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STUDY OF THE BIOACTIVE PROPERTIES OF *DAUCUS CAROTA* SUBSP. *CAROTA* EXTRACTS AND ESSENTIAL OIL

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Study of bioactive properties of *Daucus carota* subsp. *carota* extracts and essential oil

Thesis conducted under the supervision of PhDs Lígia Maria Ribeiro Salgueiro and Susana Maria de Almeida Cardoso in fulfilment of the requirements for the Master's Degree in Pharmaceutical Biotechnology and presented to the Faculty of Pharmacy of the University of Coimbra

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In front cover: *Daucus carota* subsp. *carota* mature (left) and flowering (right) umbels

"Faz tudo como se alguém te contemplasse"
Epicuro

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Abstract

Plants from the genus *Daucus* L., particularly the species *Daucus carota*, have been widely used over the millennia in folk medicine as treatment for dysentery and kidney dysfunction, amongst other ailments, These properties are based upon anecdotal evidence and many of them have not been validated using a scientific-based approach, thus justifying further investigations on the bioactive properties of these species.

The work conducted in this thesis focuses on the taxon *D. carota* subsp. *carota* that grows spontaneously in Portugal. Essential oil and phenolic-enriched extracts obtained by extraction with 70% methanol, 100% methanol and water, from the aerial parts of *D. carota* subsp. *carota,* were used to access several bioactive properties, namely antibacterial, antifungal, antioxidant, anti-inflammatory and cytotoxicity ones.

The antibacterial activity of the essential oil and the 70% methanol extract was evaluated against several pathogenic bacteria, through determination of the minimal inhibitory concentration (MIC) and minimal lethal concentration (MLC). The essential oil had a stronger activity when compared to the phenolic extract against *Staphylococcus aureus*, *Listeria monocytogenes* and *Bacillus subtilis* (MIC = 0.32 – 0.64 µL/mL versus 5-10 mg/mL). The bacteria *Escherichia coli* and *Salmonella typhimurium* were resilient against both samples.

The antifungal properties of the samples were assessed against yeasts (*Candida* spp. and *Cryptococcus neoformans*), dermatophytes and *Aspergillus* spp. which are known to cause several pathologies both in humans and animals. The MIC and the MLC values showed a significant antifungal activity of the essential oil especially against dermatophytes (MIC = 0.32 – 0.64 µL/mL) and *Cryptococcus neoformans* (MIC = 0.16 µL/mL). The 70% methanol extract only demonstrated a significant activity against dermatophytes (MIC = 1.25 – 10 µL/mL). The inhibition of the germ tube formation and the effect on preformed biofilms of *C. albicans* was also conducted for the essential oil and the results suggest that it might be a promising therapy for candidiosis.

The antioxidant capacity was also assessed in terms of radical scavenging using the ABTS cation radical scavenging assay and the ORAC assay. For the phenolic-enriched extracts the total phenolic content was also determined using the Folin-Ciocalteau assay. The total phenolic content on all tested samples was very similar between each other indicating that the phenolic compounds found in wild carrot have the same solubility in water and methanol. The ABTS and ORAC assays demonstrated that the phenolic-enriched extracts have a stronger antioxidant activity when compared to the essential oil, which was expected since phenolic

compounds are described as having more radical scavenging activity than terpenes and terpenoids.

Prior to anti-inflammatory assays, NO• scavenging potential of *D. carota* subsp. *carota* was conducted using SNAP as a NO• donor. The essential oil was unable to scavenge the NO• released by SNAP. The anti-inflammatory activity of the essential oil was also performed by evaluating the capacity to inhibit the NO release in LSP-stimulated macrophages. The results show that the essential oil was able to decrease NO production by 19.04% without cytotoxicity to macrophages.

In order to further evaluate the potential application of the essential oil in humans, the safety profile was determined using different cells (keratinocytes, alveolar epithelial cells, macrophages and hepatocytes) mimetizing the main administration routes and the metabolization in the organism (liver). The results demonstrated the essential oil in general was safe when applied in concentrations lower that 1.25 μL/mL.

The present work contributed to a better understanding on the bioactive properties of *D. carota* subsp. *carota,* and evidenced the industrial potential of this plant.

Resumo

As plantas do género *Daucus* L. são desde há séculos usadas na medicina tradicional como tratamento para disenteria, disfunção renal, entre outros problemas, particularmente a espécie *Daucus carota*. No entanto estas propriedades são baseadas no conhecimento popular e não de estudos científicos, assim justifica-se a necessidade de se realizarem estudos sobre as propriedades bioactivas desta espécie, especialmente do taxon *D. carota* subsp. *carota*, que está amplamente distribuído em Portugal

Neste trabalho utilizou-se óleo essencial e extratos enriquecidos em compostos fenólicos (extração com solução de metanol a 70% (v/v), metanol absoluto ou água), todos obtidos a partir das partes aéreas de *D. carota* subsp. *carota*. As propriedades bioativas estudadas foram as atividade antibacteriana, antifúngica, antioxidante, anti-inflamatória. Adicionalmente foi ainda avaliada a citotoxicidade do OE.

A atividade antibacteriana do óleo essencial e do extrato de metanol 70% foi determinada contra várias bactérias patogénicas. O óleo essencial demonstrou uma atividade mais intensa que o extrato contra *S. aureus*, *L. monocytogenes* e *B. subtilis* (0.32 – 0.64 µL/mL contra 5-10 mg/mL). As bactérias *E. coli* e *S. typhimurium* foram resistentes à ação tanto do óleo essencial como do extrato.

As propriedades antifúngicas das amostras foram avaliadas contra leveduras (*Candida* spp. e *Cryptococcus neofomans*), dermatófitos e *Aspergillus* spp. que são conhecidos com agentes causais de várias patologias tanto em humanos como em animais. A avaliação da concentração mínima inibitória (CMI) e da concentração mínima letal (CML) demonstrou que o óleo essencial de *Daucus carota* subsp. *carota* tem uma atividade significativa especialmente contra dermatófitos (0.32 – 0.64 µL/mL) e *C. neoformans* (0.16 µL/mL). Por sua vez o extrato de metanol 70% apenas demonstrou atividade significante contra dermatófitos (1.25 – 10 µL/mL). A inibição do tubo germinativo e o efeito em biofilme pré-formado de *C. albicans* sugere que o óleo essencial poderá ser um potencial interesse no tratamento da candidíase.

As propriedades antioxidantes das amostras foram avaliadas recorrendo à sua capacidade de capturar radicas livres (ABTS) e poder antioxidante através do método ORAC. O teor em fenólicos totais foi determinado para os vários extratos fenólicos utilizado o método de Folin-Ciocalteau. O teor em fenólicos totais foi muito semelhante entre todas as amostras, indicando que os compostos fenólicos presentes na parte aérea de cenoura selvagem apresentam igual solubilidade em água e em metanol. Os resultados obtidos para os ensaios de ABTS e ORAC demonstram que os extratos fenólicos apresentam uma maior

atividade quando comparado com o óleo essencial, o que seria de esperar uma vez que os compostos fenólicos estão descritos como sendo mais antioxidantes que os terpenos e terpenóides.

Antes da determinação das propriedades anti-inflamatórias, a capacidade do óleo essencial para capturar NO• foi avaliada usando o SNAP como dador de NO•. O óleo essencial não teve qualquer efeito na captura de NO• libertado pela ação do SNAP. A atividade anti-inflamatória do óleo essencial foi testada através da inibição da libertação de NO em macrófagos estimulados com LPS. Os resultados demonstram que o óleo essencial reduziu a produção de NO em 19.04% na concentração de 0.64 µL/mL sem demonstrar citotoxicidade para as células.

De forma a avaliar a potencial aplicação do óleo essencial em humanos, o perfil de segurança foi determinado usando diferente linhas celulares (queratinócitos, células epiteliais alveolares, macrófagos e hepatócitos) que mimetizam as principais vias de administração assim como o local de metabolização no organismo (fígado). Os resultados demonstram que o óleo essencial é seguro em concentrações abaixo de 1.25 µL/mL.

O presente estudo contribuiu para um melhor conhecimento sobre as propriedades bioativas de *D. carota* subsp. *carota* , bem como permitiu evidenciar o potencial industrial desta planta.

Palavras-chave: *Daucus carota* subsp. *carota*, óleo essencial, extratos fenólicos, propriedades antifúngicas, propriedades antibacterianas, atividade antioxidante, efeito anti-inflamatório, citotoxicidade

List of abbreviations

AAPH - 2,2'-azobis(2-amidino-propane) dihydrochloride

ABTS-[NH$_4$]$_2$ - [2,2'-azinobis(3-ethylbenzothiazoline-6- sulfonic acid] diammonium salt

ABTS$^{.+}$ - 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid

ATCC - American Type Culture Collection

CBISA - Coleção de Bactérias do Instituto Superior de Agronomia

CECT - Colécion Espanola de Culturas Tipo

CFU - Colony Forming Units

CLSI - Clinical and Laboratory Standard Institute

COX - Cyclooxygenase

CV - Crystal Violet

DE - Dry Extract

DMEM - Dulbecco's Modified Eagle's Medium

DMSO - Dimethyl Sulfoxide

DPPH$^.$ - 2,2'-diphenyl-1-picrylhydrazyl

EO - Essential Oil

EPA - Environmental Protection Agency

FDA - Food and Drug Administration

FEMA - Flavour Essence Manufacturers Association

FW - Fresh Weight

GAE - Gallic Acid Equivalent

GRAS - Generally Regarded As Safe

H$_2$O - Aqueous Extract

IFRA - International Flavour and Fragrance Association

LPS - Lipopolysaccharide

MeOH 100% - 100% Methanol Extract

MeOH 70% - 70% Methanol Extract

MIC - Minimum Inhibitory Concentration

MLC - Minimum Lethal Concentration

MTT - 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide

NO - Nitric Oxide

NOAEL - No Adverse Effect Level

NOS - Nitric Oxide Synthase

NTP - National Toxicology Program

NYP - N-acetyl-D-glucosamine

ORAC - Oxygen Radical Antioxidant Capacity

PDA - Potato Dextrose Agar

RIFM - Research Institute for Fragrance Materials

ROS - Reactive Oxygen Species

SDA - Sabouraud Dextrose Agar

SNAP - S-nitroso-N-acetyl-DL-penicillamine

TE - Trolox Equivalents

TEAC - Trolox Equivalent Antioxidant Activity

TPC - Total Phenolic Content

XTT - 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carbox-anilide

YPD - Yeast Peptone Dextrose

II. Essential Oils

I. General Introduction and Objectives

II. Essential Oils

1. General Introduction

Medicinal plants play a critical role in primary health care as up to 80% of the population, in developing countries, depends on these plants (Newman *et al.*, 2000). About 25% of prescribed medicines in developed countries derive, directly or indirectly, from plants and, if over-the-counter market is accounted, this value may ascend to 50% (Newman *et al.*, 2000). An assessment carried out by Newman (Newman *et al.*, 2007) demonstrated that 51% of the new 983 chemical entities approved as drugs from 1981 to 2006 were natural products, natural product derivatives or natural product mimics. The great importance of natural products to drug development and drug discovery justify the investigation of natural products as novel active agents (Hostettmann *et al.*, 2003). Particularly, plants represent a huge reservoir of lead compounds as many species have still not been investigated for their pharmaceutical and pharmacological potential (Hostettmann *et al.*, 2003).

The last years have seen an increasing demand for nature-based products, being the impact of the aromatic and medicinal plant market most evident on the health and agricultural sectors (Isman, 2006; Khater, 2012; Kong *et al.*, 2003). The number of people that are willing to accept such products in detriment of conventional drugs are also increasing. This acceptance is justified by a long history of use, better patient tolerance, less side-effects along with less environmental concerns (Waqar *et al.*, 2006). This trend hasn't passed unnoticed by several industries that are developing bioactive products that are biodegradable and non-toxic to both humans and animals. This development has also encouraged the development of more precise and reliable analytical methods that allow the accurate characterization of plant metabolites (Phillipson, 2007). In addition, the identification of the most promising compounds has been made possible due to the development of screening assays which have been adapted to several target organisms, including but not limited to, microorganisms (bacteria, fungi, viruses) and invertebrates (insects, crustaceous, molluscs) (Hostettmann *et al.*, 2003).

Recently, investigation on aromatic plants, particularly essential oils, has attracted several researchers (Bakkali *et al.*, 2008). These compounds have been used by local communities as they have several medicinal properties, namely bactericidal, virucidal, fungicidal, anti-parasitic and insecticidal (Bakkali *et al.*, 2008). Nevertheless, these uses are mainly based on old beliefs and faith, lacking a scientific approach to support their efficacy. *In vitro* screening programs, based on ethnobotanical approaches, have shown to be a very efficient methodology for the validation of traditional uses and the search of new active compounds (Alviano *et al.*, 2009; Zuzarte, 2012). Currently, many aromatic plants and their

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essential oils are highly valued in the pharmaceutical, agronomic, food, sanitary, cosmetic and perfume industries (Bakkali *et al.*, 2008; Zuzarte, 2012).

The Apiaceae (Umbelliferae) family comprises several aromatic plants with economic interest. Particularly, the genus *Daucus* L. includes several plants whose essential oils are greatly prized in the food and fragrance industries. Also, potential medicinal properties can be envisaged due to its use in traditional medicine to treat several ailments (Guinoiseau *et al.*, 2010). This genus includes several species that are split into 5 sections: sect. *Daucus,* sect. *Platyspermum,* sect. *Anisactis,* sect. *Chrysodaucus* and sect. *Meoides.* Sáenz Laín in 1981 described 20 species of *Daucus*, later this number ascended to 25 in a study published by Rubatzky and colleagues (Rubatzky *et al.*, 1999). Geographically, the plants from this genus can be found from the Mediterranean and North African regions across North America and Australia (Sáenz Laín, 1981). The only cultivated species of carrot is the subspecies *Daucus carota* subsp. *sativus* that resulted from a domestication process of populations of wild carrots in Central Asia (Arbizu *et al.*, 2014; Vivek *et al.*, 1998) while the subspecies *Daucus carota* L. subsp. *carota* L. is the best-known wild species of this genus (Arbizu *et al.*, 2014).

Wild carrots have been widely used in folk medicine as a therapeutic agents against several ailments (Guinoiseau *et al.*, 2010). Namely, seeds from *Daucus carota* are described as aromatic, carminative, diuretic, aphrodisiac and stimulant (Chizzola, 2010; Guinoiseau *et al.*, 2010). They are used in the treatment of dropsy, chronic dysentery, stomach disorders, hepatic injury, kidney dysfunction and worm troubles. Some authors have also described that wild carrot seeds show anti-fertility and anti-steroidogenic effects and due to this are consumed by some Indian tribes as birth control. Their effect on swelling and tumours is also described (Guinoiseau *et al.*, 2010).

In addition to the medicinal effects of seeds, the roots from *Daucus carota* are also described as having beneficial effects on several disorders, primarily due to their diuretic and ophthalmic properties (Guinoiseau *et al.*, 2010). Moreover, poultices containing roots of wild carrots are used against mammary and uterine carcinoma and skin cancer; scraped roots are known to be stimulant for indolent ulcers and grated or mashed raw roots can be safely used against threadworms, especially on children (Guinoiseau *et al.*, 2010).

Besides using plant parts *per se*, traditional medicine also employs volatile (essential oils) and non volatile extracts from *Daucus carota* (Guinoiseau *et al.*, 2010). These are used for the treatment of renal and hepatic insufficiency as well as for skin disorders (Guinoiseau *et al.*,

2010; Maxia *et al.*, 2009). Particularly, ethanolic extracts of defatted seeds are found to improve cognitive functions in mice (Chizzola, 2010).

In turn, *Daucus carota* essential oils, demonstrate a wide range of biological properties, such as antibacterial, fungicidal, hepatocellular regenerator, general tonic and stimulant. It is also used to decrease cholesterol levels and as a cicatrising agent (Guinoiseau *et al.*, 2010).

2. Objectives

Considering that *Daucus carota* is extensively used in traditional medicine as a therapeutic agent and that several studies lack subspecies identification, which is important considering the high intraspecific polymorphism in *D. carota*, the work conducted in this thesis aims to contribute to the clarification of the bioactive properties of both the essential oil and the phenolic-enriched extracts of wild carrot, D. *carota* subsp. *carota*, as follows:

➢ Evaluation of antioxidant properties using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS$^{.+}$) scavenging, oxygen radical antioxidant capacity (ORAC) and nitric oxide scavenging assays.

➢ Evaluation of antimicrobial properties against several pathogenic microorganisms, namely yeasts (*Candida albicans, C. krusei, C. tropicalis, C. guillermondi, C. parapsilosis* and *Cryptococcus neoformans*), dermatophyte strains (*Trichophyton mentagrophytes, T. mentagrophytes* var. *interdigitale, T. verrucosum, T. rubrum, Microsporum canis, M. gypseum, Epidermophyton floccosum*), *Aspergillus* strains *(A. niger, A. flavus, A. fumigatus*) as well as several bacterial strains (*Listeria monocytogenes, Salmonella typhimurium, Escherichia coli, Staphylococcus aureus,* and *Bacillus subtilis*). In addition, the ability of the most active extract to inhibit the germ tube formation of *C. albicans* and disrupt its preformed biofilms was assessed.

➢ Assessment of the anti-inflammatory properties of the most active extract on lipopolysaccharide-stimulated RAW 264.7 (macrophages) through quantification of the inhibition of nitric oxide production (Griess assay).

➢ Evaluation of the safety profile of the most active extract against several cell lines (macrophages, keratinocytes, alveolar epithelial cells and hepatocytes). This is a crucial step for a further pharmaceutical or cosmetic application.

