tested up to 400 mg.L^{-1}. Immobilisation of *Daphnia* neonates was observed after 24 hours of exposure and after 48 hours of exposure. The EC_{50} values obtained were 201.1 mg.L^{-1} after 24h and 199.7 mg.L^{-1} after 48h. The percentage of immobilised neonates is presented in figure 30 and a dose-response curve in figure 31.

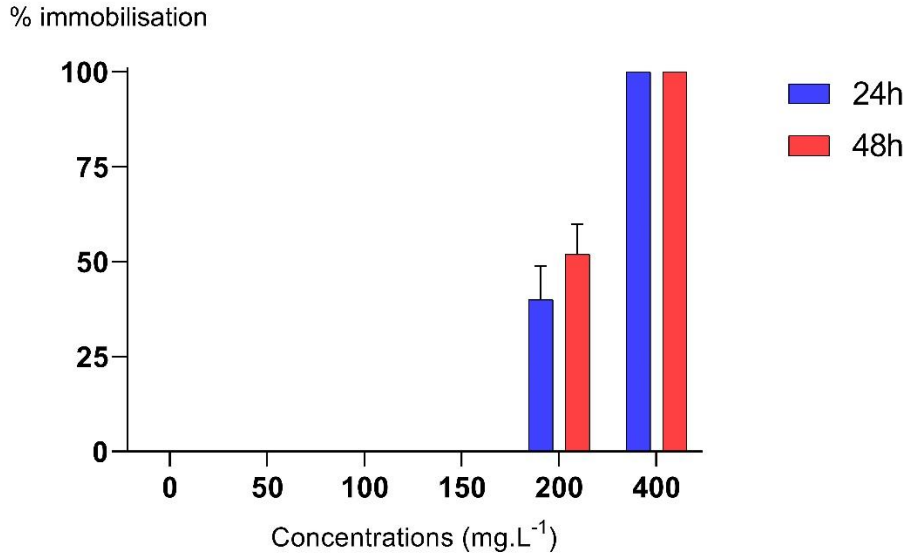

Figure 30: Percentage of immobilized *Daphnia vs* concentration of *C. ladanifer* essential oil.

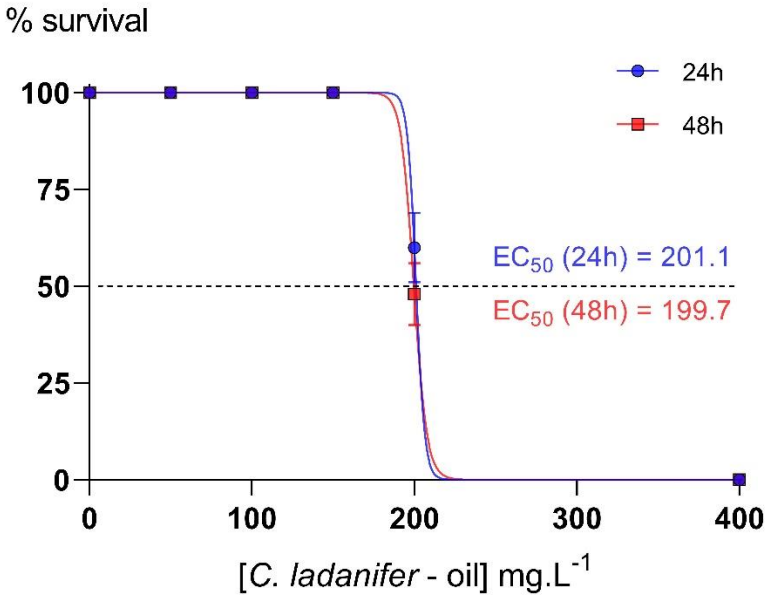

Figure 31: Dose-response curve of the effect of *C. ladanifer* essential oil in the immobilisation of *D. magna*.

4.2.2 – *Eucalyptus globulus* essential oil

The essential oil obtained from the leaves of *E. globulus* was tested for the potential toxicity towards *D. magna* neonates up to 800 mg.L^{-1}. The toxicity observed was dose and time-dependant resulting in a 48h EC_{50} value of 222.8 mg.L^{-1} and 48h EC_{50} value of 82.90 mg.L^{-1}. The percentage of immobilised neonates is presented in figure 32 and the obtained dose-response curve is presented in figure 33.

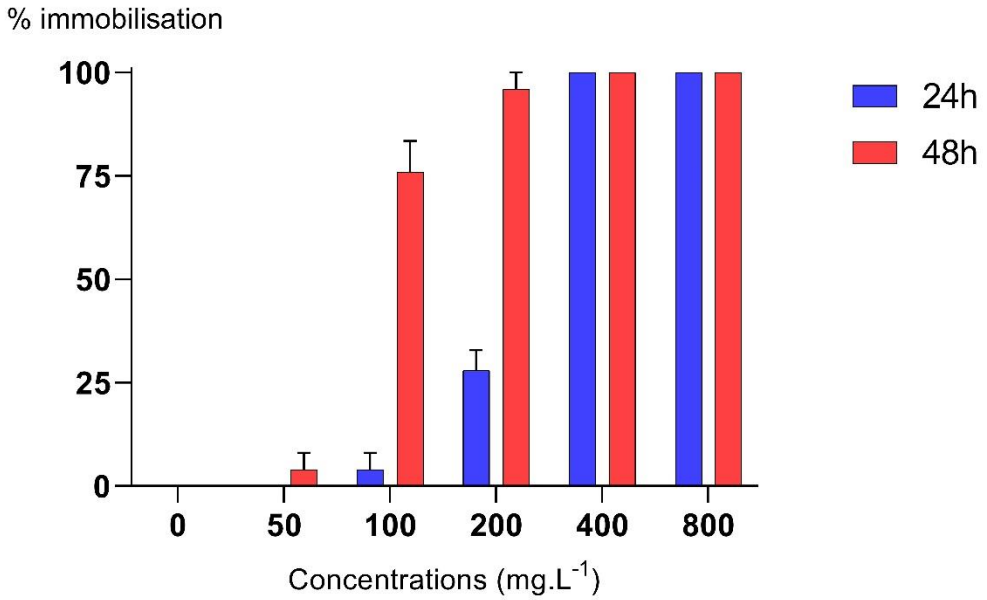

Figure 32: Percentage of immobilized *Daphnia vs* concentration of *E. globulus* essential oil.

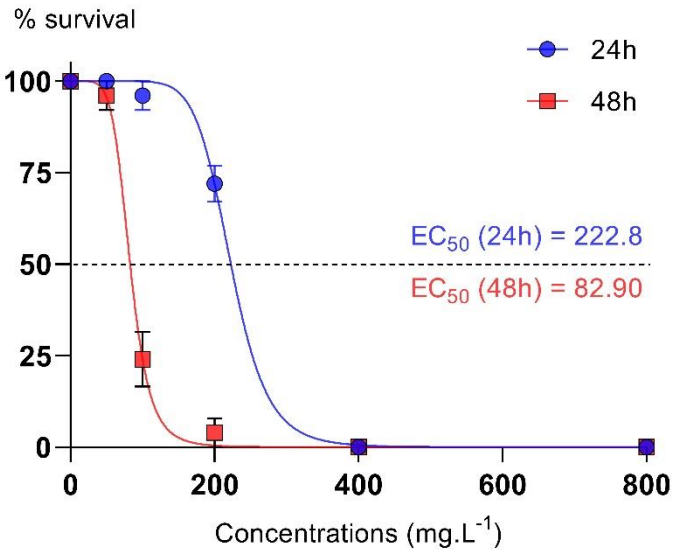

Figure 33: Dose-response curve of the effect of *E. globulus* essential oil in the immobilisation of *D. magna*.