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1. Introduction

The term "essential oil" was thought to be first used by Paracelsus von Hohenhein that named the active compound of a drug as "Quinta Essentia" in the 16th century (Perricone *et al.*, 2015; Russo *et al.*, 2015; Vergis *et al.*, 2013). Turpentine oil was mentioned as long as the Greek and Roman cultures. Also, the distillation process was described by Egyptian, Indian and Persian cultures more than 2000 years ago with the first authenticated writing about essential oil distillation being accredited to Villanova (1235-1311), a Catalan physician (Vergis *et al.*, 2013). More recently, the European Pharmacopeia 7.0 (Council of Europe, 2010) defined essential oil as a "Odorous product, usually of complex composition, obtained from a botanically defined plant raw material, by steam distillation, dry distillation, or a suitable mechanical method without heating. Essential oils are usually separated from the aqueous phase by a physical process that does not significantly affect their composition". This mixture of compounds can also be called of volatile or ethereal oil (Russo *et al.*, 2015; Vergis *et al.*, 2013). This definition excludes other aromatic/volatile extracts obtained using other extractive techniques like, solvent, supercritical fluid or microwave assisted extraction. (Council of Europe, 2010; ISO TC 54 - ISO 9235, 1997; Zuzarte *et al.*, 2015). In addition essential oils are different in terms of both chemical and physical properties when compared to fixed or fatty oils (Zuzarte *et al.*, 2015)(Zuzarte and Salgueiro, 2015). Hydrodistillation is the most commonly used methodology to obtain essential oils in a laboratorial scale. The European Pharmacopeia recommends the usage of a Clevenger apparatus for the hydrodistillation of essential oils as it allows the recycling of condensates leading to a greater yield (Asbahani *et al.*, 2015).

Essential oils can be obtained from different parts of aromatic plants and are a result of their secondary metabolism (Asbahani *et al.*, 2015; Bayala *et al.*, 2014; Russo *et al.*, 2015; Sá *et al.*, 2014; Tongnuanchan *et al.*, 2014). They are highly volatile and hydrophobic liquids, also lipophilic, soluble in organic solvents and immiscible in water (Asbahani et al., 2015; Russo et al., 2015).

Essential oils are biosynthesised in secretory structures present in several plant parts such as flowers (e.g. *Jasminum*, *Rosa*, and *Lavandula* species), flowering aerial parts (e.g. *Thymus*, *Lavandula*, *Daucus* species), buds (e.g. *Syzygium* spp.), leaves (e.g. *Eucalyptus* spp.), fruits (e.g. *Pimpinella anisum*), twigs (e.g. *Pistacia lentiscus* L.), bark (e.g. *Cinnamomum* spp.), zest/epicarp (e.g. *Citrus* spp.), seeds (e.g. *Elettaria* and *Amomum* species), wood (e.g. *Santalum* spp.), rhizome and roots (e.g. *Zingiber* spp.) (Asbahani *et al.*, 2015; Bayala *et al.*, 2014; Mann *et al.*, 2014; Perricone *et al.*, 2015; Tongnuanchan *et al.*, 2014). Yields of essential oils varies greatly with

species but also with the plant part used for oil extraction. However, the value remains usually low (about 1%) making essential oils a prized and rare substance (Asbahani et al., 2015). Aromatic species account for 10% of all known plants (Asbahani et al., 2015) and mainly belong to the following botanic families: Lamiaceae, Apiaceae, Lauraceae, Asteraceae, Rutaceae, Myrtaceae, Poaceae, Cupressaceae and Piperaceae (Asbahani et al., 2015; Zuzarte, 2012). Although they can be found worldwide most aromatic plants grow on temperate and warm climates, such as Mediterranean or tropical countries (Bayala et al., 2014; Perricone et al., 2015). Most of the aromatic species are included in the GRAS (Generally Regarded As Safe) list approved by the FDA (Food and Drug Administration) and EPA (Environmental Protection Agency) (Zuzarte, 2012).

Essential oils are synthesised and stored in specialized histological structures known as secretory structures that can be divided into two types: external or internal, depending on the location in the plant. External secretory structures can be found on the surface of plant organs whereas internal secretory structures are present inside the plant tissues. (Asbahani et al., 2015; Svoboda et al., 2000). The external structures include osmophores, glandular trichomes andepidermal cells while the internal structures are secretory cells (often idioblasts), secretory cavities and secretory ducts.

Regarding the external secretory structures, osmophores are secretory cells that are found in flower tissue and are structurally different than the envolving cells. Glandular trichomes (modified epidermal hairs) are found covering leaves, stems and some flower parts (e.g .calyx). Considering the most used terminology, these structures are divided into peltate and capitate trichomes and are characteristic of the Lamiaceae family (Svoboda et al., 2000; Zuzarte, 2012). Epidermal cells are conical epidermal cells that secrete volatile compounds and are generally found on flowers (e.g. *Rosa* spp.) and the yields in essential oil is normally very low.

Regarding internal structures: secretory ducts are canals that sometimes encompass the entire length of the plant and are delimited by secreting cells. They are found in all the members of the Apiaceae family and in some members of Coniferae and Pinaceae (Svoboda et al., 2000). Secretory cells are specialised cells that produce essential oil and accumulate it in the vacuoles. They can be found on cinnamon and laurel, amongst others. The secretory cavities are common on plants from the genera *Citrus* and *Eucalyptus* (Romane et al., 2005; Svoboda et al., 2000). These cavities are intercellular spaces often spherical in form and which are filled with essential oil that is secreted by the adjacent cells (Asbahani et al., 2015; Svoboda et al., 2000).

II. Essential Oils

Essential oils are a complex mixture of volatile compounds with low molecular weight and highly variable concentrations (Asbahani *et al.*, 2015; Perricone *et al.*, 2015; Russo *et al.*, 2015). Based on the chemical structure, the major compounds found in essential oils are generally terpenes either hydrocarbons and/or oxygenated compounds (Tongnuanchan *et al.*, 2014). The former are made of several isoprene units (C_5 units). The most common terpenes found on essential oils are monoterpenes (C_{10}, e.g. limonene, α-phellandrene) and sesquiterpenes (C_{15}, e.g. humulene, germacrene). Diterpenes (C_{20}) can be present in essential oils, but usually in very low concentrations (Tongnuanchan *et al.*, 2014). Oxygenated compounds are oxygen-containing compounds that are designated as "terpenoids" when are terpene-derived although other families can be present (Asbahani *et al.*, 2015; Bayala *et al.*, 2014; Russo *et al.*, 2015; Tongnuanchan *et al.*, 2014). The main families of oxygen-containing compounds that can be found in essential oils are: phenols (e.g. thymol, carvacrol), mono- (e.g. borneol, α-terpineol) and sesquiterpene (e.g. elemol, α-santalol) alcohols, aldehydes (e.g. citral, citronellal), ketones (e.g. menthone, camphor), esters (e.g. geranyl acetate, linalyl acetate), oxides (e.g. 1,8-cineole, linalool oxide), and lactones (e.g. psoralen, bergaptene) (Tongnuanchan *et al.*, 2014). In addition to the aforementioned compounds, some essential oils contain a special class of compounds known as phenylpropanoids (e.g. eugenol, cinnamaldehyde). These compounds are primarily found in *Sassafras* spp., *Chrysopogon zizanioides*, *Syzygium aromaticum*, *Cinnamomum verum* and other *Cinnamomum* species (Asbahani *et al.*, 2015; Vergis *et al.*, 2013).

Terpenes are formed by coupling of isoprene units (Wedler *et al.*, 2015). They are often polycyclic and result from rearrangements of acyclic precursors by terpene synthase/cyclase enzymes (Wedler *et al.*, 2015). There are two pathways that are responsible for the biosynthesis of terpenes, the mevalonate (MVA) and 2-*C*-methyl-*D*-erythritol 4-phosphate/1-deoxy-*D*-xylulose 5-phosphate (MEP/DXP) pathways (Figure 1) (Ikram *et al.*, 2015; Wedler *et al.*, 2015). Both pathways lead to the formation of isopentenyl pyrophosphate, which is the building block of these compounds (Ikram *et al.*, 2015). The MEP/DXP pathway, also known as non-mevalonate pathway, which occurs in plastids (e.g. chloroplasts) is mainly responsible for the production of mono- and sesquiterpenes as well as diterpenes (Zuzarte and Salgueiro, 2015). This pathway starts with the condensation of glyceraldehyde-3-phosphate with pyruvate which, after several enzymatic conversions, yield isopentenyl diphosphate (IDP) and dimethylallyl diphosphate (DMADP) (Ikram *et al.*, 2015; Song *et al.*, 2014; Wedler *et al.*, 2015). Conversely, the MVA pathway takes place in the cytosol and yields mainly sesquiterpenes. This synthetic pathways produces IDP by converting Acetyl-CoA into mevalonate which is further metabolized into mevalonate-diphosphate which is processed by

mevalonate diphosphate decarboxylase yielding IDP (Ikram *et al.*, 2015; Song *et al.*, 2014; Wedler *et al.*, 2015). IDP and DMADP can be interconverted into each other. DMADP is condensed with IDP to yield geranyl diphosphate (GDP). The latter can be converted in farnesyl diphosphate (FDP) at the expense of another IDP molecule. To FDP is added another IDP molecule yielding geranylgeranyl diphosphate (GGDP). GGDP can be formed directly from DMADP using 3 IPP molecules by the action of a multifunctional geranylgeranyl diphosphate synthase (Ikram *et al.*, 2015; Song *et al.*, 2014; Wedler *et al.*, 2015). GDP, FDP and GGDP are then metabolized by terpene synthases yielding mono-, sesqui- and diterpenes, respectively (Ajikumar *et al.*, 2008).
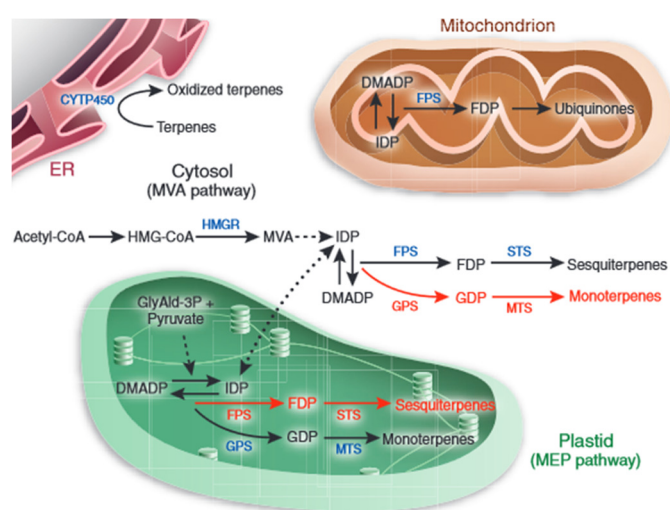
Figure 1 - Terpenoid biosynthesis in plants. Solid and dashed arrow represent single and multiple enzymatic steps, respectively. DMADP - dimethylallyl diphosphate; FDP - farnesyl diphosphate; GDP - geranyl diphosphate; GlyAld-3P - glyceraldehyde -3-phosphate; HMG-CoA – hydroxymethylglutaryl-CoA; IDP – isopentenyl diphosphate; MVA – mevalonic acid; MEP – methyl erythritol phosphate; CYTP450 – cytochrome P450 hydroxylase; FPS – FDP synthase; GPS – GDP synthase; HGMR – HMG-CoA reductase; MTS – monoterpene synthase; STS – sesquiterpene synthase. *(In: Bouwmeester, 2006)*

Phenylpropanoids, which are common compounds in some plant species namely cinnamon and clove are derived from the phenylpropanoids pathway. The shikimate pathway is the entry to this biosynthetic pathway (Vogt, 2010). After enzymatic activity shikimate originates phenylalanine, which further undergoes modifications during the general phenylpropanoid pathway. These modifications lead to the formation of *p*-coumaroyl CoA after the action of phenylalanine ammonia lyase, cinnamate 4-hydroxylase and *p*-coumaroyl CoA ligase. *p*-coumaroyl CoA is then submitted to several enzymatic and structural modifications to form several secondary metabolites, e.g. cinnamaldehyde and eugenol (Liu *et al.*, 2015; Tohge *et al.*, 2013; Vogt, 2010).

III. Phenolic-enriched extracts

III. Phenolic-enriched extracts

I. Introduction

Phenolic-enriched extracts are solutions that are rich in polyphenols, i.e. a group of secondary metabolism-derived compounds. Although they do not participate in growth and energy metabolism, polyphenols are also crucial to plant survival, as they are produced as a response to environmental, ecological or physiological stresses, e.g. UV radiation and wounding (Garcia-Salas *et al.*, 2010; Harnly *et al.*, 2007; Khoddami *et al.*, 2013). In addition, phenolic compounds play a role in the production of root nodules and in the plant immune system's response (Harnly *et al.*, 2007).

Phenolic compounds are derived from widespread precursors (AcetylCoA, shikimate and amino acids) (Harnly *et al.*, 2007). Structurally speaking, these compounds have at least one aromatic ring and one or more hydroxyl moieties that are rearranged in different forms, giving origin to the main phenolic compounds family (Garcia-Salas *et al.*, 2010; Harnly *et al.*, 2007; Khoddami *et al.*, 2013). These families are simple phenols and benzoquinones (C_6), phenolic acids (C_6-C_1), acetophenones, phenylacetic acid (C_6-C_2), cinnamic acids, phenylpropene, coumarins, chromones (C_6-C_3), naphthoquinones (C_6-C_4), xanthones (C_6-C_1-C_6), stilbenes, anthraquinones (C_6-C_2-C_6), flavonoids (C_6-C_3-C_6), lignans, neolignans ((C_6-C_2)$_2$), tannins ((C_6-C_1)$_n$) and lignins ((C_6-C_3)$_n$) (Garcia-Salas *et al.*, 2010; Harnly *et al.*, 2007).

Although there are several families, the most predominant in plants are the phenolic acids/cinnamic acids and flavonoids (general structures in Figure 2), with mean abundance of 30% and 60%, respectively (Garcia-Salas *et al.*, 2010). The former can be found as aglycones (free form), esters, glycosides and/or bound complexes. These structural differences make the phenolic and cinnamic acids more suitable for certain extraction methods and vary their susceptibility to degradation (Garcia-Salas *et al.*, 2010). In turn, the basic structure of flavonoids comprises two aromatic rings linked by three carbons, which normally form an oxygenated heterocycle and are derived from the aromatic amino acids, phenylalanine and tyrosine (Garcia-Salas *et al.*, 2010; Khoddami *et al.*, 2013). Like phenolic and cinammic acids, flavonoids can be found as aglycones, however they are normally found as glycosidic derivatives which can be responsible for the colours (blue, scarlet and orange) of leaves, flowers and fruits (Garcia-Salas *et al.*, 2010). Flavonoids can still be divided into 13 structurally different classes: chalcones, dihydrochalcones, auron, flavones, flavonols, dihydroflavonols, flavanones, flavanols (catechins), flavandioles (leucoanthocyanidins), anthocyanidins (glycosides: anthocyanins), isoflavanones, condensed tannins (proanthocyanidins) and flavonoids (Garcia-Salas *et al.*, 2010).

II. Phenolic-enriched extracts

These structural differences depend on the scale and pattern of hydroxylation, prenylation, alkalinisation and glycosylation reactions that flavonoids undergo (Khoddami *et al.*, 2013).
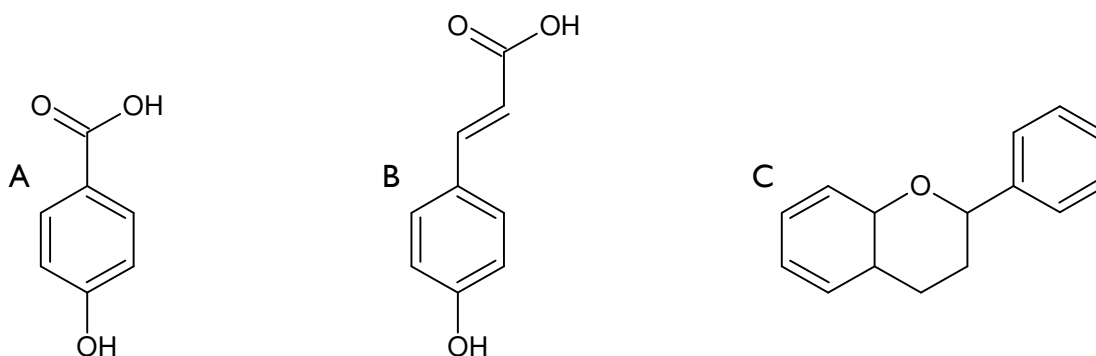
Figure 2 - General structure of main phenolic compounds found in plants: A - hydroxybenzoic acid, B - hydoxycinnamic acid, C - flavonoid

It is thought that there are more than 8000 phenolic compounds in the plant kingdom which are divided into the groups aforementioned (Garcia-Salas *et al.*, 2010). Of those, more than 450 are flavonols, 400 flavones, 350 flavanones, 300 isoflavones, 19 anthocyanidins and 350 chalcones. The glycosidic derivatives of flavonoids are accounted as more than 5000 different compounds (Harnly *et al.*, 2007).

The extraction of phenolic compounds is not so straightforward when compared to essential oils because they can be found in a panoply of conditions which affect their solubility in the solvent used during the extraction. Thus, no extraction process is able to extract all the phenolic compounds in the plant matrix (Garcia-Salas *et al.*, 2010).

The extraction of phenolic compounds from solid matrixes is normally achieved by using Soxhlet, heated reflux extraction or maceration (Garcia-Salas *et al.*, 2010; Khoddami *et al.*, 2013). The two first methods are commonly performed at high temperatures (around 90°C) during several hours while maceration can be as long as a few days. Nevertheless all these extraction methods are still widely used as they are quite simple, require cheap apparatus and have adequate phenolic extraction rates (Khoddami *et al.*, 2013).

In turn, the extraction of phenolic constituents from liquid samples is normally achieved by the use of liquid-liquid extraction. However, this method requires expensive and dangerous solvents (e.g. propanol, dichlorometane and other organic solvents) (Net *et al.*, 2015; Saraji *et al.*, 2014) and a long extraction time which can trigger the degradation of compounds, mainly because of light, air and temperature. In order to avoid these effects on the phenolic compounds, new extraction methodologies have been proposed, such as solid-phase

III. Phenolic-enriched extracts

extraction, pressurized liquid extraction, microwave-assisted extraction, ultrasound-assisted extraction and supercritical fluid extraction. In addition microextraction techniques have also been developed, such as solid-phase microextraction, single-drop microextraction, dispersive liquid-phase microextraction, amongst several others (Garcia-Salas *et al.*, 2010; Leong *et al.*, 2014; Net *et al.*, 2015; Saraji *et al.*, 2014).

IV. Morphological and chemical characterization of *Daucus* L.

IV. Morphological and chemical characterization of *Daucus* L.

1. Taxonomy

The genus *Daucus* was first described by Carl Linnaeus in his works regarding the taxonomy of the plant kingdom, the *Species Plantarum, exhibentes plantas rite cognitas, ad genera relatas, cum differentiis specificis, nominibus trivialibus, synonymis selectis, locis natalibus, secundum systema sexuale digestas*, first published in 1753 (Linnaeus, [s.d.]); the *Genera plantarum: eorumque characteres naturales secundum numerum, figuram, situm, et proportionem omnium fructificationis partium*, first published in 1737 (Linnaeus, [s.d.]) and *Systema Naturae,* first published in 1735 (Linnaeus, [s.d.]).

The genus *Daucus* belongs to the Umbelliferae (Apiaceae) family and, although widely distributed and cultivated in temperate regions of the world, its dispersion is mainly centred in the Mediterranean region, more specifically in North Africa (Sáenz Laín, 1981). Nevertheless, some species from Australia and from North America have also been described (Sáenz Laín, 1981).

Daucus L., Sp. Pl. 242 (1753)

Annual, biennial or perennial herb. Roots from taproot to napiform. Erect stem, ascendant or decumbent, glabrous, pubescent, velutinus, scabrid, hispid, more or less branched from the base or in the upper half. Basal leaves (1)2-4(5) pinnatisect, superior ones smaller. Composite umbels, occasionally with a sterile central flower, it can get to 130 rays, even or uneven, frequently strongly arched in the fructification. Numerous bracts, pinnatisect, sometime pinnatifid, rarely undivided, occasionally similar to leaves, with scabrous in the base. Bracteoles present, undivided, bifid or trifid. Calyx inconspicuous serrated, triangular. White petals, most of the time purple toned, or yellow, curved apex, external side of exterior flowers are frequently larger than the internal. Ringed stylopodium, discoidal or conical in the anthesis – conical or cylindrical in the fructification; styles more or less straight, parallel or divergent. Ellipsoidal fruits, oblong, ovoid or subspherical, somewhat compressed dorsally, rarely not compressed; mericarps with 5 primary ridges little evident and hairy, and 4 secondary ridges with a spines generally glochidial. Seeds with flat or slightly concave endosperm on the commissural face (Salvá, 2003).

The genus *Daucus* includes 5 sections, each one with their unique characteristics (Sáenz Laín, 1981; Salvá, 2003). The sect. *Daucus*, is the largest of the five and encompasses the following species: *D. carota* L., *D. gracilis* Steinh., *D. guttatus* Sm., *D. involucratus* Sm., *D.*

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jordanicus Post, *D. saharensis* Murb., *D. syrticus* Murb., *D. capillifolius* Gilli, *D. crinitus* Desf., *D. tenuisectus* Coss., *D. montevidensis* Link ex Sprengel and *D. pusillus* Michx. Amongst them, the species found in the Iberian Peninsula in accordance to Flora Iberica X are *D. carota*, *D. arcanus, D. aureus, D. crinitus, D. durieua, D. muricatus* and *D. setifolius* (Salvá, 2003). Of those spontaneous plants, the work conducted here will focus on *Daucus carota*, more specifically on the wild carrot subspecies, *D. carota* subsp. *carota*.

1.1. Daucus carota L.

Daucus carota L. is also known as wild carrot, "bird's nest", "devil's plague" and "Queen Anne's lace" (Guinoiseau *et al.*, 2010; Rokbeni *et al.*, 2013) and in Portuguese as salsa-burra, cenoura-brava or erva-coentrinha, amongst other common names (Salvá, 2003), This species has its endemic centre in the Mediterranean region however it can also be found growing wild on temperate regions of Europe, North America, Siberia, Northern and Southern India, North Africa and Southeast Asia (Guinoiseau *et al.*, 2010; Rokbeni *et al.*, 2013). The plant is a tall and robust herb with spiny fruits that can be found on dried-out fields and meadows (Chizzola, 2010; Guinoiseau *et al.*, 2010). The distribution of wild carrot in Portugal is represented in Figure 3.
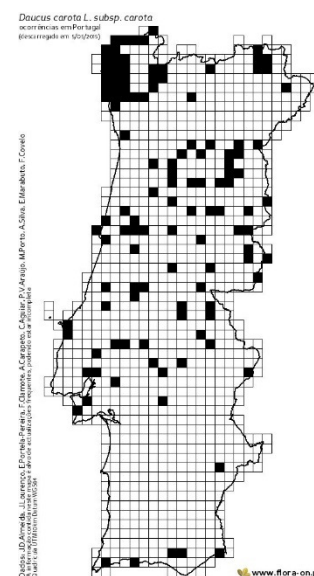
Figure 3 - Distribution map of *Daucus carota* L. subsp. *carota* in Portugal (Almeida et al., 2015)

D. carota L., Sp. Pl. 242 (1753)

Annual or biennal plant, frequently monocarpic, (3)10-170(220) cm, erect, ascendant or occasionally decumbent, branched or not since its base. Glabrous, scabrous, hispid or densely velutinus stem – reflexed or patent hair. Basal leaves 1-4(5) pinnatisects, with last order division, obovate, oblong or lanceolate to linear, mucronate, frequently peciolated and limbus from hirt to hispid, rarely glabrouscent or glabours; the upper 1-3 pinnatisect, similar to the basal, smaller in size, rarely absent or very scarse, often with last order division from linear to lanceolated, glabrous or glabrouscent, sometimes with indumentum similar to basal leaves. Umbels greatly pedunculated, rarely subsessile, 1.5-23 cm in diameter, slightly concave, flat, convex, hemispheric or globular, from strongly contracted to not contracted in the fructification, with 9-130 rays, the external of 8-110

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mm, uneven, from hirsute to scabrous, rarely glabrous or subglabrous. Bracts 5-14, with length equal or less than those of rays, apprised or reflexed, sometimes patent in the anthesis and in the fructification, pinnatisect, sometimes pinnatifid, rarely undivised, with filiform lobes, linear or lanceolated, occasionally ovoid, glabrous or glabrouscent, sometimes hirsute, with scarious and ciliated margins –rarely glabrous. Bracteoles 5-10, with length equal to that of the flowers, undivided – from linear to amply lanceolated – or occasionally 2-3-fid, glabrous, subglabrous, pubscent, scabrous or hirt, with scarious and ciliated margins – sometimes glabrous. Central sterile and purple flower present or absent. Sepals dented 0.1-0.2 mm, triangular, whitish or pale green. Petals 0.5-3.5 mm, external of exterior flowers larger than the internal – sometimes scarcely-, from cordate to ovoid, emarginated, frequently with very uneven lobules, acuminate, curved, white, rarely yellowish white, occasionally purple-, white- or dry yellow-toned. Anther yellow, occasionally white or purple. Ringed stylopodium – more or less conical in the fructification; styles 0.2-0.5 mm – 0.5-1.3 mm, parallel or scarcely divergent in the fructification. Fruits 1.5-4 mm, ovoid, oblong, elliptical or suborbicular, brown or purplish; mericarps with primary ridges with (1)2-3 rows of simple hair, white; the secondary with spine larger or smaller than the width of the mericarps, thin or thick, dilated or not, and confluent in a crest or not in the base, white, occasionally purple, with starred glochids or glochids 1-2(3) bristled.

In the Volume X of the Flora Iberica (Salvá, 2003), eleven subspecies of *D. carota* were considered and described, being five of them found in Portugal Nevertheless, the following sections will only consider subsp. *carota* since it is the species studied in this work.

Key to the subspecies *carota*: (Adapted from Flora Iberica X (Salvá, 2003))

1. Bracts with last order sublinear division or filiform, umbels, in the fructification, with rays markedly arched-convergent (strongly contracted), rarely with ray little arched-convergent; mericarp secondary ridges with thin spines, scarcely dilated and only confluent in one crest at the base

    2.

    Bracts with last order ovoid divisions, lanceolate, or linear-lanceolate (wide), rarely sublinear; umbels, in the fructification, with rays little arched-convergent or more or less straight (little contracted); secondary ridges in the mericarp with

thick spines, dilated and confluent in a crest at the base or thin, scarcely dilated and only confluent in a crest at a base   2.

2. Root non napiform, fibrous, whitish, wild plants   3.

3. Plant (4)25-110 cm; basal leaves 1-3(4) pinnatisect, umbel with (1.5)3-7(11)cm in diameter in the anthesis; fruits 1.8-3.2 mm   subsp. *carota*

Wild carrot is a variable biennial plant that reproduces solely by seeds (Figure 4). The flowering periods ranges from June to August while the seed maturation phase occurs from August to September. During the first year of growth the formation of a rosette of lobed, deeply dissected leaves can be observed, conversely the leaves on the flowering stems, which are formed during the second year of growth, are oblong and alternate. (Guinoiseau *et al.*, 2010). The flowering umbel of *D. carota* subsp. *carota* is represented in Figure 5 where it can be seen the characteristic dark flower in the middle.

Figure 5 - Seeds from *Daucus carota* subsp. *carota*. Found at http://www.flora-on.pt/#/haH2l

Figure 4 - *Daucus carota* subsp. *carota* umbel, in middle it can be seen the central darkish flower. Found at http://www.flora-on.pt/#/hC8go

2.   Nutritional and phytochemical components of carrot root

Carrot roots have a moisture/water content that varies from 86 to 90% (Guinoiseau *et al.*, 2010; Sharma *et al.*, 2012). This vegetable is a good source of minerals, protein and carbohydrates as well as vitamins and other phytonutrients (Sharma *et al.*, 2012). The content on carbohydrates ranges from 5 to 10%, with reducing sugars summing up 1.67 to 3.35% and non-reducing 1.02 to 1.18% (Sharma *et al.*, 2012). The most common

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sugars on carrots are sucrose, fructose, glucose and xylose (Guinoiseau *et al.*, 2010; Sharma *et al.*, 2012). Carrots are also rich in dietary fibres, with mean values of insoluble fibres ranging from 1.2 to 2.4%, from which 71.7%, 13% and 15.2% consists of cellulose, hemicellulose and lignin, respectively. In addition, carrots are also a source of soluble fibres, such as pectins (Sharma *et al.*, 2012) and a primary source of pro-vitamin A yielding 14-17% of total vitamin A consumption (Alasalvar *et al.*, 2001; Nicolle *et al.*, 2004). Carotenoid concentration in carrots varies from 5.33 to 54.8 mg/100g (Sharma *et al.*, 2012). Six types of carotenoids have been described, α-, β-, γ- and ζ-carotene, lycopene and β-zeacarotene (Alasalvar *et al.*, 2001; Nicolle *et al.*, 2004), being α- and β-carotene (structure shown in figure 6) the most predominant and most biologically relevant, which activity resembles 50% and 100% of that of pro-vitamin A, respectively (Alasalvar *et al.*, 2001; Sharma *et al.*, 2012).
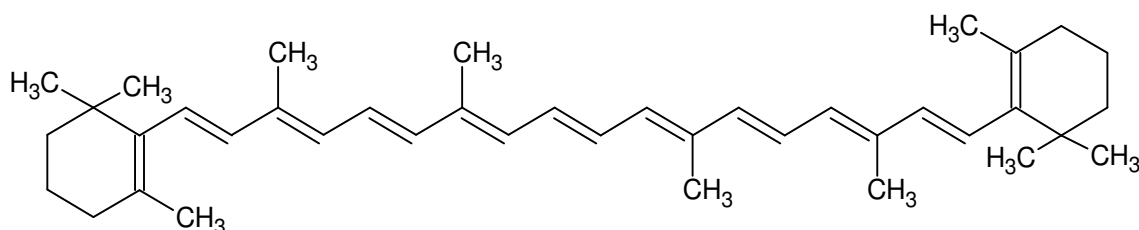
Figure 6 - β-carotene structure

Among phenolic compounds, carrot roots are mostly rich in anthocyanins. The content of these phenolic compounds is largely variable showing only traces on pink carrots and 16-71 mg/100g in orange carrots, 179 mg/100g in black carrots and 100-243 mg/100g in purple carrots (Leja *et al.*, 2013; Sharma *et al.*, 2012). The most common anthocyanins found in carrots are cyanidin-3-(2-xylosylgalactoside) (Figure 7A1), cyanidin-3-xylosylglucosylgalactoside (Figure 7A2) and cyanidin-3-ferulylxyloglucosylgalactoside (Figure 7A3) (Sharma *et al.*, 2012). Moreover, minor antocyanins have also been described, namely peonidin (Figure 7A4) and pelargonidin (Figure 7A5) glycosides (Kammerer *et al.*, 2003, 2004)

Regarding other phenolic compounds in carrot roots, it has been shown that they are mostly rich in hydroxycinnamic acids esterified with quinic acid (Kammerer *et al.*, 2004) and are free of flavonols, although apigenin (Figure 7B1), kaempferol (Figure 7B2)

and luteolin (Figure 7B3) glycosides derivatives can also be found (Carlos, 2014). Chlorogenic acid, an caffeyolquinic acid isomer, accounts from 42.2% to 61.8% of total phenolic compounds in carrots (Carlos, 2014). Specifically, orange, purple, yellow and white carrots collected from United Kingdom are rich in caffeyolquinic acid (Figure 7C1) isomers, *p*-coumaroylquinic acid (Figure 7C2) isomers and feruloylquinic acid (Figure 7C3) isomers as well as the aglycone of caffeic acid (Alasalvar *et al.*, 2001). These phenolic acids have also been described in black carrot (*Daucus carota* subsp. *sativus* var. *atrorubens* Alef.). In addition, carrots are also a source of polyacetylenes, namely falcarinol and falcarindiol (Carlos, 2014). Moreover, coumarins and oxygenated acyclic diacethylene derivatives have been isolated from *Daucus carota* subps. *carota* roots (Guinoiseau *et al.*, 2010).

A1 – R_1: 2-xylosylgalactoside, R_2: OH
A2 – R_1: xylosylglucosylgalactoside, R_2: OH
A3 – R_1: ferulylxyloglucosylgalactoside, R_2: OH
A4 – R_1: H, R_2: OCH$_3$
A5 – R_1: H, R_2: OH

C1 – R_1: caffeic acid
C2 – R_1: *p*-coumaric acid
C3 – R_1: ferulic acid

B1 – R_1=R_2: H
B2 – R_1: OH, R_2: H
B3 – R_1: H, R_2: OH
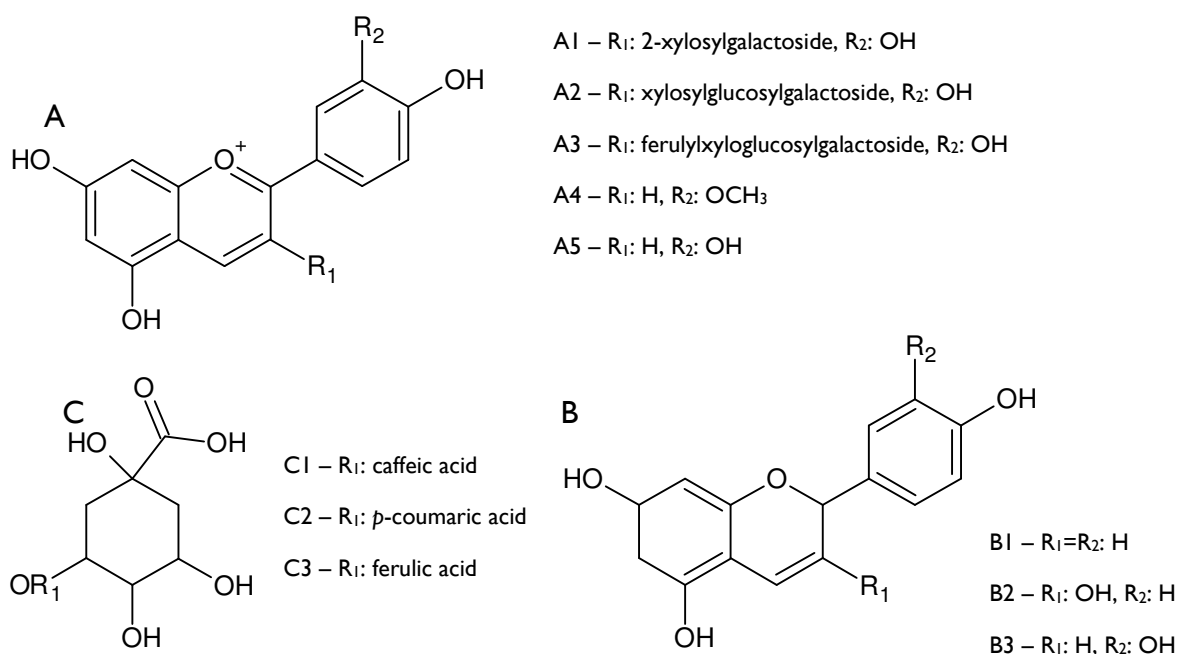
Figure 7 - Major phenolic compounds found in carrot: A - anthocyanins, B - quinic acid derivatives, C - flavones

3. *Daucus carota* essential oil

Daucus carota essential oil can be obtained from different plant parts, such as seeds, aerial parts or roots (Guinoiseau *et al.*, 2010). It is however known that the essential oil yield decreases when moving from seeds to aerial parts (umbels and leaves) and to roots (Guinoiseau *et al.*, 2010). Previous studies have described that seed essential oil yield is

IV. Morphological and chemical characterization of *Daucus* L.

highly variable with values ranging from 0.05% to 7.15% while oil isolated from roots of *Daucus* species have a very low yield (0.004%) (Guinoiseau et al., 2010).