4.2.3 - *Thymbra capitata* essential oil

The essential oil from the aerial parts of *T. capitata* caused immobilization on the *Daphnia* with an EC_{50} value of 12.5 $mg.L^{-1}$ after 24h of exposure, and EC_{50} value of 10.81 $mg.L^{-1}$ after 48h of exposure. The percentage of immobilisation is presented in figure 34. A dose-response curve is presented in figure 35.

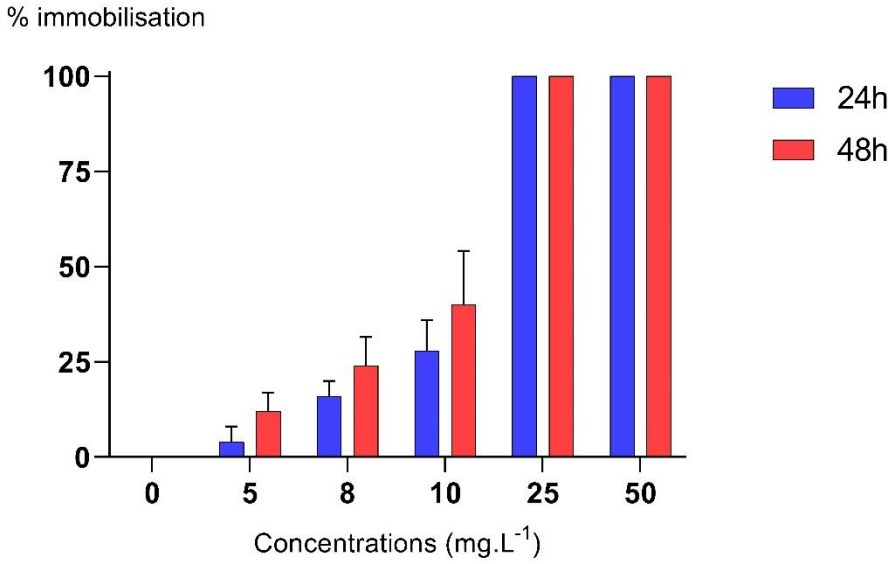

Figure 34: Percentage of immobilized *Daphnia vs* concentration of *T. capitata* essential oil.

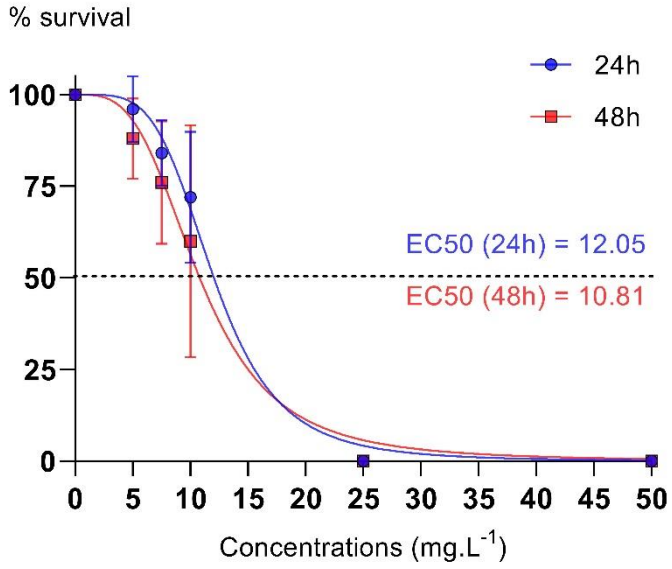

Figure 35: Dose-response curve of the effect of *T. capitata* essential oil in the immobilisation of *D. magna*.

4.2.4 – *Syzygium aromaticum* essential oil

The essential oil obtained from *S. aromaticum* caused immobilisation of *D. magna* neonates at low concentrations. The toxicity observed was dose and time-dependant, resulting in a 24h EC_{50} value of 11.63 $mg.L^{-1}$ and 48h EC_{50} of 2.418 $mg.L^{-1}$. Percentage of immobilisation observed is presented in figure 36 and a dose-response curve in figure 37.

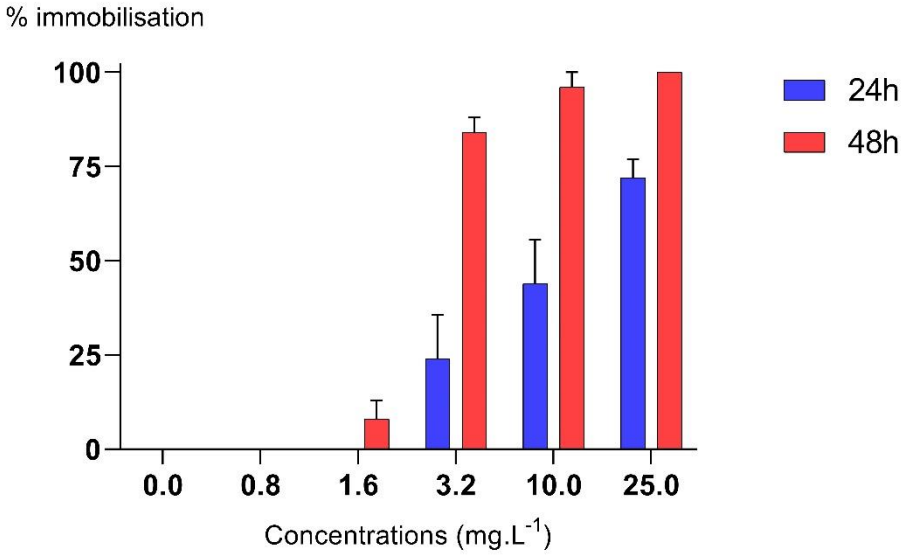

Figure 36: Percentage of immobilized *Daphnia vs* concentration of *S. aromaticum* essential oil.

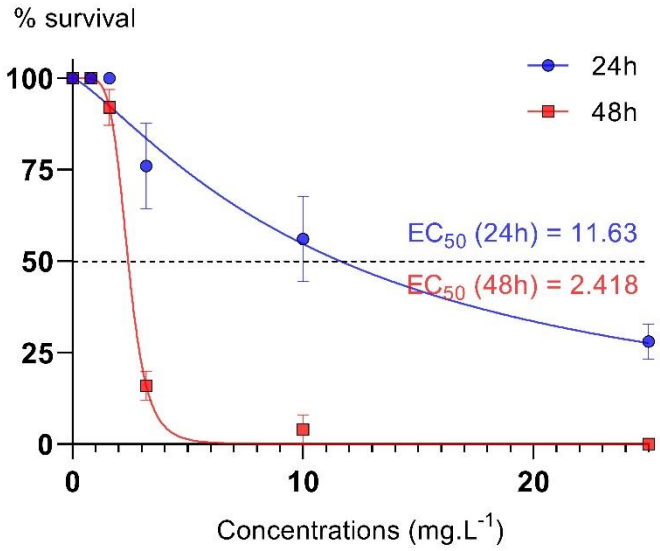

Figure 37: Dose-response curve of the effect of *S. aromaticum* essential oil in the immobilisation of *D. magna*.

4.2.5 – *Humulus lupulus* chloroformic extract (flowers)

Of all the different studied extracts obtained from *H. lupulus*, only the chloroformic extract from the flowers caused observable toxic effects to the *D. magna* neonates, although at high concentrations. The observed toxicity was dose and time-dependant, resulting in a 24h EC_{50} of 1086 $mg.L^{-1}$ and 48h EC_{50} of 905.7 $mg.L^{-1}$. Percentage of immobilised neonates is presented in figure 38 and a dose response curve is presented in figure 39.

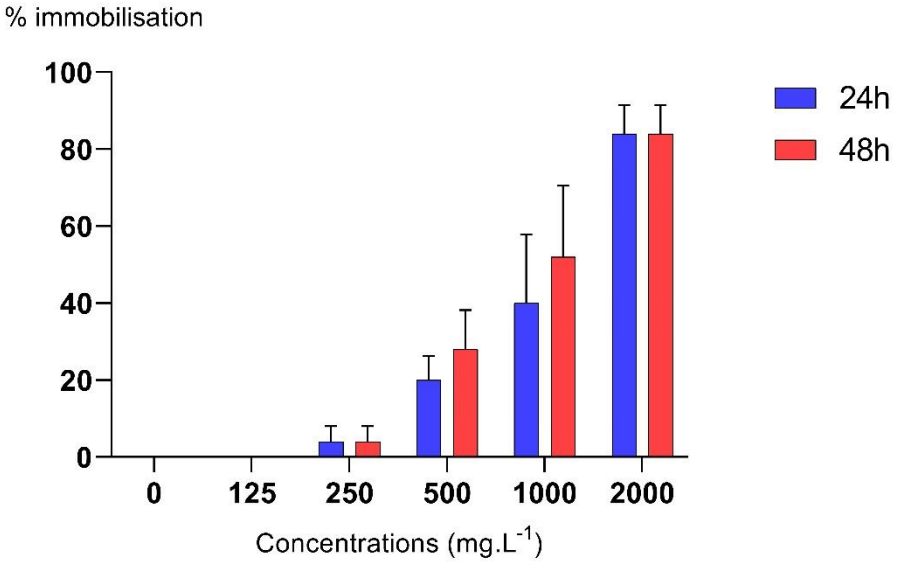

Figure 38: Percentage of immobilized *Daphnia vs* concentration of *H. lupulus* chloroformic extract (flowers).

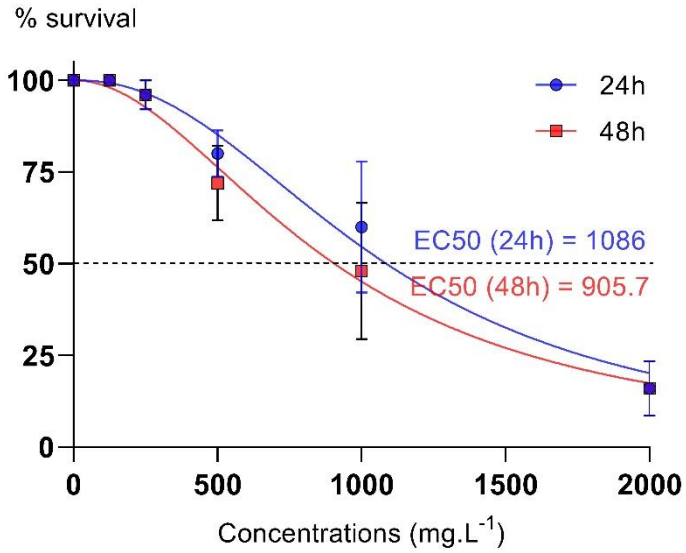

Figure 39: Dose-response curve of the effect of *H. lupulus* chloroformic extract (flowers) in the immobilisation of *D. magna*.

4.2.3 Test substances that did not cause immobilisation to *D. magna*.

The essential oil obtained from the aerial parts of *H. italicum* did not cause immobilisation of the *D. magna* neonates after 48 hours of exposure up to the maximum concentration tested (800 $mg.L^{-1}$). The hydrolate from *H. italicum* also did not show any observable effects to the neonates after 48h of exposure up to 2000 $mg.L^{-1}$. The different extracts from *H. lupulus* (mix hydrolate and flower hydrolate, mix chloroformic, mix ethanolic, mix hydro-ethanolic, mix methanolic and flowers methanolic) did not cause observable toxicity to the *Daphnia* neonates up to the highest concentrations tested. The same was observed for all the other hydrolates tested (*C. ladanifer*, *C. lusitanica*, *E. purpurea*, *M. chamomilla*, *O. basilicum* and *T. citriodorus*).

4.3 Classification

The studied essential oils from *E. globulus*, *T. capitata* and *S. aromaticum* can be classified as hazardous to the aquatic environment as the 48h EC_{50} values obtained are below 100 $mg.L^{-1}$. The *S. aromaticum* essential oil is classified in the Acute 2 category (48h EC_{50} > 1 and ≤ 10 $mg.L^{-1}$) and the essential oils from *T. capitata* and *E. globulus* are classified in the Acute 3 category (48h EC_{50} > 10 and ≤ 100 $mg.L^{-1}$). The *C. ladanifer* essential oil and the chloroformic extract from the flowers of *H. lupulus* have a 48h EC_{50} value above 100 $mg.L^{-1}$, as so, they are not classifiable as being hazardous to aquatic environments. The same can be said for all the other extracts from *H. lupulus* as they caused no observable effect at least up to 400 $mg.L^{-1}$. All the hydrolates studied are also not classifiable as being hazardous to aquatic environments as all of them caused no observable effects up to concentrations high above the classification limit. A comparison between obtained 48h EC_{50} values and the correspondent classification is shown in figure 40.

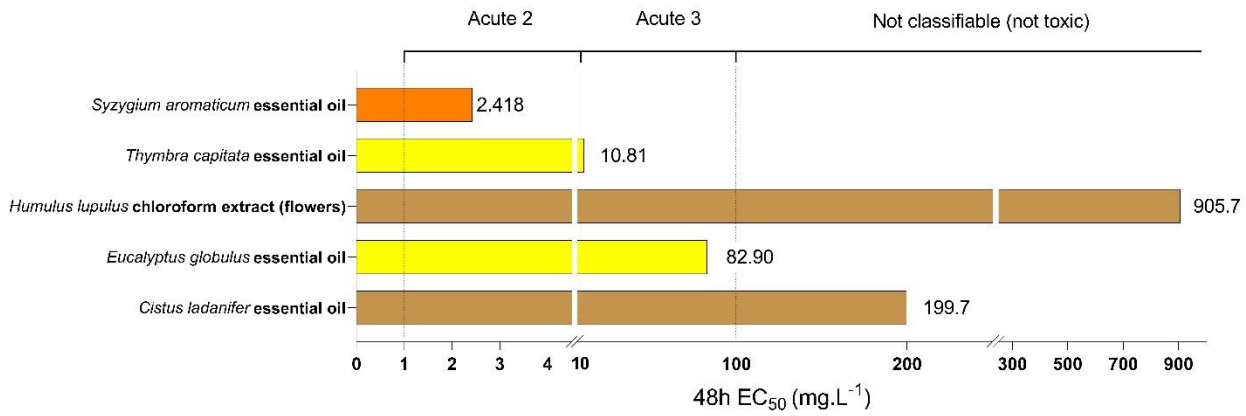

Figure 40: comparison between obtained 48h EC_{50} values for immobilisation of *D. magna* of the essential oils from *C. ladanifer*, *E. globulus*, *T. capitata* and *S. aromaticum* and the *H. lupulus* chloroformic extract, and their classification regarding toxicity, as proposed by the GHS.