Moreover, the chemicalcomposition of carrot seed essential oil is greatly variable. The most common families of secondary metabolites found are terpenes, both mono and sesquiterpenes, as well as phenylpropanoids (Guinoiseau *et al.*, 2010). Five main compositions can be described. Three are characterized by the existence of a terpenic main component, namely carotol, geranyl acetate or sabinene (Figure 8), while the fourth family is described as having these three components in an equal ratio (Guinoiseau *et al.*, 2010) and the fifth family is characterized by having the phenylpropanoid *(E)*-methylisoeugenol (Figure 8) as a main component (Guinoiseau *et al.*, 2010).
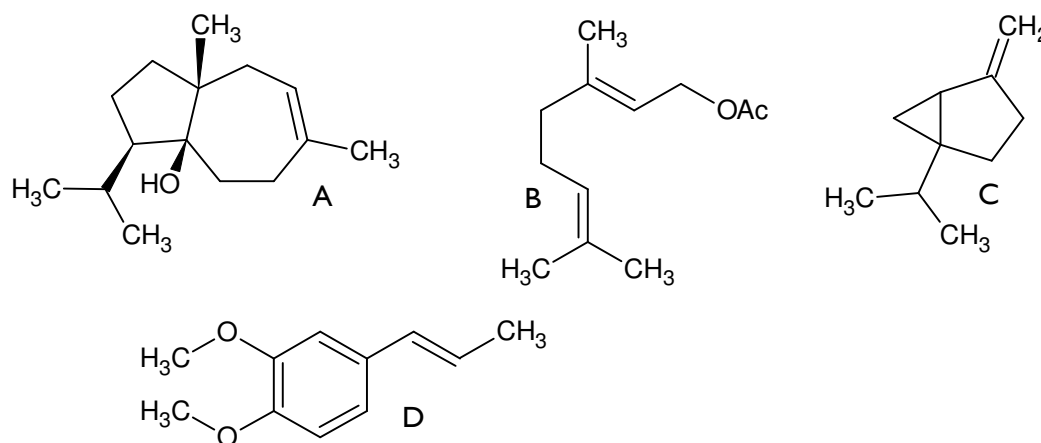
Figure 8 - Structures of major components of *Daucus* essential oil: Carotol (A), Geranyl acetate (B), Sabinene (C), (E)-methylisoeugenol (D)

Carotol rich-oils: Carotol is described as one the major components on both cultivated (*D. carota* subsp. *sativus*) and wild carrot (different subspecies of *D. carota*) seed oil. Oils from India (70%), Pakistan (62.8-77.5%), Egypt (65.8-67.2%), France (69.7-73.1%) and Turkey (68.8%) are described as having a carotol content higher than 60% (Guinoiseau *et al.*, 2010).

Geranyl acetate rich-oils: Geranyl acetate is the major component in essential oils distilled from samples from Portugal (65%, *D. carota* subsp. *carota*), Spain (51.7-76.9%, *D. carota* subsp. *gummifer*), Germany and Russia (Guinoiseau *et al.*, 2010).

Sabinene rich-oils: This compound has been described as the major compound in essential oils from Lithuania (28.2-37.5%, *D. carota* subsp. *carota*) (Guinoiseau *et al.*, 2010).

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Oils that contain the three above compounds in an equal ratio: Some oils are described as having all three, or at least two out of three, of the reported compounds with equal abundance. For example the content on carotol, geranyl acetate and sabinene in industrial oil from France is 18%, 17% and 15%, respectively and the contents of carotol, geranyl acetate and sabinene in essential oil from *D. carota* subsp. *sativus* from Germany are 24.1%, 18.3% and 10.1% (Guinoiseau *et al.*, 2010).

Phenylpropanoid rich-oils: Some oils major components are phenylpropanoids instead of terpenes. Essential oil from Lebanese *D. carota* subsp, *maximus* is characterized by *(E)*-methylisoeugenol (37.2%) and *β*-asarone (17.7%), whereas Corsican essential oil is rich in *(E)*-methylisoeugenol (21.8-41.6%) and elemicin (4.8-16.3%) (Guinoiseau *et al.*, 2010).

Besides these five most common carrot seed oils, there are some oils that have a composition that is characterized by different monoterpenes, such as, geraniol or neryl acetate or by sesquiterpenes, e.g. β-bisabolene or γ-bisabolene (Guinoiseau *et al.*, 2010).

Essential oils that are obtained from aerial parts of the plant (umbels, flowers, stems and leaves) have a composition that is, similar to what was described for seed oils, being also highly variable between samples (Guinoiseau *et al.*, 2010). In fact, some samples are characterised as having sabinene, α-pinene, both monoterpenes, or trans-asarone, a member of the phenylpropanoid family (Guinoiseau *et al.*, 2010). Contrarily, most samples are described as having a mixture of compounds for instance, carotol/himachalenol/β-bisabolene or shyobunone/preisocalamendiol (Guinoiseau *et al.*, 2010).

Very few studies have been carried out using carrot root oils (Guinoiseau *et al.*, 2010). Terpinolene has been described as the major compound in the essential oil from roots of *D.carota* subsp. *sativa* whereas theoil isolated from roots of wild *D. carota* subsp. *maritimus* were characterized by two phenylpropanoids, dillapiole (46.6%) and myristicine

namely, carotamine (an aromatic peptide) was isolated from an aqueous ethanolic extract from the aerial parts of *Daucus carota* var *boissieri* (Guinoiseau *et al.*, 2010) and the aqueous extracts from seeds were a source of crotonic acid (Jasicka-Misiak *et al.*, 2004). Nevertheless, studies regarding the phenolic composition of *Daucus carota* extracts are very sparse and therefore no section on this matter was considered.

V. **Bioactivities and cytotoxicity of *Daucus carota* subsp. *carota* essential oil and phenolic-enriched extracts**

V | Bioactive activities and cytotoxicity of *Daucus carota* subsp. *carota* essential oil and phenolic-enriched extracts
1. Essential oil and phenolic-enriched extract

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1. Essential oil and phenolic-enriched extract

The work conducted in this thesis consisted in the evaluation of the bioactive properties of both essential oil and phenolic-enriched extracts obtained from *Daucus carota* subsp. *carota*, commonly known as wild carrot. In this section the extraction techniques used as well as the major compounds identified and extraction yields are highlighted. In the following sections studies regarding the antibacterial, antifungal, antioxidant and anti-inflammatory properties of the essential oil and/or the phenolic-enriched extracts as well as their cytotoxicity are presented.

1.1. Material and methods

1.1.1. Plant material
Aerial parts of *Daucus carota* subsp. *carota* were collected from Serra da Lousã, Coimbra, Portugal. The plant material was air dried and then submitted to different extraction processes in order to obtain the essential oil (distillation) or phenolic-enriched extracts (solvent extraction).

1.1.2. Distillation and oil characterization
Hydrodistillation was performed in a circulatory Clevenger-type apparatus according to the procedure described in the European Pharmacopoeia during 4 h (Council of Europe, 2010).

Chemical characterization of the essential oil from this species was previously conducted in our laboratory using both gas chromatography (GC) and gas chromatography-mass spectroscopy (GC-MS). The volatile compounds were identified by their retention indices (using SPB-1 and SupelcoWax-10 columns) and their mass spectra. Relative amounts of individual components were calculated based on GC peak areas without a FID response factor correction, according to Cavaleiro et al (Cavaleiro *et al.*, 2004)(2004).

1.1.3. Solvent extraction and characterization of the phenolic profile
Prior to extraction, the dried matter was grounded and 10 g of the grounded sample was dissolved in 500 mL of three distinct solvents: distilled water, methanol or a hydromethanol solution at 70% (v/v). The aqueous extract was obtained using boiling water under magnetic agitation for 20 min, while the other two extracts were obtained with magnetic agitation at room temperature for 1 h. Extractions were performed twice in the same material after a cheesecloth filtration and under the same conditions. Prior to both extractions, the extracts were mixed and submitted to evaporation. The solvent was removed

in a rotator evaporator under vacuum at 40 °C and distilled water was added until the solvent was thoroughly evaporated to a final volume approximately of 50 mL, followed by freezing and lyophilisation. The dried powder material of the distilled water extract (H_2O), methanol 100% extract (MeOH 100%) and methanol 70% (v/v) extract (MeOH 70%) was separately stored in screw-tight flasks and in a desiccator in the dark until further analysis.

The phenolic profile of the polar extracts were obtained by HPLC-DAD-ESI-MS^n, according to Catarino et al. (2015). The identification of individual phenolic compounds was achieved by comparison of their retention times, UV–Vis spectra and MS^n spectra data with those of the closest available reference standards and data reported in the literature. The identification of those compounds for which no standard compounds were available was achieved only by comparison of the data reported in literature.

1.2. Results

1.2.1. Essential Oil

1.2.1.1. Identification of the major compounds

The essential oil was obtained with a yield of 0.9% (v/w). In table 1 the major compounds identified in *D. carota* subsp. *carota* essential oil are presented. Amongst them the most preeminent is geranyl acetate (29%; Fig 9A) and α-pinene (27%; Fig. 9B). In fact, this composition is as expected since the essential oils obtained from wild carrot in the Iberian Peninsula belong to the geranyl acetate chemotype, characterized by high amounts of geranyl acetate, however other compounds like α-pinene can also be found in significant amounts (Guinoiseau *et al.*, 2010).
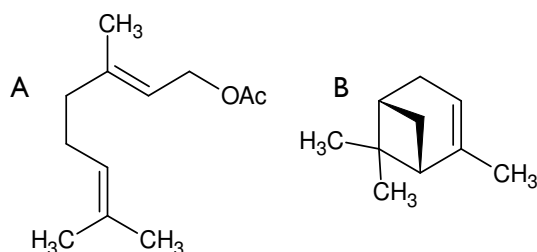
Figure 9 - Major compounds found in *D. carota* subsp. *carota* essential oil: A- geranyl acetate; B- α-pinene

Table 1 -Main compounds identified in *Daucus carota* subsp. *carota* essential oil

Compound	Relative abundance (%)	Identification
geranyl acetate	29	RI[a], GC-MS

α-pinene	27	RI[a], GC-MS
11αH-himachal-4-ene-1-β-ol	9.2	RI[a], GC-MS
limonene	9	RI[a], GC-MS

[a] – Retention index determined using SPB-1 and SupelcoWax-10 columns

Previous studies concerning several *Daucus carota* subspecies have demonstrated that the essential oil composition is greatly variable not only amongst subspecies but also when different plant origins are compared. Also, the plant part used for oil extraction is an important aspect to consider. For example, *D. carota* subsp. *carota* mature umbels essential oil from Cantanhede (Central Portugal) is characterized by high amounts of geranyl acetate (65%) and α-pinene (13%) while Sardinian essential oil is rich in β- bisabolene (51.0%), E-methylisoeugenol (10.0%) and 11αH-himachal-4-ene-1-β- ol (9.0%) (Maxia *et al.*, 2009). Moreover, the essential oil from leaves and fruits of *D. carota* subsp. *carota* growing in Vienna (Austria) is characterized by high amounts of α-pinene (20.9 – 44.8%) and sabinene (11.3 – 46.6%) (Chizzola, 2010).

Regarding different subspecies, the essential oil from ripe umbels of *D. carota* subsp. *gummifer* from São Pedro de Moel (Portugal) is mainly composed by geranyl acetate (37%), α-pinene (30.9%) and carotol (11%) (Valente *et al.*, 2015); essential oil obtained from ripe umbels of *D. carota* subsp. *halophilus* growing in Algarve (Portugal) is rich in elemicin (26.0 – 31.0%) and sabinene (27.6 – 29.0%) (Tavares *et al.*, 2008) whereas Tunisian *D. carota* subsp. *maritimus* flowers yields an essentiall oil that is characterized by high amounts of sabinene (51.6%), terpinen-4-ol (11.0%), p-cymene (4.2%) and eudesm-6-en-4-ol (3.6%) (Jabrane *et al.*, 2009).

1.2.2. Phenolic-enriched extracts

1.2.2.1. Extraction yield and identification of major compounds

The extraction yields of the different phenolic-enriched extracts are summarized in table 2, where it can be observed that the H_2O extract is the one with the highest extraction yield (23.82%), while MeOH 100% only represented 9.41% of the dried plant material.

Table 2 - Extraction conditions and yield of the 3 distinct phenolic-enriched extracts

Sample	Extraction Conditions	Yield (%)

1. Essential oil and phenolic-enriched extract

MeOH 100%	Room temperature with magnetic stirring for 1h	9,41
H2O	Boiling water with magnetic stirring for 20 min	23,82
MeOH 70%	Room temperature with magnetic stirring for 1h	17,98

The chromatographic analysis of the three phenolic extracts, revealed that the composition was very similar between them. In figure 10 a representative chromatogram is shown. As seen in table 3, the major compounds identified were caffeoylquinic acid isomers (Figure 11A) and dicaffeoylquinic acid isomers (Figure 11B) as hydroxycinnamic acids and rutin (Figure 11C) and quercetin-hexoside (Figure 11D) belonging to the flavonoids family.
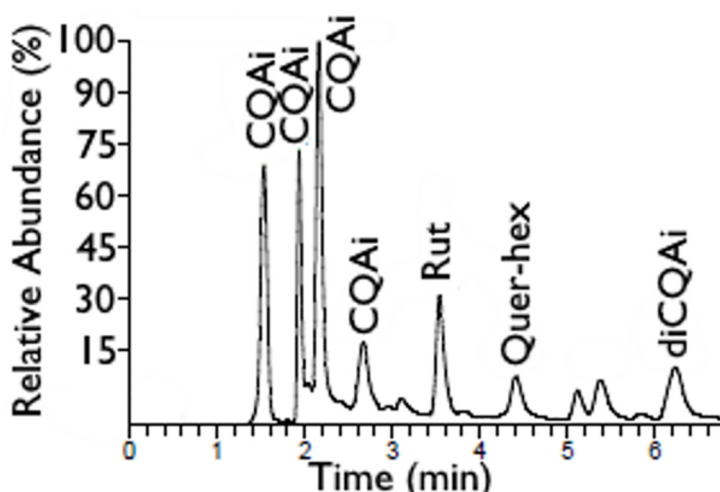
Figure 10 - Representative chromatogram of the *D. carota* subsp. *carota* phenolic extracts studied at 280 nm. CQAi – Caffeoylquinic acid isomer, Rut – Rutin, Quer-hex – Quercetin-hexoside, diCQAi – dicaffeoylquinic acid isomer

To the author´s knowledge, studies regarding the phenolic characterization of carrot only used roots. Therefore, the characterization of extracts obtained from the aerial parts are herein presented for the first time. Kammerer and colleagues identified in black carrot (*Daucus carota* ssp. *sativus* var. *atrorubens* Alef.) root methanolic extract several caffeoylquinic acid isomers and well as dicaffeoylquinic acids amongst other hydroxycinnamic acids, however they didn't identify any of the referred flavonols pointed out in the present study (Kammerer *et al.*, 2004).