Chapter 5 - Discussion

Essential oils and other plant extracts are generally regarded as safe products, but there is very little data available about their potential toxic effects to ecosystems. Despite this general perception as being safe, some of these products have been reported to be toxic to aquatic organisms. In this work, it has been shown that some essential oils and can cause effects to the aquatic organism *Daphnia magna* and some of them (*S aromaticum*, *T. capitata* and *E. globulus*) can be classified as hazardous to aquatic environments (figure 39). The analysis of the effects observed are presented in the following sections, divided by the type of test substance (essential oils, extracts and hydrolates).

5.1 Essential oils

The highest acute toxicity to *D. magna* was observed with the essential oil obtained from *S. aromaticum*, followed by the essential oil from *T. capitata*, the essential oil from *E. globulus* and finally the essential oil from *C. ladanifer*. Of them all the least toxic essential oil was the one obtained from the aerial parts of *H. italicum*, which showed no observable effects (immobilisation) up to 800 $mg.L^{-1}$, meaning that up to this concentration this essential oil does not seem to pose risk to *D. magna* organisms, but still above 800 $mg.L^{-1}$ the effects are not known.

5.1.1 *Syzygium aromaticum* essential oil

The highest toxicity was observed with the *S. aromaticum* (clove) essential oil. The 24h EC_{50} value obtained was 11.63 $mg.L^{-1}$ and the 48h EC_{50} value was 2.418 $mg.L^{-1}$. The immobilisation was dose and time-dependent. The chemical analysis of the essential oil (pers. comm., IPCB) showed that its main composition is eugenol (66.12%) and eugenol acetate (24.66%). Small amounts of caryophyllene are also present (8.34%).

Eugenol, eugenol acetate and β-caryophyllene have been reported as the major compounds in essential oils obtained from *S. aromaticum* in several studies (Batiha et al., 2020). An essential oil obtained from the flower buds (Algeria) was reported to contain 78.72% of eugenol (Selles et al., 2020), and another study also reported eugenol as the main component (53.23%) of an essential oil obtained from the floral bud of *S. aromaticum* from Brazil (Teles et al., 2021). The abundance of eugenol in clove essential oils has been attributed to many bioactive properties of this product (Batiha et al., 2020).

Eugenol is registered in the ECHA (European Chemicals Agency) database (EC number: 202-589-1 | CAS number: 97-53-0) and is reported to be acutely toxic to *D. magna* with a 48h

EC_{50} of 1.13 $mg.L^{-1}$. The test is reported to have been performed according to the OECD guideline 202 in a static system. Moreover, acute toxicity to fish (*D. rerio*) is also reported (96h LC_{50} of 13 $mg.L^{-1}$) and it is considered harmful to this organism. It is also considered harmful to the freshwater algae *Desmodesmus subspicatus* (72h EC_{50} for algal growth of 24 $mg.L^{-1}$) (https://echa.europa.eu/pt/registration-dossier/-/registered-dossier/13694/6/2/4, assessed in 12-04-21). One study reported that concentrations above 50 $\mu L.L^{-1}$ of clove oil caused immobilisation, heart rate inhibition and heart muscle contraction as well as a decrease in swimming activity of *D. magna* (Bownik, 2015). Another study reported a 48h EC_{50} of clove oil to *D. magna* of 2.8 $mg.L^{-1}$ (You et al., 2011).

Comparing our 48h EC_{50} results (~2.5 $mg.L^{-1}$) to the EC_{50} reported by You, C. et al (2.8 $mg.L^{-1}$), they are very similar. It would be interesting to know about the chemical composition of the essential oil tested in the reported study to compare the observed toxicity and the relative amounts of compounds present, but this information is not reported by the authors.

Considering the reported EC_{50} and LC_{50} values of eugenol in the ECHA database to different aquatic organisms, it is safe to say that this product can cause harmful acute effects to aquatic systems at low concentrations. Despite all of this, eugenol is extremely volatile, and it has been shown to be readily biodegraded in water (Baker and Grant, 2018), so, although it is acutely toxic to aquatic systems, it is to be expected the effects not to be long lasting. As already mentioned, although eugenol is the most abundant compound in the clove essential oil, eugenol acetate and caryophyllene are also present. Caryophyllene is reported to be acutely toxic to *D. magna* at very low concentrations (concentrations above 0.17 $mg.L^{-1}$ are reported to cause immobilisation) (https://echa.europa.eu/pt/registration-dossier/-/registered-dossier/20872/6/1, assessed in 12-04-21). Regarding eugenol acetate, the 48h EC_{50} value for immobilisation of *D. magna* is reported to be 24.33 mg.L-1 according to two reliable QSAR (Quantitative Structure-Activity Relationship) results (https://echa.europa.eu/pt/registration-dossier/-/registered-dossier/21436/6/2/4, assessed in 12-04-21). QSAR modelling has been recommended when available data is enough to predict the effect of a substance in a way of reducing the need for animal testing.

Despite all the available information regarding the acute toxicity of the main compounds, the essential oil is a mixture of chemicals that sometimes can act over organisms in a different way than each compound in isolation.

5.1.2 *Thymbra capitata* essential oil

The essential oil obtained from the aerial parts of *Thymbra capitata* caused the second highest toxicity to *Daphnia magna*. The EC_{50} values obtained varied from 12.4 mg.L-1 after 24h of exposure and 11.2 mg.L-1 after 48h of exposure. The chemical analysis (pers. comm., IPCB)

showed a high content of carvacrol in the composition of the essential oil. In total, 99.47% of the composition was identified, being carvacrol, by far, the most abundant compound making up 79.95% of the total composition. The second most abundant component was γ-terpinene (6.10%) followed by ρ-cimene (5.51%). The high content of carvacrol could be the explanation for the toxicity observed at low concentrations.

A recent study focused on the potential toxic effects of essential oils used in aquaculture on *D. magna* as a non-target organism. The essential oils from *Lippia alba*, *Lippia gracilis*, *Lippia sidoides*, *Mentha arvensis*, *Mentha piperita*, *Ocimum gratissimum* and *Piper callosum* showed toxicity ranging from slightly toxic to moderately toxic (48h EC_{50} values ranging from 10 to 100 $mg.L^{-1}$ for slightly toxic and 1 to 10 $mg.L^{-1}$ for moderately toxic as classified by the authors). Interestingly the highest toxicity was observed with the essential oil from *L. gracilis* (48h EC_{50} of 3.59 $mg.L^{-1}$) and its main compounds were carvacrol (38.9%), ρ-cymene (12.7%) and γ-terpinene (11.9%) (Miura et al., 2021).

Carvacrol is registered in the ECHA (European Chemicals Agency) database (EC number: 207-889-6 | CAS number: 499-75-2) as being toxic to aquatic life (96h LC_{50} to *Danio rerio* of 6.17 $mg.L^{-1}$; 48h EC_{50} for immobilisation to *D. magna* of 6.06 $mg.L^{-1}$; 72h EC_{50} for algal growth of *Raphidocelis subcapitata* of 4.05 $mg.L^{-1}$) (https://echa.europa.eu/pt/registration-dossier/-/registered-dossier/23562/6/1 accessed in 07.04.21). Moreover, carvacrol is considered not readily biodegradable and according to CLP (Directive 1272/2008/EC), the aquatic hazard of this substance is classified as Chronic Category 2, meaning the effects in the environment can be long lasting. Therefore, it is recommended to avoid releasing this substance or solutions of this substance into lakes, streams, ponds, or public waters. Considering the 48h EC_{50} value obtained and all the available information regarding the reported toxic effects of carvacrol, the most abundant compound present in the studied *T. capitata* essential oil, it can be said that this essential oil has the potential to cause environmental damages, especially to aquatic systems where it can affect populations of microalgae, crustaceans, and fish. Therefore, special precautions should be taken to avoid intentional or accidental releases of this product into aquatic systems.

5.1.3 *Eucalyptus globulus* essential oil

The essential oil from *E. globulus* showed the highest time-dependent toxicity with a 24h EC_{50} of 222.8 $mg.L^{-1}$ and 48h EC_{50} of 82.90 $mg.L^{-1}$. Data from the literature reports the main compound of essential oils obtained from *E. globulus* to be 1,8-cineole or eucalyptol as it is more commonly referred. Harkat- Madouri et al (2015) reported 55.29% of 1,8-cineole in an essential oil obtained from the leaves of *E. globulus* from Algeria. Spathulenol (7.44%) and α-terpineol (5.46%) were also identified (Harkat-Madouri et al., 2015). A commercial *E. globulus* essential oil

from India was also reported to be rich in 1,8-cineole (45.4%) and other compounds such as limonene (17.8%) and ρ-cymene (9.5%) were also identified (Tyagi and Malik, 2011).

Although eucalyptol is usually the most abundant compound in essential oils from the leaves of *E. globulus*, the relative amount of other compounds can vary according to the region the plant grows and the stage of leaves development in which the plant material is collected (Salehi et al., 2019). The chemical composition of our studied essential oil is not available by the time of the submission of this dissertation as this analysis is performed by other colleagues involved in the InovEP project.

Extracts from *E. globulus* (including essential oils) are registered in the ECHA database (EC number: 283-406-2 | CAS number: 84625-32-1) as being toxic to aquatic organisms with long-lasting effects under the chronic 2 category (https://echa.europa.eu/pt/registration-dossier/-/registered-dossier/14864/6/1, assessed in 12-04-21). The effect concentration (EC) was estimated according to the additivity formula (equation 2) as recommended in the Regulation (EC) No 1272/2008 (CLP) where:

$$\frac{\sum Ci}{L(E)C50m} = \sum_{\eta} \frac{Ci}{L(E)C50i}$$

Equation 2: additivity formula used to estimate the effect concentration.

Ci = concentration of component i (weight percentage);

L(E)C50i = LC50 or EC50 for component i, in $mg.L^{-1}$;

η = number of components

L(E)C50m = L(E)C50 of the part of the mixture with test data.

The obtained 48h EC_{50} value by using the additivity formula was 1.02 $mg.L^{-1}$ for aquatic invertebrates. This approach can be used when there is available data on the $L(E)C_{50}$ values of specific compounds present in complex mixtures and the relative amount of each compound to estimate an effect concentration of a mixture. Despite the usage of the additivity formula to predict the effect concentration of the essential oil, it was ultimately considered not realistic "based on individual toxicities of the major constituents, the $L(E)C_{50}$ values for Eucalyptus oil were estimated to be 0.90 $mg.L^{-1}$ for fish, 1.02 $mg.L^{-1}$ for aquatic invertebrates and 1.64 $mg.L^{-1}$ for algae, and the NOEC value for Eucalyptus oil was estimated to be 0.88 $mg.L^{-1}$ for algae (based on growth rate). Considering these results, Eucalyptus oil should be classified as Aquatic Acute 1 and Aquatic Chronic 3, according to the 2nd ATP of the Regulation (EC) No 1272/2008. However, this constituent approach was considered as a worst-case and finally not realistic." Therefore acute toxicity tests were performed with the predicted most sensitive species (fish) with intriguing results as reported: "As a validation that additivity approach is a real worst case, the experimental

studies show 96h-LC_{50} of 18mg.L^{-1} and 42 mg.L^{-1} for both *Eucalyptus* crude oil and *Eucalyptus globulus* oil respectively". (https://echa.europa.eu/pt/registration-dossier/-/registered-dossier/14864/6/1, accessed in 13-04-2021). Although many mathematical models have been proposed to estimate the effect of mixtures of chemicals, sometimes the experimental results are different from the ones predicted (Brezovšek et al., 2014; Tichý et al., 2002). These deviations between predicted and observed responses can be attributed to many factors, such as a too simple model or the inability of the model to capture interactions between chemicals. Moreover, as mentioned by Rider, C. et al "Much of the toxicology work on mixtures has focused on low numbers of chemicals (binary and ternary combinations), with less work addressing chemical mixtures containing 10–20 constituents", as it is often the case of essential oils (Rider et al., 2018). The 48h EC_{50} value for immobilisation of *D. magna* of our studied *E. globulus* essential oil was 82.90 mg.L^{-1} which is very far from the predicted EC_{50} value using the additivity formula that is reported in the ECHA database.

5.1.4 *Cistus ladanifer* essential oil

The essential oil from *C. ladanifer* was, out of all the essential oils that caused immobilisation, the one with the lowest observable effects to *D. magna*. Analysing the chemical composition of the essential oil (pers. comm., IPCB) it is noticeable the complex composition of it, being the major compounds present α-pinene, camphene and 2,2,6-trimethylcyclohexanone. These three major components together do not reach 50% of the total composition. Thirty-four other components are present in relative concentrations below 5% each, and 7.43% of the composition was not identified.

A recent study assessed the chemical composition of Portuguese *C. ladanifer* essential oils and also reported α-pinene as the major compound (13.2-27.9%) (Tavares et al., 2020a). This compound is reported to have toxic effects on *D. magna* with a 24h LC_{50} of 68 mg.L^{-1} and 48h LC_{50} of 41 mg.L^{-1}. The second most abundant compound (camphene) is also reported to have toxic effects on *D. magna*: 24h LC_{50} of 46 mg.L^{-1} and 48h LC_{50} of 22 mg.L^{-1} (Hazardous Substances Data Bank (HSBD) accessed on 29-01-2021). For 2,2,6-Trimethylcyclohexanone there is no available data regarding the toxicity of this compound to *D. magna*. The toxicity observed (although only at high concentrations) of the *C. ladanifer* essential oil could be attributed to the presence of these two compounds that together represent 42.49% of the essential oil composition.

In the ECHA database, an essential oil obtained from the stems and leaves of *C. ladanifer* is registered under the EC number: 946-570-2. In the ecotoxicological information, this essential oil is reported to have a 48h EC_{50} of 63.2 mg.L^{-1} for the immobilisation endpoint of *D. magna*. This assessment is reported to have been carried according to the OECD guideline 202 in a semi-static condition (https://echa.europa.eu/pt/registration-dossier/-/registered-dossier/21887/6/2/4 accessed in 07.04.21). Comparing to the 48h EC_{50} value obtained in our

study (199.7 mg.L^{-1}), the reported EC_{50} in the ECHA database is much lower. These differences in observed toxicity to *D. magna* can result from chemical differences between essential oils. One study reported that the major compounds present in an essential oil obtained from *C. ladanifer* collected in eastern Morocco were camphene (15.5%) and borneol (11.1%) (Zidane et al., 2013). Another study reported viridiflorol and *ɣ*-gurjunene as the main compounds (28.2% and 14.61%, respectively) of an essential oil of *C. ladanifer* collected in the Taza region of Morocco (Benali et al., 2020). These differences in major compounds present in the essential oils can lead to different toxic effects according to the compounds present and their relative quantity.