V | Bioactive activities and cytotoxicity of *Daucus carota* subsp. *carota* essential oil and phenolic-enriched extracts

I. Essential oil and phenolic-enriched extract

Table 3 - Identification of LC-DAD-ESI/MS^n of the major components of *D. carota* subsp. *carota* extracts

RT (min)	λmax	Compound (MW)	Main MS^n fragments	Identification
1.53	221, 325	354	MS^2[353]: 191, 179, 173	Caffeoylquinic acid isomer
1.94	220, 322	354	MS^2[353]: 191, 179, 135	Caffeoylquinic acid isomer
2.16	221, 316	354	MS^2[353]: 191, 179, 173	Caffeoylquinic acid isomer
2.67	217, 328	354	MS^2[353]: 191, 173, 111	Caffeoylquinic acid isomer
3.54	257, 354	610	MS^2[609]: 301	Rutin (quercetin-3-O-rutinoside)
4.41	256, 351	464	MS^2[463]: 301	Quercetin-hexoside
6.23	215, 327	516	MS^2[515]: 353	Dicaffeoylquinic acid isomer
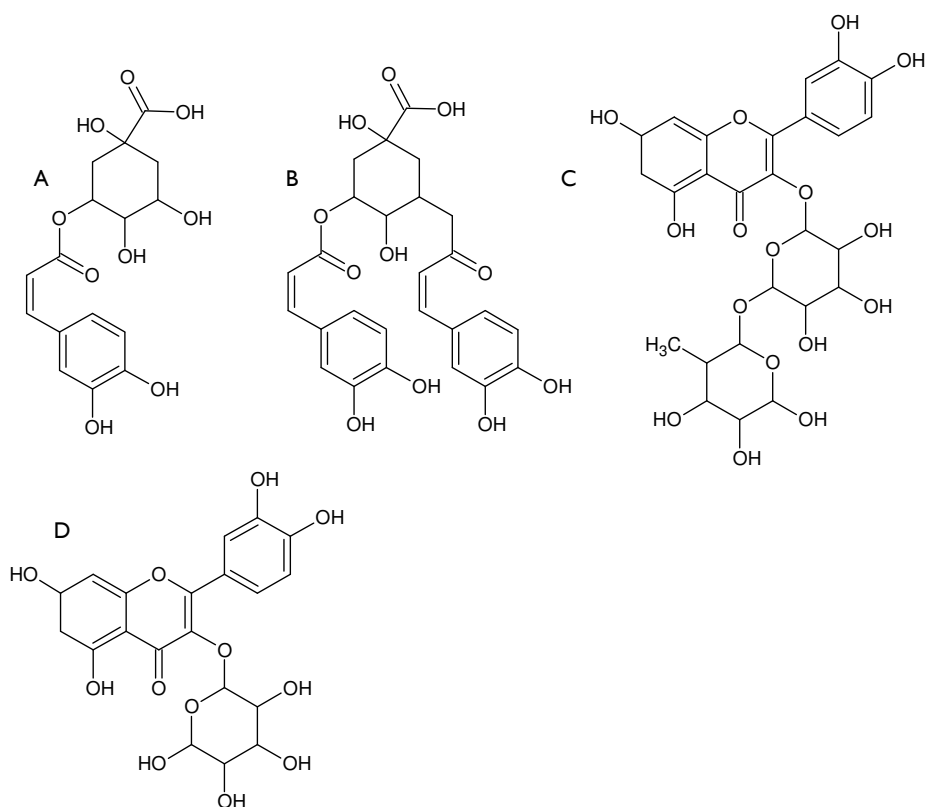
Figure 11 - Phenolic compounds found in *D.carota* subsp. *carota* extracts. A - Caffeoylquinic acid, B - dicaffeoylquinic acid, C - Rutin, D - Quercetin-hexoside

2. Antibacterial activity

2.1. Introduction

The incidence of bacterial infections has been rising over time due to the increase in population's age, increase in the usage of biomaterials, a higher rate of organ transplantations and, most importantly, the increase of bacteria resistance to antibiotics (Oosten, van *et al.*, 2015). The severity of these infections varies from self-limiting to potentially life-threatening, resulting in major morbidity and mortality rates, thus increasing the overall healthcare costs (Oosten, van *et al.*, 2015). Since the discovery of penicillin and the following boom in the antibiotic field, these drugs have been widely used and were even considered "miracle drugs" which led to the belief that bacterial infections were utterly defeated (Penesyan *et al.*, 2015). However, the overuse of antibiotics also led to the appearance of even more pathogenic bacteria that created resistance to these inhibitory effects. The most common bacterial infections are caused by *Listeria monocytogenes., Staphylococcus aureus, Salmonella thypimurium*, *Escherichia coli* and *Bacillus* spp.. A brief summary of these infections regarding their causal agent, main symptoms and current treatment is presented.

i) Listeriosis

This infection is caused by the Gram-positive bacterium *Listeria monocytogenes*, a foodborne pathogen described in 1980 (Noordhout *et al.*, 2014). In 2000, the prevalence of this disease, in the USA, was of 4 per million inhabitants. In Europe this number varies from 0.1 to 11.3 per million inhabitants, and circa 20% are neonatal infections (Lamont *et al.*, 2011). Infections by *L.. monocytogenes* in healthy patients may cause febrile gastroenteritis which typically is mild and self-limiting. However, when the patient belongs to a risk group (pregnant women, elderly, immunocompromised person, unborn babies or neonates), listeriosis may cause several illnesses, e.g. severe sepsis, meningitis or encephalitis which lead to lifelong consequences or, in the most severe cases, death (Noordhout *et al.*, 2014). The reported cases of listeriosis are divided into three clinical syndromes: maternofetal listeriosis, blood stream infection and meningoencephalitis (Hernandez-Milian *et al.*, 2014; Swaminathan *et al.*, 2007). The fatality rate of this bacterial infection is about 20-30% of the cases despite availability of adequate treatment (Swaminathan *et al.*, 2007).

The first-line treatment for *L.. monocytogenes* infections is a β-lactam antibiotic, usually ampicillin. Since penicillin-derived antibiotics are known to be bacteriostatic, some treatments employ combinations with other compounds such as aminoglycosides (e.g. gentamicin) (Hernandez-Milian *et al.*, 2014; Swaminathan *et al.*, 2007).

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2. Antibacterial activity

ii) *Staphylococcus aureus* infections

Staphylococcus aureus is a commensal yet pathogenic Gram-positive bacterium. Nearly 30% of the population is colonized by this bacteria (Lister *et al.*, 2014; Tong *et al.*, 2015). This pathogen is the preeminent cause for bacteraemia and infective endocarditis in addition to osteoarticular, skin and soft tissue, pleuropulmonary and device-related infections (Tong *et al.*, 2015).

S. aureus bacteraemia is the best described infection. This disease has an incidence of 10-30 per 100000 person-year in the industrialized world, while in the non-industrialized word the incidence is still unknown due to the lack of population-based studies (Tong *et al.*, 2015). The risk factors for this pathogeny is age, with higher incidence in the first year of life and with advanced aging; the gender is also important with a ratio male-to-female of approximately 1.5, with the reason for this difference remaining unknown (Tong *et al.*, 2015). Ethnicity is also an important factor however, the reason for this is not fully explained. Likewise, HIV infection is a very significant risk factor with HIV-infected patients having an incidence 24 times greater than non-HIV-infected patients (Tong *et al.*, 2015). Haemodialysis patients also have a greater risk of contracting *S. aureus* bacteraemia (Tong *et al.*, 2015). In cases of bacteraemia caused by methicillin-sensitive *S. aureus,* the first-line therapy consists of β-lactam antibiotics while in cases of methicillin-resistant *S. aureus* the therapy is based upon vancomycin and daptomycin (Tong *et al.*, 2015).

iii) Salmonellosis

Salmonella enterica serovar typhimurium (*S. typhimurium*) is a Gram-negative bacterium that causes severe inflammation of the intestinal mucosa which leads to gastroenteritis (Patel *et al.*, 2014). This serovar as well as others that cause non-typhoidal *Salmonella* infections, are primarily transmitted in industrialized countries by food contaminated with animal feces (Bula-Rudas *et al.*, 2015; Crump *et al.*, 2015; Kariuki *et al.*, 2015). However, it can also be transmitted by direct contact with infected animals, e.g. reptiles, amphibians, live poultry, and pet rodents (Bula-Rudas *et al.*, 2015; Crump *et al.*, 2015). The prevalence of these syndromes worldwide is thought to be 93.8 million cases of which 155000 result in death (Kariuki *et al.*, 2015). These infections, in immunocompetent and healthy patients, normally cause a self-limiting enterocolitis with diarrhoea (Bula-Rudas *et al.*, 2015; Crump *et al.*, 2015; Kariuki *et al.*, 2015). However, approximately 6% of the cases lead to bloodstream infections, being the risk higher

in some groups, e.g. immunocompromised individuals, infants, and elderly people (Crump *et al.*, 2015; Kariuki *et al.*, 2015).

The usage of antimicrobial agents to treat cases of *Salmonella* infections is dependent on the local epidemiology and the resistance pattern of the infectious agent. The most used agents are ampicillin, amoxicillin, tetracycline, macrolides, trimethoprim-sulfamethoxazole, chloramphenicol and fluoroquinolones (Bula-Rudas *et al.*, 2015).

iv) *Escherichia coli* infections

E. coli is a Gram-negative bacterium that belongs to the *Enterobacteriaceae* family. This is a commensal bacterium in the gastrointestinal tract that normally is harmless to the host. However, *E. coli* has several serotypes that are highly pathogenic and responsible for infections in the gastrointestinal tract as well as in the extraintestinal locals such as the urinary tract, bloodstream and central nervous system (Croxen *et al.*, 2013).

In turn enteric *E.coli* is responsible for diarrhoea, however the type of diarrhoea as well as the associated symptoms, outcomes, sites of infection and disease can differ. There are seven described pathotypes of enteric *E. coli*, namely enteropathogenic, enterohemorragic, enterotoxigenic, enteroagregative, diffusely adherent, enteroinvasive and adherent invasive *E. coli* (Allocati *et al.*, 2013; Bettelheim *et al.*, 2015; Croxen *et al.*, 2013).

The pathogenic serotypes can be divided into enteric *E. coli* or extraintestinal *E. coli*. The latter are responsible for infections outside the gastrointestinal tract, of which one pathotype causes lower urinary tract and systemic infections and is known as uropathogenic *E. coli*, the other infects the central nervous system causing neonatal meningitis being known as neonatal meningitis *E. coli* and the third is known as avian pathogenic *E. coli*, because it is thought to cause a foodborne disease (Allocati *et al.*, 2013).

The treatment of *E. coli* infections, both enteric and extraintestinal, is normally achieved by oral rehydration therapies, however in some persistent cases antimicrobial therapy may be needed (Croxen *et al.*, 2013). In these cases, the therapy requires the administration of first-line antibiotics such as ampicillin, trimethoprim-sulfamethoxazole, tetracyclines and fluoroquinolones (Kong *et al.*, 2015).

v) *Bacillus* spp.

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2. Antibacterial activity

Bacillus subtilis is a Gram-positive bacterium that is ubiquitous in soil and gastrointestinal tract. This species is only pathogenic in immunocompromised patients. In healthty patients, the species can be used to restore the balance in the microbial flora in the intestinal tract (Oggioni *et al.*, 1998). Due to this, the section regarding *B. subtilis* is not as exhaustive as the remaining pathogenic bacteria.

The boost of bacterial resistance to conventional antibiotics has encouraged scientists to discover new antibacterial agents with a broader spectrum of action (Nazzaro *et al.*, 2013). Since immemorial times, plant-derived products, such as essential oils and phenolic-enriched extracts, have been used in folk medicine to control bacterial infections (Dorman *et al.*, 2000; Faleiro M.L., 2011; Nazzaro *et al.*, 2013). Several authors have described the antibacterial activity of essential oils and their isolated compounds against different pathogenic bacteria (e.g. Chalchat *et al.*, 2000; Filipowicz *et al.*, 2003; Sonboli *et al.*, 2005). The mechanism of action that causes the bacteriostatic and/or bactericidal effects of essential oils have also been described by some authors (Dorman *et al.*, 2000; Nazzaro *et al.*, 2013). These studies have demonstrated that Gram-negative bacteria are more resilient to essential oils than Gram-positive ones (Nazzaro *et al.*, 2013). This susceptibility of Gram-positive bacteria may be explained by differences in the cell wall composition. It is known that 90-95% of the cell wall in Gram-positive bacteria is peptidoglycan which allows hydrophobic compounds to penetrate the cell and act on the cell wall as well as on the cytoplasm (Nazzaro *et al.*, 2013). Conversely, the cell wall in Gram-negative bacteria is far more complex. In these bacteria the cell wall-peptidoglycan is only 2-3 nm thick and is bounded to an outer membrane (Nazzaro *et al.*, 2013). This outer membrane is a bilayer of phospholipid and several proteins amongst which the lipopolysaccharide is a good example and very important to bacteria virulence. The outer membrane is almost fully impermeable to hydrophobic compounds (e.g. essential oils) however some compounds can slowly enter the cell via the abundant porins found on the membrane (Nazzaro *et al.*, 2013). Both the presence of lipopolysaccharide and the impermeability to hydrophobic compounds are responsible for the resistance to essential oils and other antimicrobial agents (Nazzaro *et al.*, 2013). However this resistance can be overcome by permeabilisers, compounds (e.g. small terpenoids and phenolic compounds) that are able to disrupt the outer membrane by disintegrating the lipopolysaccharide layer (Puupponen-Pimiä *et al.*, 2005).

2.2. Material and Methods

2.2.1. Essential oil, phenolic-enriched extracts and reference compound

The essential oil from *Daucus carota* subsp. *carota* and the 70% methanol phenolic-enriched extract were used to determine the antibacterial activity of this species. Regarding the phenolic extracts, their chemical composition was very similar with minor quantitative differences. Thus, their bioactivity is expected to be similar and, in the present study, only one of the phenolic-enriched extracts (70% methanol) was selected to evaluate the antibacterial and antifungal activities. The reference compound used was the antibiotic ampicillin purchased as Ampicillin sodium salt from Fluka BioChemika (Buchs, Switerzland)

2.2.2. Bacterial strains

The antibacterial activity was carried out against both Gram-negative and Gram-positive bacteria. Most of these strains were obtained from the American Type Culture Collection (*Salmonella thyphimurium* ATCC 14028; *Staphylococcus aureus* ATCC 6538; *Escherichia coli* ATCC 25922; *Bacillus subtilis* ATCC 6633), one strain was obtained from the *Coleção de Bactérias do Instituto Superior de Agronomia* (*Listeria monocytogenes* CBISA 3183) and one was isolated from meat (*Bacillus subtilis*).

2.2.3. Antibacterial activity

The macrodilution method was used for the determination of the Minimum Inhibitory Concentration (MIC) and Minimum Lethal Concentration (MLC) of both the essential oil and the phenolic-enriched extract as described in the CLSI reference M07-A9 (CLSI, 2012). Briefly, serial doubling dilutions of the oil were prepared in dimethyl sulfoxide (DMSO, Sigma Life Science, Sigma-Aldrich, MO, USA)). Dilutions ofethe phenolic-enriched extract were prepared in Mueller Hinton broth (Oxoid, Hampshire, England), with concentrations ranging from 0.08 to 20 μL/mL. Recent cultures of each strain were used to prepare the cell suspensions (1.2×10^5 CFU/mL)) and cells concentration was confirmed by viable count on Mueller Hinton Agar (Oxoid, Hampshire, England). All tests were performed in Mueller Hinton Broth medium and the test tubes were incubated aerobically at 37 °C for 24h and then MICs were registered. To evaluate MLCs, 20 μL of broth were taken from each negative tube after MIC reading, and cultured in Mueller Hinton Agar plates and then incubated as referred above. Mueller-Hinton broth sterility was also evaluated for each strain. DMSO innocuity was also determined. All tests were performed in duplicate. The results are shown as mean of three independent experiments.

2.3. Results and Discussion

2.3.1. Antibacterial activity of the essential oil

Table 4 respresents the MIC and the MLC values of the essential oil and methanolic extract of *Daucus carota* subsp. *carota* against several bacterial strains. As expected, the essential oil demonstrated a stronger inhibitory effect against Gram positive bacteria (MIC 0.32-0.64 µL/mL) than against Gram negative positive bacteria (>10 µL/mL). The Gram positive bacteria *S. aureus* and both *B. subtilis* strains showed a higher susceptibility to the essential oil's inhibitory effect (MIC 0.32 µL/mL and MLC 0.64 µL/mL).

Table 4. Antibacterial activity (MIC and MLC) of essential oil and MeOH 70% extract of *Daucus carota* subsp. *carota* for several bacterial strains.

Strains	Essential Oil		MeOH 70%		Ampicillin	
	MIC[a]	MLC[a]	MIC[b]	MLC[b]	MIC[c]	MLC[c]
Bacillus subtilis (isolated from meat)	0.32	0.64	5	10	0.06	4
Bacillus subtilis ATCC 6633	0.32	0.64	5	10	0.06	0.025
Escherichia coli ATCC 25922	>10	>10	>10	>10	8	16
Listeria monocytogenes CBISA 3183	0.64	>10	>10	>10	2	16
Salmonella thyphimurium ATCC 14028	>10	>10	>10	>10	4	8
Staphylococcus aureus ATCC 6538	0.32	0.64	5	10	0.25	0.5

[a] MIC and MLC were determined by a macrodilution method and expressed in µL/mL.
[b] MIC and MLC were determined by a macrodilution method and expressed in mg/mL.
[c] MIC and MLC were determined by a macrodilution method and expressed in µg/mL.
Results were obtained from three independent experiments performed in duplicate.

The results obtained for *Daucus carota* subsp. *carota* essential oil (rich in geranyl acetate, α-pinene and limonene) are quite interesting since it has been reported that essential oils rich in ketones and esters (e.g. α-thujone, geranyl acetate) retain a weak antibacterial activity. In fact, previous studies have demonstrated that geranyl acetate has a rather low growth inhibition against a wide range of bacteria both Gram negative and Gram positive (Dorman *et al.*, 2000; Kakarla *et al.*, 2009). Also, limonene, a cyclic terpene, demonstrated a very weak activity against several bacterial strains, e.g. *S. aureus*, *E. coli*, and *B. subtilis* (Caven-Quantrill *et al.*, 2007; Dorman *et al.*, 2000; Filipowicz *et al.*, 2003; Sonboli *et al.*, 2005; Verbenaceae *et al.*, 2006). Conversely, α-pinene, a monoterpene found in many essential oils, showed a strong effect on the inhibition of the growth of a wide variety of bacteria (Dorman *et al.*, 2000;

Filipowicz *et al.*, 2003; Orhan *et al.*, 2012; Verbenaceae *et al.*, 2006). Although the activity of geranyl acetate and limone are weak, α-pinene has a very good activity, thus the activity of *D. carota* subsp. *carota* essential oil might be explained by the high amounts of α-pinene (27%). The differences observed between the activity of isolated compounds and the whole essential oil might be due to the presence of minor compounds that can einfluence the activity of α-pinene.