5.1.5 *Helichrysum italicum* essential oil

The *H. italicum* essential oil was the only one that did not cause any immobilisation to *D. magna* up to the highest concentration tested (800 mg.L^{-1}). The chemical composition of the essential oil showed a high complexity of compounds present (pers. comm., IPCB). The most abundant compound was γ-curcumene followed by (-)-italicene and neryl acetate was the third most abundant compound. These three compounds together make up only 40% of the total identified composition. Twenty-four other compounds were identified at small percentages (below 10%).

Essential oils obtained from *H. italicum* usually differ in their chemical composition. One study reported neryl acetate (28.2%) and γ-curcumene (18.8%) as the main compounds of an essential oil obtained from *H. italicum* collected in Montenegro (Kladar et al., 2015), while in another study, another essential oil from *H. italicum* from the same country revealed β-eudesmene (21.65%), β-bisabolene (19.90%) and α-pinene (16.90%) as the three major compounds (Oliva et al., 2020). As another example of how the chemical composition of essential oils from this plant can be very different, an essential oil from *H. italicum* collected in north Algeria was reported to have α-cedrene (13.61%), α-curcumene (11.41%) and geranyl acetate (10.05%) as the three major compounds (Djihane et al., 2017). Despite the differences in the chemical composition, what these reported essential oils all have in common is the relatively low percentage that the major compounds are present in. The same trend was observed with the essential oil studied in this work. This can be the explanation of the lack of observable effects (immobilisation) in the acute toxicity tests with *D. magna*. Moreover, there is no available data (as far as my knowledge goes) regarding toxicological effects of the three major compounds identified (γ-curcumene, (-)-italicene and neryl acetate) to *D. magna* or any other aquatic invertebrate. These results suggest that the essential oil from the aerial parts of *H. italicum* here studied poses low to no risk to *D. magna*, although effects at concentrations above the ones here studied remain unknown.

5.2 Extracts

In this work, six extracts obtained from *H. lupulus* were studied regarding their acute toxicity to *Daphnia magna* (table 8).

Of all the *H. lupulus* extracts, only the chloroformic extract obtained from the flowers caused observable effects to *Daphnia magna*. This extract was tested up to very high concentrations (2000 $mg.L^{-1}$) as it was available in more quantity. The other extracts showed no observable effects up to the highest concentrations tested, which were always above 400 $mg.L^{-1}$ (4 times above the concentration that can be still considered toxic in the GHS as shown in table 1). Figure 40 shows a comparison between the 48h EC_{50} values obtained and also the respective classification according to the GHS.

Despite the results here presented regarding the acute toxicity of our studied extracts, some of these products have been reported to have toxic effects on *D. magna*. For example, an aqueous extract from the bark of Thuya (*Tetraclinis articulata*, Cupressaceae) was acutely toxic to *D. magna* with a 48h EC_{50} for immobilisation of 6.49 $mg.L^{-1}$. The extract also caused a decrease in the number of neonates, indicating long-term effects to the population of this organism (Montassir et al., 2017). To the extreme, plants that are known to produce toxic metabolites, and have been exploited due to their toxicity, such as *Tephrosia vogelii* (Fabaceae) and *Hura crepitans* (Euphorbiaceae) can have very harmful effects on non-target organisms. The first has been commercially exploited in India and other tropical regions as a fish poison and insecticide (Bravim dos Santos et al., 2021; Touqeer et al., 2013). An aqueous extract from the leaves of *T. vogelii* showed remarkable toxicity to *D. magna* with a 24h EC_{50} of 0.47 µg.L-1 (Li et al., 2015). *H. crepitans* is a plant known for the toxic latex it produces which is rich in huratoxin, a lectin that inhibits protein synthesis (Vassallo et al., 2020a). An aqueous extract from the bark of this plant also showed high acute toxicity to *D. magna* with a 48h LC_{50} of 36 $\mu g.L^{-1}$ (Iannacone et al., 2014).

5.2.1 *Humulus lupulus* chloroformic extract from the flowers

The chloroformic extract obtained from the flowers caused observable effects to *D. magna* although at very high concentrations, way above the maximum limits set by the GHS of the United Nations (UN, 2019). The extract was tested up to 2000 $mg.L^{-1}$ and when compared to the chloroformic extract obtained from the aerial parts of *H. lupulus*, which did not cause observable effects up to the same concentration, we can assume that possibly there could be differences in the chemical composition of both extracts. It has been reported that the cones (flowers) from hop are rich in oils, resins and polyphenols such as flavonols, flavan-3-ols, phenolic carboxylic acids and other polyphenols as resveratrol (Almaguer et al., 2014; Astray et al., 2020). In fact, it was verified through the chemical analysis that the extract from the flowers contained a higher amount of rutin when compared to the extract from the aerial parts (2.282 µg/mg of extract

from the flowers vs 1.092 μg/mg of extract from the aerial parts). Small quantities of resveratrol were also identified in the extract from the flowers (0.041 μg/mg of extract from the flowers), while it was not identified in the extract from the aerial parts (pers. comm., IPCB). The toxicity observed could also be due to the possible presence of residues of the solvent that was used to perform the extraction (chloroform is reported to have a 48h EC_{50} of 65.7 $mg.L^{-1}$ to *D. magna* (Gersich et al., 1986)). Unfortunately, as far as my knowledge goes, there is no available data regarding the possible toxic effects of rutin or resveratrol on *D. magna* organisms for comparison.

5.3 Hydrolates

Hydrolates, also known as hydrosols or aromatic waters are sub-products obtained from the distillation process used to isolate essential oils from plants. For plants that contain very low amounts of essential oil, a hydrolate is usually the only product that can be obtained from the distillation process (Catty, 2001; Price and Price, 2004). All the hydrolates tested showed no observable effects up to the highest concentrations tested. As hydrolates are the leftover product from the distillation process they do not contain high quantities of compounds, and these are usually the less hydrophobic compounds that are present in the essential oils. Despite this, several studies have addressed the bioactive properties of hydrolates to valorise this product that has been usually regarded as waste, in a circular economy approach. There are studies, for example, that show that hydrolates can be effectively used as antimicrobials (Di Vito et al., 2021; Sainz et al., 2019; Tavares et al., 2020a). These studies showed that volatiles present in hydrolates can be more effective in inhibiting microbial growth than essential oils because they are active at lower concentrations. This more hydrophilic nature, the lower abundance in compounds and lower concentrations of compounds can be the explanation for the lack of acute toxicity observed towards *D. magna*. As it has been shown in this study, the essential oil from *T. capitata* showed toxic effects to *Daphnia magna* (all organisms were immobilized at the concentration of 25 $mg.L^{-1}$), while the respective hydrolate caused no immobilisation up to 400 $mg.L^{-1}$, a concentration 16 times higher. The hydrolate from *C. ladanifer* also showed no effects to *D. magna* up to 2000 $mg.L^{-1}$, while the essential oil caused 100% immobilisation at the concentration of 400 $mg.L^{-1}$.