2.3.2. Antibacterial activity of the hydromethanolic extract

The antibacterial activity of the methanol 70% (v/v) extract was weaker than that described for the essential oil however the pattern was similar i.e., Gram negative bacteria (MIC>10 mg/mL) were more resilient than Gram positive ones (MIC 5 mg/mL). Similarly to what was described for the essential oil, the bacteria *S. aureus* and both *B. subtilis* strains were the most susceptible to the extract (MIC 5 mg/mL and MLC 10 mg/mL).

Considering previous studies on the major compounds of this extract: caffeoylquinic acid isomers, rutin, quercetin-hexoside and dicaffeoylquinic acid, some studies have been conducted. Namely, 3-/4-/5-Caffeyolquinic acid showed a MIC ranging from 0.1-0.2 mg/mL against *S. aureus* and *E. coli* (Xia *et al.*, 2011). In another study, 5-Caffeyolquinic acid demonstrated a MIC of 5-10 mg/mL against *S. aureus* and *E. coli* (Bajko *et al.*, 2015). The flavonoid glycoside, rutin (quercetin-3-O-rutinoside) was able to inhibit *S. typhimurium* and *E. coli* (Srinivas *et al.*, 2012). At 50 μg this flavonoid showed an inhibition zone of 0.6 mm for *S. aureus* (Soberón *et al.*, 2014). The MIC of rutin for *E. coli*, *S, aureus* and *B. subtilis* ranged from 4 to 16 mg/mL (Orhan *et al.*, 2010; Wang *et al.*, 2013). Therefore, the activity of the isolated major compounds *D. carota* subsp. *carota* hydromethanolic extract is weaker than the extract *per se*. This difference might be explained by the presence of minor phenolic compounds that synergistically work with these major compounds.

The antibacterial activity of *Daucus carota* has also been described for isolated compounds from other aromaticextracts in addition to those found in theessential oil (Bergonzelli *et al.*, 2003; Glisic *et al.*, 2007; Rossi *et al.*, 2007; Staniszewska *et al.*, 2005).

Carrot fruit supercritical fluid extract as well as the essential oil (major components carotol and sabinene) are described as having a minimum inhibitory concentration (MIC) ranging from 80 to 1280 μg/mL for Gram positive bacteria (*S. aureus, B. cereus,* and *B. subtilis Rhodococcus equi, Listeria monocytogenes* and *Enterococcus faecalis*) while for Gram negative

bacteria the MIC is higher than 1280 μg/mL (*E. coli, P. aeruginosa* and *Salmonella enteritidis*) (Glisic *et al.*, 2007; Guinoiseau *et al.*, 2010). Similarly, oils from herb, flowering and mature umbels demonstrate a higher inhibitory effect against Gram positive than against Gram negative bacteria (Guinoiseau *et al.*, 2010; Staniszewska *et al.*, 2005).

Oils obtained from flowers and roots of *D. carota* subsp. *maritimus* were also described as having a bactericidal activity against Gram negative strains, *Pseudomonas aeruginosa, E. coli, Klebsiella pneumoniae* and *S. typhimurium* (MIC ranging from 1.25 to 5 g/L). Flower oil dominated by sabinene (51.6%), terpinen-4-ol (11.0%) and eudesm-6-en-4-ol (3.6%) had a higher inhibitory activity against *E. coli* growth when compared with root oils. Conversely, root oil majorly constituted by two phenyl propanoids, dillapiole (46.6%) and myristicine (29.7%), was more active than flower oil against *S. pneumonia* and *Shigella* spp. (Jabrane *et al.*, 2009).

The results obtained in the present study are quite promising for infections caused by Gram positve bacteria since these bacteria are more susceptible to the action of both *Daucus carota* subsp. *carota* essential oil and phenolic-enriched extract.

3. Antifungal activity

3.1. Introduction

Fungi are the causative agent on several and serious pathogenic infections whose prevalence has increased over the last decades, with particular focus on high-risk patients (Muñoz *et al.*, 2012; Pfaller *et al.*, 2006; Sipsas *et al.*, 2012; Zuzarte, 2012). The severity of fungal infections is dependent on the virulence of the strain, the host reaction to the metabolites synthetized by the fungi, the local of infection along with some environmental factors (Romani, 2007; Weitzman *et al.*, 1995; Zuzarte, 2012). Fungal infections are often disfiguring, recurrent and chronic and normally imply long-term treatments (Shehata *et al.*, 2008). These infections can be treated by a wide range of commercially available antifungal agents. Nevertheless, the increased resistance of several strains to antifungals and associated side effects, ranging from mild reactions to hepatotoxicity, neurotoxicity, nephrotoxicity and hematologic reactions, are responsible for unsuccessful treatments (Andriole, 2000; Gupta *et al.*, 2008; Rosso, 2000; Zuzarte, 2012). Candidosis, cryptococcosis, dermatophytosis and aspergillosis are exemples of the most common and most threatening fungal infections that are even more threatening on immunocompromised patients (Zuzarte, 2012). A brief summary on these infections pointing out their casual agents, symptoms and treatments is presented.

i) Candidosis

This fungi-related disease outcomes when the host suffers an infection from yeasts of the genus *Candida* (Zuzarte, 2012). These species are commensal microorganisms of the oral cavity, gastrointestinal tract and vagina (Mårdh *et al.*, 2003, 2002). In the last few years, the number of *Candida* infections have significantly increased, being *C. albicans*, *C. glabrata* and *C. parapsilosis* the main agents of nosocomial fungal bloodstream infections (Hajjeh *et al.*, 2004). The most common manifestation of these infections is vulvovaginal candidosis, affecting 70-75% of women and it is a recurrent infection in 40-50% of the cases (Costa-De-Oliveira *et al.*, 2008). The main symptoms of vulvovaginal candidosis include vaginal irritation, vulvar burning, pruritus and vaginal discharge (Palmeira-de-Oliveira *et al.*, 2009; Zuzarte, 2012). The last years have also seen an increase in recurrence cases of vulvovaginal candidosis, being associated to relapses of persistent strains after long-term treatments (Murina *et al.*, 2011; Zuzarte, 2012). This disease has several risk factors that increase the chances of contracting it, the most preeminent and common factors are, pregnancy, diabetes mellitus, oral contraceptive use,

antibiotics, tight-fitting cloths, synthetic underwear, dietary excesses or deficiencies, intense sexual activity and the use of female hygiene products (Sobel, 2007).

Only a few of the commercially available antifungals are effective against *Candida* spp. infections, with amphotericin B, fluconazole and itraconazole being the most widely used (Carrillo-Muñoz *et al.*, 2006).

ii) The pathogenic mechanisms behind *Candida* infections are still unclear but have been associated to germ tube formation. Blastoconidia are involved in asymptomatic colonization of the vagina, while germ tube and hyphae are found on symptomatic cases of these infections. In addition, the organization in biofilms are thought to be responsible for the observed resistance to several antifungal agents thus reducing the effectiveness of these drugs (Kuhn *et al.*, 2002). This resistance is assumed to be associated with phenotypic modifications on fungal cells alongside with expression of resistance genes that occur during biofilm formation (Chandra *et al.*, 2001; Douglas, 2003). Hence, germ tube inhibition and/or disruption of biofilm organization appear to be a promising target for the control of these infections, avoiding dissemination of the infectious agent.Cryptococcosis

Cryptococcosis is an invasive fungal infection caused by yeasts of the genus *Cryptococcus*, mainly *C. neoformans* and *C. gattii*. Clinical manifestations of this type of infection range from asymptomatic infection to severe pneumonia and respiratory failure (Brizendine *et al.*, 2011). Managing this disease can be rather difficult as the treatment, which can be either with amphotericin B, flucytosine or fluconazole, varies with the characteristics of the affected population (HIV-seropositive, organ transplant recipients or non-transplant/non-HIV) (Desalermos *et al.*, 2012). Immunocompromised patients are a higher risk group as the infection by *Cryptococcus* may spread to the brain leading to neurological disturbances such as meningoencephalitis (Lin *et al.*, 2006), a disease responsible for more than 600,000 deaths per year worldwide (Desalermos *et al.*, 2012).

iii) Dermatophytosis

Fungi from the genus *Trichophyton, Microsporum* and *Epidermophyton* are responsible for common infections of keratinized tissues (skin, hair and nails) of both humans and animals usually called dermatophytosis (Dahdah *et al.*, 2008; Hainer, 2003; Shehata *et al.*, 2008). The

infection caused by these fungi may vary from mild to very intense. These infections are normally non-invasive, however in immunocompromised patients they can rapidly progress to life-threating infections (Zuzarte, 2012). Moreover, zoophilic and geophilic dermatophytes are accountable for severe inflammatory reactions owed to delayed hypersensitivity responses to fungi proteases. Additionally, pediatric and geriatric populations have seen an increase in the number of infections by these agents (Monod, 2008; Mukherjee *et al.*, 2003).

Cutaneous dermatophytosis are recognized by their scaly patches, with central clearing and sharply demarcated, annular, erythematous, advancing margins, sometimes presenting vesicles, blisters and pustules (Dahdah *et al.*, 2008). Distinction between these infections and other clinical conditions with similar symptomatology (e.g. nummular eczema, subacute cutaneous lupus erythematosus, herpes simplex, varicella zoster virus) is not an easy task (Ziemer *et al.*, 2007).

Prescribed drugs for dermatophytosis includes both oral and topical formulations, that derive from two antifungal drug families: azoles (e.g. clotrimazole, miconazole) and allylamines (e.g. terbinafine and naftifine) (Gupta *et al.*, 2008; Zuzarte, 2012). Superficial mycosis such as *tinea pedis, tinea mannum, tinea corporis* and *tinea crurise* normally respond to topical treatments (Andrews *et al.*, 2008; Dahdah *et al.*, 2008; Gupta *et al.*, 2008). Amorolfine and butenafine, derivatives from morpholine, have also been described as antifungal agents (Gupta *et al.*, 2008). In cases where the infection area is too wide or the infections are severe or persistent, systemic therapies should be considered. This is also true for *tinea unguium* and *tinea capitis* against which terbinafine, itraconazole, fluconazole, griseofulvin and ketoconazole have been deemed more adequate. Nevertheless, systemic formulations may present major side-effects including hepatotoxicity, neurotoxicity, nephrotoxicity and hematologic reactions, and in some cases, uncommon skin problems may appear (Andrews *et al.*, 2008; Gupta *et al.*, 2008; Zuzarte, 2012). In addition, drug interactions must also be evaluated since they are responsible for the reduced effectiveness of these therapies (Andriole, 1999; Rosso, 2000).

iv) Aspergillosis

When *Aspergillus* spp. infect the alveolar tissues in the lungs the resultant disease is known as aspergillosis. These diseases include pulmonary aspergilloma, invasive aspergillosis and allergic bronchopulmonary aspergillosis (Khan *et al.*, 2011). *A. fumigatus* and *A. niger* are the main strains responsible for aspergillosis, while *A. flavus* and *A. clavatus* are considered less

harmful. Invasive aspergillosis is becoming a key health concern as it frequently leads to the death of immunocompromised patients (Pfaller *et al.*, 2010). The significance of this condition has been growing with the increased numbers of patients with impaired immune system due to several conditions (e.g. cancer, organ transplantation) alongside with those with chronic obstructive pulmonary disease (Kousha *et al.*, 2011). Furthermore, the resistance of *Aspergillus* strains to antifungal compounds has increased over the last 20 years since its usage has also become more disseminated (Hadrich *et al.*, 2012). Voriconazole and amphotericin B deoxycholate are the most commonly used antifungal drugs in these cases (Walsh *et al.*, 2008).

Likewise, food, commodities and raw materials are vulnerable to contamination by *Aspergillus* spp., a major cause of food spoilage, particularly in tropical countries as these fungi produce mycotoxins (Hedayati *et al.*, 2007; Whitfield, 2003; Zuzarte, 2012). They are able to cause food decomposition and produce allergenic compounds that might occur before evident fungal growth (Pirbalouti *et al.*, 2011).

Given that the incidence of fungal infections has increased worldwide, there is an urgency to search for more effective and less toxic antifungal agents as alternatives to the synthetic ones. For centuries, essential oils have been extensively used as antifungals in traditional remedies (Ríos *et al.*, 2005). Recently, several studies have corroborated the huge potential of these products as antifungal agents (Bakkali *et al.*, 2008; Pina-Vaz *et al.*, 2004; Pinto *et al.*, 2006; Reichling *et al.*, 2009). Hence, it is not surprisingly that essential oils are an important group of natural products that can be used for the development of broad-spectrum, safer and cheaper antifungal agents. Although many studies report the antifungal activity of essential oils, the interactions between the oil and the microorganism, which are responsible for the inhibitory activity, are still poorly understood. In fact, studies regarding the mechanism of action of oils and extracts are sparse, thus hampering its potential utilization for industrial processes.

Although some studies regarding the antifungal activity of *D. carota* subsp. *carota* essential oil have already been conducted, the mechanism of action underlying the inhibitory effect is still greatly unknown. Hence, the aim of this study is to f demonstrate the inhibitory effects on fungi [yeasts (*Candida* spp. *Cryptococcus neoformans*), dermatophytes (*Trichophyton., Microsoporum* and *Epidermophyton* species) and other filamentous fungi (*Aspergillus* spp.)] as well as to elucidate the mechanism of action underlying the antifungal effect by studying the

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inhibitory capacity of the essential oil on *C. albicans* germ tube formation as well as its effect on preformed *C. albicans* biofilms.

3.2. Materials and Methods

3.2.1. Essential oil, phenolic-enriched extracts and reference compounds

The essential oil and the phenolic-enriched extract (MeOH 70%) used in the antifungal activity assay are the same as described in the previous section (2. Antibacterial activity). The reference compounds used were Fluconazole, provided by Pfizer as the pure powder and amphotericin B purchased from Sigma (80.0% purity).

3.2.2. Fungal strains

The antifungal activity of essential oils and phenolic-enriched extract was tested against yeasts and filamentous fungi: three *Candida* reference strains were obtained from the American Type Culture Collection (*C. albicans* ATCC 10231; *C. tropicalis* ATCC 13803; *C. parapsilosis* ATCC 90018) and two clinical strains were isolated from recurrent cases of vulvovaginal candidosis (*C. krusei* H9; *C. guillermondii* MAT23); one *Cryptococcus neoformans* reference strain was from the Colección Española de Culturas Tipo (*C. neoformans* CECT 1078); four dermatophytes strains were obtained from the Colección Española de Culturas Tipo (*T. rubrum* CECT 2794; *T. mentagrophytes* var. *interdigitale* CECT 2958; *T. verrucosum* CECT 2992; *M. gypseum* CECT 2908), the remaining dermatophytes were clinically isolated from nails and skin (*T. mentagrophytes* FF7; *M. canis* FF1; *E. floccosum* FF9); two reference *Aspergillus* strains were obtained from the American Type Culture Collection (*A. niger* ATCC 16404 and *A. fumigatus* ATCC 46645) and one *Aspergillus* strain was from a clinical origin and was obtained from bronchial secretions (*A. flavus* F44). All strains were cultured in Sabouraud Dextrose Agar (SDA) or Potato Dextrose Agar (PDA) before each test in order to ensure optimal growth conditions.

3.2.3. Antifungal activity

The macrodilution method was used to determine the Minimum Inhibitory Concentration (MIC) and Minimum Lethal Concentration (MLC) of the essential oil and the phenolic-enriched extract as described in the CLSI references M27-A3 (CLSI, 2008) and M38-A2 (CLSI, 2008) for yeasts and filamentous fungi, respectively. Briefly, serial doubling dilutions of phenolic-enriched extract and essential oil were made on RPMI 1640 broth (with L-glutamine, without bicarbonate, pH 7.0, BioChrom Ag., Berlin, Germany) or dimethyl sulfoxide (DMSO, Sigma-Aldrich, MO, USA), respectively, with concentrations ranging from 10 to 0.02 µL/mL for the essential oil and 10 to 0.02 mg/mL for the phenolic extract. Recent cultures of each strain

were used to prepare the cell suspensions (1-–2×10^3 cells/mL for yeasts and 1-–2×10^4 cells/mL for filamentous fungi) and cells concentration was confirmed by viable count on Sabouraud agar (Oxoid, Hampshire, England). All tests were performed in RPMI medium and the test tubes were incubated aerobically at 35 °C for 48 h/72 h (*Candida* spp. and *Aspergillus* spp./*Cryptococcus neoformans*) and at 30 °C for 7 days (dermatophytes) and then MICs were registered. To evaluate MLCs, 20µL of broth were taken from each negative tube after MIC reading, and cultured in Sabouraud dextrose agar plates and then incubated as referred above. RPMI sterility and DMSO innocuity were evaluated for each strain. All tests were performed in duplicate and the results are shown as the mean value of three independent experiments.3.2.4. Mechanism of action

The mechanism of action underlying the inhbibitory effect of the the essential oil on yeast virulence factors was considered by evaluating the effect of the oil on the germ tube formation of *C. albicans* as well as its effect on preformed biofilms.

3.2.4.1. Inhibition of germ tube formation

The capability to inhibit the formation of the germ tube on *C. albicans* was tested for the essential oil accordingly to the method described by Pinto and colleagues (Pinto *et al.*, 2009). The essential oil's serial doubling dilutions were made on DMSO starting on the MIC and 10µL of each dilution was added to sterile test tubes. The yeast *C. albicans* after an overnight incubation on SDA at 35°C was suspended on NYP medium (*N*-acetyl-D-glucosamine (Sigma) 10^{-3} M; Yeast Nitrogen Base (Difco) 3.35 g/L; Proline (Fluka) 10^{-3} M; NaCl 4.5 g/L, pH 6.7±0.1) and adjusted to the turbidity of the tube 0.5 of McFarland Scale (1.5 x 10^6 cells/mL). Then 990µL of inoculated NYP was added to the test tubes that were incubated for 3h at 37°C. Following this period 100 cells were counted on a haemocytometer and non-filamentous cells were distinguished from filamentous ones. Filamentous cells are characterized by a cylindrical filament where no constriction is observed on its base. All the cells that failed to exhibit these characteristics were considered non-filamentous. The innocuity of DMSO was tested and all tests were performed in duplicate. Results are shown as a mean ± standard deviation of three independent determinations.