Chapter 6 - Conclusions

Plants have been used by mankind for centuries as a source of therapeutic agents. They are used in traditional medicine and by many industries to obtain valuable compounds of commercial interest. Essential oils and other plant extracts are nowadays important products used in pharmaceuticals, cosmetics, food and textiles. Recently, a new global trend for more natural products has led to an increase in the demand by customers and industries. More attention has been directed to hydrolates as a source of therapeutical agents. These by-products of the distillation process to obtain essential oils were disregarded for many years as just a leftover of the process with no real interest, but today and in a context of circular economy, they have been reported to possess important properties that can lead to the development of new industrial applications.

As the demand for plant-based products continues to increase, the InovEP Project aims to provide scientific data that can be used by industries to develop new applications of natural products obtained from plants such as essential oils, hydrolates and extracts. In the European Union, regulations regarding the introduction of new products on the market, particularly REACH, require intense research regarding their safety. Therefore, in this work, the eco-safety of essential oils, hydrolates and extracts obtained from *Cistus ladanifer*, *Cupressus lusitanica*, *Echinacea purpurea*, *Eucalyptus globulus*, *Hamamelis virginiana*, *Helichrysum italicum*, *Humulus lupulus*, *Matricharia chamomilla*, *Ocimum basilicum*, *Thymbra capitata*, *Thymus citriodorus* and *Syzygium aromaticum* was evaluated using the model organism *Daphnia magna* as recommended by international regulatory agencies to perform reproducible, comparable and robust aquatic toxicity tests.

The acute toxicity tests performed provide an insight into the possible effects that these products can have in the environment, particularly in aquatic systems. The essential oils caused the highest effects regarding immobilisation of *D. magna* organisms. The highest observable toxicity was caused by the essential oil obtained from *S. aromaticum*, and this could be linked to the high content of eugenol identified. The second highest observed effects were caused by the essential oil obtained from *T. capitata* which has a high amount of carvacrol, a phenolic monoterpenoid that is reported to be toxic to aquatic life. The essential oil from *E. globulus* also caused observable effects to *D. magna* that can be caused by the reported most abundant compound usually present in eucalyptus essential oils – 1,8 – cineole or eucalyptol. The essential oil from *C. ladanifer* caused the lowest observable immobilisation of all the essential oils tested in this work. The effects were observed at relatively high concentrations of the essential oil. Interestingly, the essential oil from *H. italicum* did not cause any immobilisation of *D. magna* up to the highest concentration tested and it was the only essential oil that caused no effects in the test organism. Of all the extracts tested from *H. lupulus*, only the chloroformic extract obtained from the flowers of this plant caused observable effects (immobilisation) to *D. magna*, although at very high concentrations. Regarding the hydrolates, none of them showed observable effects

(immobilisation) to *D. magna* up to the highest concentrations tested which can be attributed to the lower concentrations of compounds that characterise these products.

To classify the toxicity observed, the GHS proposed by the UN can be used based on the 48h EC_{50} values obtained for immobilisation of the test organisms, in this case, the crustacean *D. magna*. Using this classification, the essential oils from *S. aromaticum* (acute 2), *T. capitata* and *E. globulus* (acute 3) can be classified as acutely toxic to aquatic systems. The essential oil from *C. ladanifer* and all the extracts from *H. lupulus* as well as all the hydrolates tested cannot be classified as the obtained 48h EC_{50} values are above the proposed classification limit of 100 $mg.L^{-1}$. Therefore, precautions should be taken to avoid accidental or intentional contamination of water bodies when producing and handling essential oils from *S. aromaticum*, *T. capitata* and *E. globulus* as they can have effects on aquatic organisms in the same trophic level as *D. magna*. The essential oil from *C. ladanifer*, the extracts from *H. lupulus* and all the hydrolates tested seem to pose low to no risk to *D. magna* as far as observed regarding the acute exposure. Still, and following the precautionary principle, the same precautions should be taken as the complete effects on the environment are not known.

To gain a better understanding of the effects that the studied essential oils, extracts and hydrolates can have in other trophic levels (e. g. microalgae and fish) and other systems (e. g. marine or terrestrial), acute toxicity tests could be performed using other recommended model organisms for each system. Chronic toxicity tests could also be performed to analyse the effects that long-term exposure can have on organisms particularly when compounds that are known to have low biodegradability are present.

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Annexes

1. Publication 1: Article
   Fernandes A, Pereira C, Coelho S, Ferraz CA, Sousa ACA, Pastorinho MR, Pacheco MJ, Ciríaco L, Lopes A (2020) Ecotoxicological evaluation of methiocarb eletrochemical oxidation. Applied Sciences

2. Publication 2: Article
   Ferraz CA, Sousa ACA, Caramelo D, Delgado F, Palmeira de oliveira A, Pastorinho MR **(submitted)** Characterisation of essential oils and hydrolates from *Cistus ladanifer*, *Helichrysum italicum*, *Ocimum basilicum* and *Thymbra capitata*: chemical profile and eco-safety evaluation with the freshwater crustacean *Daphnia magna*. Industrial Crops and products

3. Publication 3: Review article
   Ferraz CA, Palmeira de Oliveira A, Pastorinho MR, Sousa ACA **(submitted)** Ecotoxicity of plant extracts and essential oils: a review. Environmental Pollution

4. Oral communication in international congress
   Ferraz CA, Sousa ACA, de Oliveira AP, Pastorinho MR (2021) Contributions towards the ecotoxicological evaluation of plant extracts and essential oils. Natural Products Application: Health, Cosmetics and Food, 4-5 February 2021 Online Edition

5. Posters
   Ferraz C, Pais RT, Gaspar C, Palmeira de Oliveira A, Sousa AC, Pastorinho MR (2019) Acute toxicity of plant extracts towards *Daphnia magna*. III International Congress in Health Sciences Research – Trends in Aging and Cancer, 14-16 November 2019, Covilhã, Portugal, p. 89

Acute toxicity of plant extracts towards *Daphnia magna*

Celso A. Ferraz [1], Ricardo T. Pais [1], Carlos Gaspar [1], Ana Palmeira de Oliveira [1], Ana Catarina Sousa [1,2,3], M. Ramiro Pastorinho [1,2,4]

[1]Health Sciences Research Centre (CICS), University of Beira Interior, 6200-506 Covilhã, Portugal

[2]NuESA-Health and Environment Study Unit, Faculty of Health Sciences, University of Beira Interior, 6200 -506 Covilhã, Portugal

[3]CICECO – Aveiro Institute of Materials, Chemistry Department, University of Aveiro, 3810-193 Aveiro, Portugal

[4]Department of Biology, University of Évora, 7002 -554 Évora, Portugal

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INTRODUCTION

- Plants are an important source of bioactive compounds of high economic interest, being used in various types of industries.
- Gum rockrose (*Cistus ladanifer*), Mediterranean thyme (*Thymbra capitata*), Basil (*Ocimum basilicum*), Curry plant (*Helichrysum italicum*) and Hop (*Humulus lupulus*) are plants already used in the cosmetics and the perfume industries.
- Considering that extracts of these plants will potentially be released into the environment, it is necessary to assess the risk associated with their introduction to the ecosystem. Yet, no ecotoxicological data on the effects of these extracts is available.


Cistus ladanifer

Thymbra capitata

Ocimum basilicum

Helichrysum italicum

Humulus lupulus

OBJECTIVE

- To evaluate the toxic potential of five plants' extracts (*C. ladanifer, T. capitata, O. Basilicum, H. italicum, H. lupulus*) towards the model organism *Daphnia magna*.