3.2.4.2. Effect on preformed C. albicans biofilm

The effect of the essential oil on preformed *C. albicans* biofilm was evaluated using the method described by Taweechaisupapong (Taweechaisupapong *et al.*, 2012) with some

modifications. Briefly, a loop of SDA culture of *C. albicans* ATCC 10231 grown for 24h at 37°C was suspended in Yeast Peptone Dextrose (YPD) broth (1% yeast extract, 2% peptone, 2% dextrose) and was incubated for 24h at 37°C. The YPD was removed and the cells were thoroughly washed twice with sterile PBS pH 7.4 (0.8% NaCl, 0.02% KH_2PO_4, 0.31% $Na_2HPO_4 \cdot 12H_2O$, 0.02& KCl). Between each washing step the suspension was submitted to centrifugation for 10 min at 3000g. Cell density was adjusted to approximately 1×10^6 CFU/mL which was confirmed by cell counting in a haemocytometer. Then, 100 µL was added to selected wells in a 96-well microtiter plate and was incubated for 24h at 37°C. Then, the wells were washed three times with PBS pH 7.4. 100 µL of essential oil double serial dilutions (10 – 1.25 µL/mL) prepared in RPMI was added to the selected wells and incubated for a further 24h at 37°C. Negative and positive controls were also prepared by adding sterile RPMI broth and inoculated RPMI broth into selected wells. Results are shown as mean ± standard deviation of three independent determinations. All determination were made in duplicate.

3.2.4.2.1. Biofilm mass quantification by crystal violet assay

The biofilm mass quantification using crystal violet assay was executed based upon the method described by Raut (Raut *et al.*, 2013) with some modifications. Briefly, at the end of the steps described above the medium was removed and the cells were fixated to the well with 99% methanol for 15 min. The supernatant was removed and the wells were air-dried for 1-2 min. 100 µL of 0.02% crystal violet (CV) solution was added and left to stain the cells for 15 min. The excess of CV was removed by washing the wells 2-3 times with distilled and sterile water. Absorbed stain was released from cells with 150 µL of 33% acetic acid and the mixture was transferred to new wells. Optical density (OD) was registered at 620 nm on a microtiter plate reader. Biofilm mass was determined by comparing the OD of treated samples with the OD of control. Results are presented as mean ± standard deviation of three independent determinations performed in duplicate.

3.2.4.2.2. Biofilm viability by XTT metabolization assay

This viability assay was perfomed as described by Saharkhiz (Saharkhiz *et al.*, 2012) with some modifications. Briefly, by the end of the procedure described in section 3.2.4.2. the medium was removed and the wells were thoroughly washed with PBS pH 7.4. To a 1 mg/mL solution of XTT, menadione (10 mM in acetone) was added to a final concentration of 4 µM. 100 µL of this final solution was added to the selected wells that were incubated for 2h at 37°C in the dark. The absorbance was read at 490 nm and biofilm viability was determined by

comparing the absorbance of treated samples with those of untreated ones. Results are shown as mean ± standard deviation of three independent determinations performed in duplicate.

3.2.5. Statistical analysis

The statistical analysis comparing the control group with the different treatment groups was performed using an ordinary one-way ANOVA with Dunnett's multiple comparison test with significance levels $*p<0.05$, $**p<0.01$, $***p<0.001$ and $****p<0.0001$. These tests were conducted in GraphPad Prism version 6 (GraphPad Software, San Diego, CA, USA).

3.3. Results and discussion

3.3.1. Essential oil

3.3.1.1. Antifungal activity

The antifungal activity (MIC and MLC) of the essential oil for all tested fungal strains is summarized in table 5. The most susceptible fungal strain tested was *Cryptococcus neoformans* with a MIC and MLC of 0.16 µg/mL. For *Candida* species, the MIC ranged from 0.32 to 5 µL/mL while MLC varied from 0.32 to >10 µL/mL. The most susceptible strain was *C. guillermondii* (MIC and MLC of 0.32 µL/mL). For dermatophytes MIC ranged between 0.32 and 0.64 µL/mL and MLC oscillated between 0.32 and 1.25 µL/mL, being *E. floccosum* and *T. rubrum* the most susceptible (MIC and MLC = 0.32 µL/mL). The essential oil was able to inhibit the growth of *A. fumigatus* and *A. niger* (MIC = 2.5 and 1.25, respectively) however at the tested concentrations it failed to kill the fungi. Against *A. flavus* the oil was ineffective at all the tested concentrations. Since the values of MIC and MLC are the same for most of the strains it can be said that the essential oil had a fungicidal activity against those strains.

Table 5. Antifungal activity (MIC and MLC) of essential oil and MeOH 70% extract of *Daucus carota* subsp. *carota* for *Candida* spp., *Cryptococcus neoformans*, dermatophyte and *Aspergillus* strains.

Strains	Essential Oil		MeOH 70%		Fluconazole		Amphotericin	
	MIC[a]	MLC[a]	MIC[b]	MLC[b]	MIC[c]	MLC[c]	MIC[c]	MLC[c]
C. albicans ATCC 10231	5	5	>10	>10	1	>128	NT	NT
C. guillermondii MAT23	0.32	0.32	>10	>10	8	8	NT	NT
C. krusei H9	5	5	>10	>10	64	64-128	NT	NT
C. parapsilosis ATCC 90018	10	>10	>10	>10	<1	<1	NT	NT
C. tropicalis ATCC 13803	5	>10	>10	>10	4	>128	NT	NT
C. neoformans CECT 1078	0.16	0.16	5	>10	16	128	NT	NT
E. floccosum FF9	0.32	0.32	1.25	2.5	16	16	NT	NT
M. canis FF1	0.64	0.64	5	5	128	128	NT	NT
M. gypseum CECT 2908	0.64	0.64	5	5	128	>128	NT	NT
T. mentagrophytes FF7	0.64	0.64	10	10	16-32	32-64	NT	NT
T. mentagrophytes var. *interdigitale* CECT 2958	0.64	1.25	5	5	128	>128	NT	NT
T. rubrum CECT 2794	0.32	0.32	5	5	16	64	NT	NT
T. verrucosum CECT 2992	0.64	0.64	>10	>10	>128	>128	NT	NT
A. flavus F44	>10	>10	>10	>10	NT	NT	2	8
A. fumigatus ATCC 46645	2.5	>10	>10	>10	NT	NT	2	4
A. niger ATCC 16404	1.25	>10	>10	>10	NT	NT	01/fev	4

[a] MIC and MLC were determined by a macrodilution method and expressed in µL/mL.

[b] MIC and MLC were determined by a macrodilution method and expressed in mg/mL.

[c] MIC and MLC were determined by a macrodilution method and expressed in µg/mL.

Results were obtained from three independent determinations performed in duplicate.

3. Antifungal activity

As far as we know, only one study has been conducted reporting the antifungal activity of *D. carota* subsp. *carota* essential oil against *Candida* spp., *Cryptococcus neoformans*, *E. floccosum*, *Trichophyton* spp., *Microscoporum* spp. and *Aspergillus* spp. (Maxia *et al.*, 2009). In this study the essential oil composition was similar to that reported in the present study (geranyl acetate and α-pinene as major compounds) however α-pinene was more abundant in the essential oil used in this study, thus the weaker activity of Maxia's study might be explained.

Previous studies have shown that *C. albicans* is susceptible to oils distilled from carrot fruits as well as supercritical carbon dioxide extract (MIC of 640 μg/mL); oils obtained from herb, blooming and mature umbels demonstrate a MIC in the range of 4 and 5 μL/mL (Glisic *et al.*, 2007; Staniszewska *et al.*, 2005).

Essential oils isolated from flowering and mature umbels of other subspecies of *Daucus carota* from Portugal, namely *D. carota* subsp. *halophilus,* and *D. carota* subsp. *gummifer* have both been evaluated against yeasts (*Cryptococcus* and *Candida* strains), dermatophytes (*Trichophyton*, *Microsporum* and *Epidermophyton* strains) as well as *Aspergillus* spp. (Maxia *et al.*, 2009; Tavares *et al.*, 2008; Valente *et al.*, 2015). These studies demonstrated a similar pattern on inhibition as shown in this work, with dermatophytes and *Ceryptococcus neoformans* being the most susceptible to the action of *D.carota* essential oil.

 Previously the antifungal activity of the major compounds of *D. carota* subsp. *carota* oil (geranyl acetate, α-pinene and limonene) was assessed. Geranyl acetate was able to inhibit the growth of two *Aspergillus* strains *(A. flavus* and *A. fumigatus)* using the well diffusion method (Kakarla *et al.*, 2009) whereas using the macrodilution method, this compound demonstrated good antifungal effects against dermatophytes and *Cryptococcus neoformans*, however it had a weak performance in inhibiting the growth of *Candida* strains and *Aspergillus* spp. (Gonçalves *et al.*, 2012; Pinto *et al.*, 2013). Similarly, α-pinene showed inhibitory effects activity against *C. albicans* and *Cryptococcus neoformans* (Matsuzaki *et al.*, 2013; Rivas da Silva *et al.*, 2012). Moreover, Pinto and colleagues (Pinto *et al.*, 2013) demonstrated that this compound exhibits a strong fungistatic and fungicidal activity, being this effect preeminent for *Candida* and *Aspergillus* spp.. Several authors have also described the antifungal activity of limonene against several fungi strains (Chee *et al.*, 2009; Marei *et al.*, 2012; Pinto *et al.*, 2013; Singh *et al.*, 2010; Soković *et al.*, 2009; Ünal *et al.*, 2012). Therefore the activity of these major compounds of *D. carota* subsp. *carota* essential oil may explain the fungistatic and fungicidal activity of this oil.

The difference observed between the essential oil and the isolated compounds may be due to the presence of minor compounds that might work synergistically or antagonistically with these major compounds.

3.3.1.2. Mechanism of action

.3.1.2.1 Inhbition of germ tube formation in *Candida albicans*

The effect of sub-inhibitory concentrations of the essential oil of *D. carota* subsp. *carota* essential oil on the inhibition of *C. albicans* germ tube formation is summarized in table 6. The essential oil was able to achieve more than 50% inhibition at concentrations as low as 0.04 µL/mL (MIC/128).

Table 6. Influence of sub-inhibitory concentrations of the essential oil of *Daucus carota* subsp. *carota* on germ tube formation of *C. albicans* ATCC 10231.

	C. albicans ATCC 10231 (% of filamentous cells)
Control[a]	100
MIC	0
(Conc.[b]**)**	(5)
MIC/2	0.59 ± 1.0
(Conc.[b]**)**	(2.5)
MIC/4	0.88 ± 1.54
(Conc.[b]**)**	(1.25)
MIC/8	1.63 ± 2.82
(Conc.[b]**)**	(0.64)
MIC/16	2.52 ± 4.36
(Conc.[b]**)**	(0.32)
MIC/32	2.90 ± 1.25
(Conc.[b]**)**	(0.16)
MIC/64	21.49 ± 10.89
(Conc.[b]**)**	(0.08)
MIC/128	44.44 ± 8.60
(Conc.[b]**)**	(0.04)
MIC/256	68.54 ± 5.09
(Conc.[b]**)**	(0.02)

[a] Samples with 1% (v/v) DMSO.

[b] Concentration in µL/mL.

3.3.1.2.2 Effect of *Candida albicans* biofilm

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Figures 12 and 13 show the effect of the essential oil on preformed *C. albicans* biofilm. It can be observed that all the tested concentrations were able to reduce biofilm biomass and viability. The crystal violet method quantifies the biomass of the biofilm by staining it with the dye whereas the XTT assay evaluates cell viability by analysing the formation of a water soluble crystal whose formation occurs after mitochondrial metabolization. Results show that all the tested concentrationsof the essential oil (10-1.25 µL/mL) significantly reduced the biomass of the biofilm with *p-value* of 0.0001 and 0.001 for concentrations of 10-2.5 and 1.25 µL/mL, respectively. At the maximum concentration tested (10 µL/mL) the biomass was 52.41% of that of the control. Conversely, at the lowest concentration tested the biomass was 77.04% of that of the control. The XTT results were in accordance with those observed in the crystal violet assay. However, the differences were less significantly when compared to crystal violet assay, *p-values* of 0.05, 0.01 and 0.001 for 2.5, 5 and 10 µL/mL, respectively. At 10 µL/mL the cell survival was 68.98% of that of the control whereas at the lowest concentration (1.25 µL/mL) cell survival was of 91.27% which wasn't considered statistically significant.
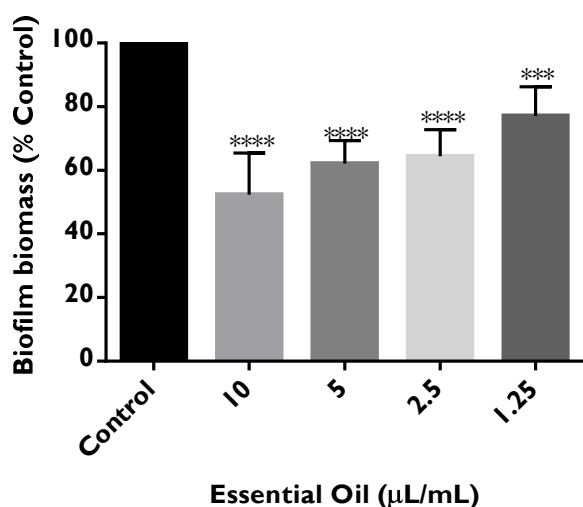
Figure 12. Biofilm biomass after treatment with essential oil using the crystal violet assay. Biofilm biomass was determined using the formula $(Abs_{620}sample/Abs_{620}control)*100$. Results are shown as mean ± standard deviation of at least three independent determinations carried out in duplicate. ***$p<0.001$, ****$p<0.0001$, compared to control using One-way ANOVA followed by a Dunnett's multiple comparison test.

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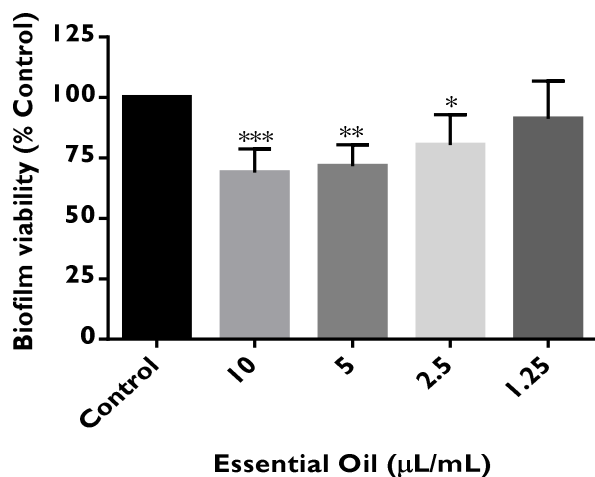
Figure 13. Biofilm viability after treatment with essential oil using the XTT viability assay. Biofilm viability was determined using the formula (Abs$_{490}$sample/Abs$_{490}$control)*100. Results are shown as mean ± standard deviation of at least three independent determinations carried out in duplicate. ***p<0.001, ****p<0.0001, compared to control using one-way ANOVA followed by a Dunnett's multiple comparison test).

As far as it is known, studies on the mechanism of action of *D. carota* subsp. *carota* essential oil or its isolated major compounds are inexistent. Therefore, the present study is the first on the mechanism of action of *Daucus carota* subsp. *carota* oil

In fact, studies regarding the effect of the essential oil of *Daucus* spp. on germ tube formation and biofilm disruption are unknown, however some authors have described those effects on other Apiaceae, namely *Coriandrum sativum* (Bersan *et al.*, 2014; Freires *et al.*, 2014), *Carum copticum* (Khan *et al.*, 2014) and *Ferulago capillaris* (Pinto *et al.*, 2013).

When compared to previous studies reporting the antifungal activity of *Daucus carota* subspecies, the present study in addition to the antifungal activity also points out a potential mechanism of action to explain the antifungal activity of the oil. Of noteworthy attention is the effect of the essential oil on the inhibition of germ tube formation and disruption of preformed biofilms in *C. albicans*. It is known that both the dimorphic transition (yeast to filamentous form) and biofilm formation are virulence factors for *C. albicans* (Mayer *et al.*, 2013; Mitchell, 1998). The essential oil demonstrated a strong inhibitory effect on the inhibition of the germ tube formation at concentrations thirty-two times lower than MIC (about 97% inhibition) which demonstrate potential to be used in the treatment of candidosis nevertheless concentrations as low as one hundred and twenty eight times below (MIC/128) were able to inhibit the filamentous cells formation by more than 50% (55.56%). The effect of the oil on the preformed biofilm was also interesting since a decrease in both biofilm biomass and viability

3. Antifungal activity

was observed at concentrations twice lower than the MIC (2.5 µL/mL, biomass of 64.41% and viability of 80.24% when compared to control), thus making it a useful treatment of medical devices with *C. albicans* biofilm. Therefore, essential oil from *D. carota* subsp. *carota* is a promising natural product to be used as an antifungal agent against these fungi that are pathogenic to humans.