MATERIALS AND METHODS

Extracts


Cistus ladanifer
• Essential oil.


Thymbra capitata
• Essential oil.
• Hydrolate.


Ocimum basilicum
• Hydrolate.


Helichrysum italicum
• Essential oil.


Humulus lupulus
• MeOH mix; EtOH mix.
• Aqueous mix.
• Flower MeOH; Aqueous flower.

D. magna culture


7.6 – 8.0


20±1° C


16/8 (light/dark)


Raphidocelis subcapitata **(Diet)**

Acute toxicity tests


x5/well

Range of concentrations customized for each extract

Immobilization at 24 and 48h

EC_{50}

(concentration estimated to immobilize 50% of individuals)

Validation test

$K_2Cr_2O_7$

0.2 - 0.4 - 0.8 - 1.6 - 3.2 ($mg.L^{-1}$)


Immobilization at 24h

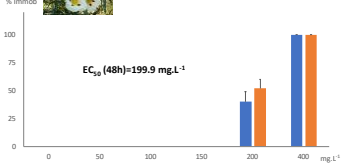
EC_{50} = 1.71±1.3x10^{-9}

Certified range

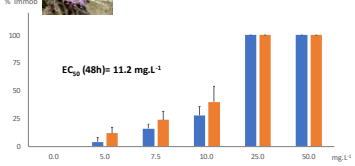
EC_{50} = 0.6 – 2.1 $mg.L^{-1}$

RESULTS and DISCUSSION


Cistus ladanifer **(Essential oil)**




Thymbra capitata **(Essential oil)**



Daphnia magna immobilization(%) after 24 (blue bars) and 48h of exposure (orange bars). The vertical lines correspond to the standard error of the mean.

No immobilization was observed for:


Thymbra capitata
• **Up to 400 $mg.L^{-1}$:** Hydrolate


Ocimum basilicum
• **Up to 2 $g.L^{-1}$:** Hydrolate


Helichrysum italicum
• **Up to 800 $mg.L^{-1}$:** Essential oil


Humulus lupulus
• **Up to 400 $mg.L^{-1}$:** MeOH mix; EtOH mix; Aq. mix; Flower MeOH
• **Up to 500 $mg.L^{-1}$:** Aqueous flower

- The EC_{50} varied from **200.8 ± 3.28x10^{-5} $mg.L^{-1}$** at 24h and **199.9 ± 5.94x10^{-5} $mg.L^{-1}$** at 48h for *C. ladanifer* essential oil.
- For *T. capitata* the EC_{50} varied from **12.4 ± 0.4731 $mg.L^{-1}$** at 24h and **11.2 ± 0.4095 $mg.L^{-1}$** at 48h.
- These results suggest that *T. capitata* essential oil is more toxic to *D. magna* than the *C. ladanifer* essential oil.
- For *T. capitata* (in the form of hydrolate), *O. basilicum, H. italicum* and *H. lupulus*, no immobilization was observed up to the highest concentrations tested, suggesting that these extracts present none to low risk for *D. magna*.
- The results obtained demonstrate that all extracts tested are generally less toxic than those reported in the literature for other plants [1-4].

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Ferraz C, Pais RT, de Oliveira AP, Sousa ACA, Pastorinho MR (2019) Evaluation of the ecotoxicity of plant extracts in *Daphnia magna*. XIV Annual CICS-UBI Symposium 2019, 4-5 July 2019, Covilhã, Portugal, p. 125

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Evaluation of the ecotoxicity of plant extracts in *Daphnia magna*

Celso A. Ferraz[1], Ricardo T. Pais[1], Ana Palmeira de Oliveira[1], Ana Catarina Sousa[1,2,3], M. Ramiro Pastorinho[1,2,4]

[1]Health Sciences Research Centre (CICS), University of Beira Interior, 6200 -506 Covilhã, Portugal
[2]NuESA-Health and Environment Study Unit, Faculty of Health Sciences, University of Beira Interior, 6200 -506 Covilhã, Portugal
[3]CICECO – Aveiro Institute of Materials, Chemistry Department, University of Aveiro, 3810 -193 Aveiro, Portugal
[4]Department of Biology, University of Évora, 7002-554 Évora, Portugal

Introduction

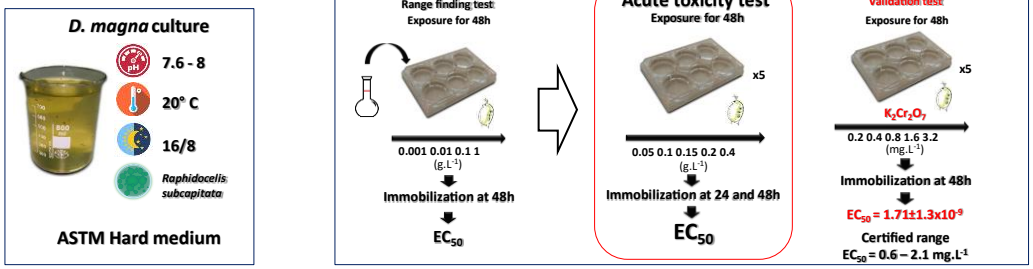
- Plants are an important source of bioactive compounds of high economic interest, being used in various industries [1].

- Gum rockrose (*Cistus ladanifer*) is an autochthonous plant that is used in cosmetics and perfumery, particularly in the form of essential oil [2].
- Although *C. ladanifer* extracts are used with industrial purposes, there is no data related to its ecotoxicological effects.
- Considering that these coumpounds will potentially be released into the environment, it is necessary to assess the risk associated with their introduction to the ecosystem.

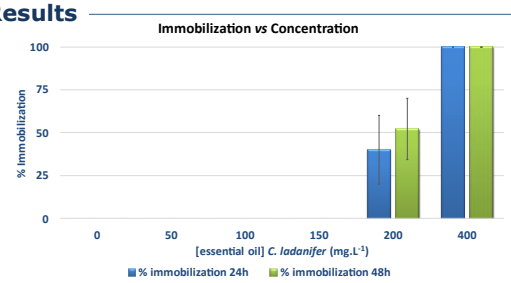
- Risk evaluation can be performed using the model organism *Daphnia magna*.
- *D. magna* is a small crustacean, recommended by regulatory agencies for ecotoxicological tests [3].
- Its life cycle, in ideal conditions, is carried through parthenogenesis resulting in genetically identical offspring [4].

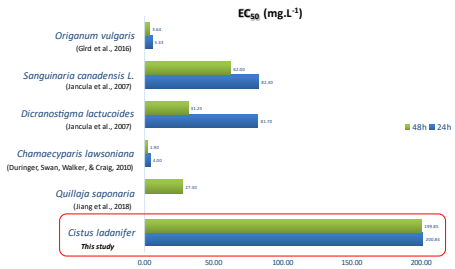
What are the toxicological effects of *C. ladanifer* extracts?

Materials and Methods



Results





Conclusions

The EC_{50} (the concentration estimated to immobilize 50 % of individuals) varied between **200.8 ± 3.28x10^{-5} mg.L^{-1}** at 24 hours and **199.9 ± 5.94x10^{-5} mg.L^{-1}** at 48 hours.
The results obtained for *C. ladanifer* suggest that this extract is less toxic than those reported in the literature for other plants.

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FCT COMPETE 2020 PORTUGAL 2020

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