3.3.2. 70% Methanol Extract

3.3.2.1. Antifungal activity

The antifungal activity (MIC and MLC) of the methanolic extract against yeasts, dermatophytes and *Aspergillus* strains is summarized in table 5. The extract was ineffective against all tested *Candida* strains, yet was able to inhibit the growth of *Cryptococcus neoformans* with a MIC of 5 mg/mL and a MLC > 10 mg/mL. Regarding the activity against dermatophytes, the extract was able to inhibit the growth of all tested strains (MIC = 1.25-10 mg/mL and MLC = 2.5 – 10 mg/mL) excluding *Trichophyton verrucosum*. All the tested concentrations had no effect on the growth of *Aspergillus* strains.

Previously, the antifungal activity of the major compounds present in the hydromethanolic extract was assessed by several authors. Bajko and colleagues (Bajko *et al.*, 2015) reported a MIC =10 mg/mL for 5-caffeoylquinic acid against *C. albicans*. In another study the MIC of 3-/4- and 5-caffeoylquinic acid was described as ranging from 0.1 to 0.2 mg/mL for *C. albicans* and *A. niger* (Xia *et al.*, 2011). The flavonoid rutin was also described as fungistatic for two *Candida* strains, namely *C. albicans* (MIC=16 mg/mL) and *C. krusei* (MIC=32 mg/mL) (Orhan *et al.*, 2010). Analysing the activity of the major isolated compounds it would be expected that the activity of the extract itself would be weaker, however the MIC values were lower than the ones described for the major compounds. This variation might be explained by the synergetic effect between phenolic compounds found in the whole extract.

4. Antioxidant activity

4.1. Introduction

In aerobic cells, mitochondria are the main site for the generation of reactive oxygen species (ROS) since univalent reduction of triplet-state molecular oxygen yields superoxide anion ($O_2^{\cdot-}$). ROS can also be produced by other enzymes such as xanthine oxidase and NADPH-oxidases. Although superoxide anion itself has a low reactivity, this species can be easily converted via enzymatic or non-enzymatic reactions into highly reactive species (e.g. hydroxyl radical and peroxynitrite radical) (Catarino *et al.*, 2015).

In order to maintain the redox homeostasis, cells have several mechanisms that control the balance between ROS and reactive nitrogen species (RNS) generation and elimination. This control is achieved by the action of both enzymatic and non-enzymatic antioxidant agents. For instance, superoxide anion is converted into oxygen and hydrogen peroxide by superoxide dismutase and the latter is further converted into water and oxygen by the enzyme catalase. Food is a source of dietary/non-enzymatic antioxidants such as polyphenols, vitamin C and E, as well as carotenoids (Catarino *et al.*, 2015; Huang *et al.*, 2005).

These systems can, however, be disrupted in some cases, such as smoking and UV exposure (Catarino *et al.*, 2015; Rahimuddin *et al.*, 2007). In these conditions the cells enter an oxidative stress state which triggers signalling cascades and induces the production of reactive species that enter in a vicious cycle as the overproduction of one species increases the production of another species (Catarino *et al.*, 2015). As result, these ROS and RNS will react with macromolecules (e.g. DNA, lipids, proteins) leading to several pathogenic conditions (Catarino *et al.*, 2015) such as Alzheimer's disease, Parkinson's disease, atheroscleorosis, cancer, arthritis, immunological incompetence and other neurodegenerative disorders (Badarinath *et al.*, 2010; Rahimuddin *et al.*, 2007; Yu *et al.*, 2005)

Antioxidant agents may perform both a direct or indirect effect. Direct effects are measured by their ability to scavenge free radicals, to quench ROS, to chelate metal ions and to inhibit lipid peroxidation (Catarino *et al.*, 2015; Tongnuanchan *et al.*, 2014). Conversely, indirect effects are mainly due to modulation of key antioxidant enzymes or interaction with receptors important to signalling pathways (Catarino *et al.*, 2015). The radical scavenging action of antioxidant agents are explained mainly by two mechanisms, namely Hydrogen Atom Transfer (HAT) and Single Electron Transfer (SET) (Catarino *et al.*, 2015; Karadag *et al.*, 2009). In HAT reactions, the hydrogen from the compounds passes to the radical resulting in the formation of a non-reactive compound. On the other hand, SET is explained by the

transference of an electron between the antioxidant agent and the radical causing its reduction (Catarino *et al.*, 2015). The most generalist HAT-based methods for the evaluation of antioxidant capacity are based upon an oxidizable molecular probe in the presence of a free radical generator and the antioxidant agent (Karadag *et al.*, 2009). Oxygen radical absorbance capacity (ORAC) and trapping antioxidant parameter (TRAP) are commonly used in HAT-based antioxidant methods that measure the capability to inhibit peroxyl radical-induced oxidations. Conversely, in SET-based methods only an antioxidant and a probe is present as in these methods the probe is the oxidant agent that will substract an electron from the agent causing a colour shift (Huang *et al.*, 2005; Karadag *et al.*, 2009). Trolox equivalent antioxidant capacity (TEAC), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) scavenging assay and ferric ion reducing antioxidant power (FRAP) are examples of SET-based methods.

The antioxidant ability of *D. carota* essential oils and extracts has been assessed in chemical models, namely using the DPPH$^{\bullet}$ (2,2'-diphenyl-1-picrylhydrazyl) scavenging assay and ABTS$^{\bullet+}$ (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) decolourization assay, metal chelating power and oxygen radical absorbing capacity (ORAC) (Guinoiseau *et al.*, 2010). Overall, the antioxidant potential of the samples was attributed to the presence of both nutritional (e.g. vitamins A, C and E) and non-nutritional (e.g. phenolic compounds, carotenoids) antioxidants (Guinoiseau *et al.*, 2010).

4.2. Material and Methods

4.2.1. Essential oil and phenolic-enriched extracts
The essential oil and the phenolic-enriched extracts (MeOH 70%, MeOH 100% and H$_2$O) were obtained as described before in section 1 of chapter V.

4.2.2. Reagents
Gallic acid, ABTS-[NH$_4$]$_2$ ([2,2'-azinobis(3-ethylbenzothiazoline-6- sulfonic acid] diammonium salt), the standard Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and Folin-Ciocalteu reagent were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium dihydrogen phosphate anhydrous was obtained from Panreac (Barcelona, Spain). AAPH (2,2'-azobis(2-amidino-propane) dihydrochloride) and fluorescein were from Acros Organics (Geel, Belgium).

4.2.3. Folin-Ciocalteu reagent based assay

Folin-Ciocalteu reagent (FCR) based assay is a SET-based method as an electron is transfered between the compound and molybdenum (Karadag *et al.*, 2009). This method can be considered an antioxidant assay as it *de facto* measures the reducing power of the sample (Huang *et al.*, 2005). However, it is more promptly used for the determination of the phenolic content in the samples. Due to this, it is rarely used in essential oils since they are rich in terpenes and terpenoids and not in polyphenols as verified in other extracts. FCR is a widely used method as it is easy, cheap and very reproducible. In addition, a very strong correlation between FCR and other antioxidant assays (e.g. DPPH, TEAC, FRAP) is found (Huang *et al.*, 2005; Karadag *et al.*, 2009). However this method has some drawbacks, namely lack of standardization as the reaction is dependent on the reactivity of the standard compound, i.e. the more hydroxyl moieties the higher the absorbance. Another drawback is that although it measures total phenolic content, the reaction can also occur in the presence of non-phenolic compounds, thus leading to erroneous conclusions (Karadag *et al.*, 2009).

The total phenolic content of the phenolic-enriched extracts was determined using the method described by Singleton and Rossi (Singleton *et al.*, 1965) with some modifications. Briefly, to 100µL of extract solution (1 mg/mL), 1500µL of distilled water and 100µL of Folin Ciocalteu reagent were added. Following a 2 min incubation, 300µL of sodium carbonate 17% (w/v) was added to the test tubes and mixed in a vortex agitator and the reaction was carried out on a water bath at 40°C for 30 min. The sample absorbance was read at 765 nm on a spectrophotometer against a negative control, which was made by using 100µL of distilled water instead of the sample. The total phenolic content was determined using a standard curve made with gallic acid (20-200 µg/mL) and the results were expressed as µg of gallic acid equivalent per milligram of dried extract (µg GAE/mg DE). Results are presented as mean values ± standard deviation of three independent assays. All determinations were performed in duplicate.

4.2.4. Antioxidant properties

4.2.4.1. ABTS radical cation assay
This method, also known as Trolox equivalent antioxidant capacity (TEAC), measures the capacity of the compound to reduce the absorbance of an intensely coloured solution of

2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS$^{\cdot+}$) (Karadag *et al.*, 2009; Wood *et al.*, 2006). The ABTS is a peroxidase substrate and can also be chemically oxidised into the radical in the presence of peroxyl radicals that can be formed in the presence of potassium persulfate (Karadag *et al.*, 2009). This method is a rather simple method thus making it widely used for the determination of antioxidant capacity (Huang *et al.*, 2005; Wood *et al.*, 2006). In addition the ABTS salt can be solubilized in either aqueous or organic solvents allowing the evaluation of both lipophilic and hydrophilic antioxidants (Huang *et al.*, 2005; Karadag *et al.*, 2009). Nevertheless this method has some limitations, namely it evaluates the capacity to react with the radical instead of the inhibition of the oxidative process. Also, the TEAC values can be false as some compounds take long periods of time to achieve the end-point. Moreover, the ABTS radical is a synthetic compound not found in biological systems (Karadag *et al.*, 2009). In this assay the reduction of the radical is achieved by either electron or H atom transfer (Wood *et al.*, 2006),

ABTS$^{\cdot+}$ decolourization assay was performed as described by Re et al. (Re *et al.*, 1999). The ABTS$^{\cdot+}$ stock solution was generated by reacting 7 mM (ABTS-[NH$_4$]$_2$) with 2.45 mM di-potassium persulfate (K$_2$S$_2$O$_8$) in the dark at room temperature for 12-16h. The working solution was prepared daily by diluting the stock solution with absolute ethanol until an absorbance of 0.700±0.03. 1mL of ABTS$^{\cdot+}$ was added to 10µL (for MeOH 70% and MeOH 100%) or to 30µL (H$_2$O) of 1mg/mL extract solutions, or to 100µL of 20mg/mL of an essential oil solution made in DMSO. Exactly after 20 min, the absorbance was read at 734 nm in a spectrophotometer against a blank (absolute ethanol). The antioxidant power of samples was expressed as IC$_{50}$ (µg/mL). All determinations were made in duplicates. Data are shown as mean values ± standard deviation of three independent assays.

4.2.4.3. Oxygen radical antioxidant capacity assay

Oxygen radical antioxidant capacity (ORAC) evaluates the capacity of the antioxidant agent to inhibit peroxyl radical-induced oxidations and is based upon H-atom donation for chain breaking (Huang *et al.*, 2005; Karadag *et al.*, 2009; Wood *et al.*, 2006). This methods is preferable as peroxyl radicals are the most common free radicals found in biological systems (Wood *et al.*, 2006). This is a fluorescent method in which the antioxidant agent is incubated with fluorescein (a fluorescent probe) at 37 °C and then AAPH (a peroxyl radical generator) is added and the fluorescence is measured for 60 min (Karadag *et al.*, 2009). The decrease in the fluorescence is a signal of oxidative damage by the action of peroxyl radicals (Karadag *et*

al., 2009). In the presence of an antioxidant agent this decay is delayed (Karadag *et al.*, 2009). ORAC is advisable for antioxidant compounds that have no lag phase. Also, different free radical generators can be used, e.g. AAPH as peroxyl generator or Cu^{2+}-H_2O_2 as a hydroxyl generator (Karadag *et al.*, 2009). However, the original method is only able to evaluate the antioxidant capacity of hydrophilic agents, whereas to detect lipophilic agents several modifications to the original methods must be made, e.g. using a solubility enhancer and a lipid soluble radical generator (Huang *et al.*, 2005; Karadag *et al.*, 2009)

Oxygen radical antioxidant capacity (ORAC) was determined using the method described by Garret et al. (Garrett *et al.*, 2010) with some modifications. Briefly, a working solution of fluorescein 10 nM was made by diluting a stock solution of 100 µM in phosphate buffer (75 mM, pH 7.4) and a Trolox solution (200-25 µM) was made by diluting a stock solution of 1 mM in phosphate buffer. 150µL of fluorescein was pipetted to a 96-ewell plate and 25 µL of Trolox or sample (0.5 – 0.01 mg/mL for extracts and 10 – 0.32 mg/mL for essential oil) was added. For negative and positive controls, 25 µL of phosphate buffer was used. This mixture was incubated at 37°C for 10 min for temperature stabilization as AAPH-dependent peroxyl radical formation is optimal at this temperature. After that, 25 µL of AAPH (153 mM) was added to all wells except that of negative control that contained 25 µL of phosphate buffer. The fluorescence was immediatly read on a plate reader every 1 minute with 5 seconds shaking prior to each reading, in a total of 60 min. The emission wavelength was set at 530/20 nm and excitation wavelength at 485/20 nm. Area under the curve (AUC) was determined by the following equation (1)

$$AUC = 1 + \sum_{t_0 = 60\ min}^{t_i = 60\ min} \frac{A_i}{A_0} \quad (1)$$

Where A_0 is the fluorescence at t=0 min and A_i is the fluorescence at t=i. Results are expressed as Trolox Equivalent (TE)/mg DW of each sample range which is determined by the equation (2) where mSample is the slope of the linear regression analysis of the sample and mTrolox is the slope of the linear regression analysis of Trolox:

TE (range of concentration) = mSample/mTrolox (2)

Results are shown as mean ± S.E.M of at least three independent experiments. All determinations were made in duplicate.

4.2.5. Statistical analysis

The statistical analysis comparing the control group with the different treatment groups was performed using an ordinary one-way ANOVA with Dunnett's multiple comparison test with significance levels $*p<0.05$, $**p<0.01$, $***p<0.001$ and $****p<0.0001$. These tests were conducted in GraphPad Prism version 6 (GraphPad Software, San Diego, CA, USA).

4.3. Results and Discussion

4.3.1. Folin-Ciocalteu reagent based assay

The total phenolic content (TPC) of the extracts was determined using the Folin-Ciocalteu method. The results showed that TPC content was very similar in all three samples showing no statistical differences between them. The TPC of aqueous extract was 118.1 ± 9.7 µg GAE/mg DW while (hydro)methanolic extracts had a very similar TPC value i.e. 134.2 ± 10.3 and 134.6 ± 1.9 µg GAE/mg DW for MeOH 70% and MeOH 100%, respectively (Figure 14).
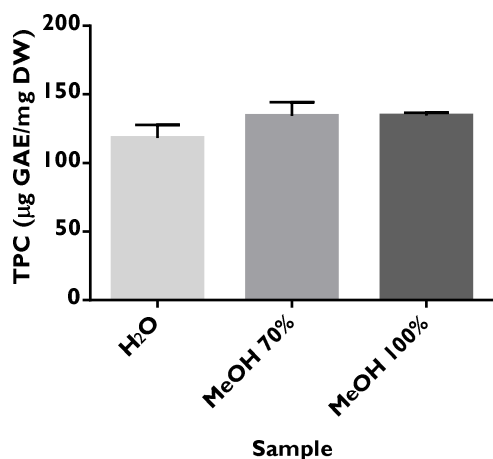
Figure 14 - Total phenolic content (TPC) of phenolic-enriched extracts. Results are shown as mean ± S.E.M of three independent determinations made in duplicate.

To the author´s knowledge, there are no studies regarding the total phenolic content of the aerial parts of carrot, being the reported data limited to carrot roots. Leja et al. (Leja *et al.*, 2013) assessed the TPC in hydroacetone extracts from roots of white, yellow, orange, red and purple carrots and the values were 18, 21.5, 29.3, 31 and 245.7 µg GAE/100 mg FW. Algarra and colleagues (Algarra *et al.*, 2014) described that TPC was 9.4, 187.8 and 492.0 µg

GAE/100 mg FW for 50% ethanol extracts obtained from orange, black and purple carrots, respectively. Koley and colleagues (Koley *et al.*, 2014) tested several 80% acetone extracts from Indian cultivars with red, orange and purple carrots and TPC values ranged from 6.41 to 290.3 µg/100 mg FW. TPC of 80% ethanol carrot root extracts varied from 21 to 87 mg GAE/100 g FW (Singh *et al.*, 2012). Since these values were expressed as µg GAE/100 mg FW and the herein obtained are presented in µg GAE/mg DW, a direct comparison between them is not possible.

4.3.2. ABTS$^{.+}$ scavenging ability

Figure 15 show the ABTS$^{.+}$ scavenging capacity of the tested samples expressed as IC_{50}. Among them, the essential oil had the weakest activity (IC_{50}=1924 ± 124.2 µg/mL. Conversely, the phenolic-enriched extracts had much better activity, being the aqueous extract the weakest amongst them (IC_{50}=142.2 ± 23.4 µg/mL). Again, both methanolic extracts have a very similar activity, IC_{50}= 44.6 ± 3.2 and 42.54 ± 1.7 µg/mL for 70% and 100% methanol extracts, respectively.

Up to date, no studies of TEAC using extracts or essential oils from aerial parts of *D. carota* have been described, with investigations focusing primarily on carrot roots. Koley and colleagues (Koley *et al.*, 2014) analysed several red, orange and purple carrot cultivar extracts and demonstrated that the TEAC of those samples ranged between 1.63 and 57.53 µmol Trolox/g FW. In another study, 80% methanol extracts of carrot roots showed a TEAC ranging from 0.170 to 1.480 mmol TE/100 g DW (Morales-Soto *et al.*, 2014). Yu and colleagues (Yu *et al.*, 2005) obtained a methanol extract of carrot seed oil which had an activity of 8.90 µmol TE/g oil and TEAC of 80% acetone extracts of red, purple and orange carrot roots ranged from 1.63 to 57.53 µmol TE/g FW (Koley *et al.*, 2014).
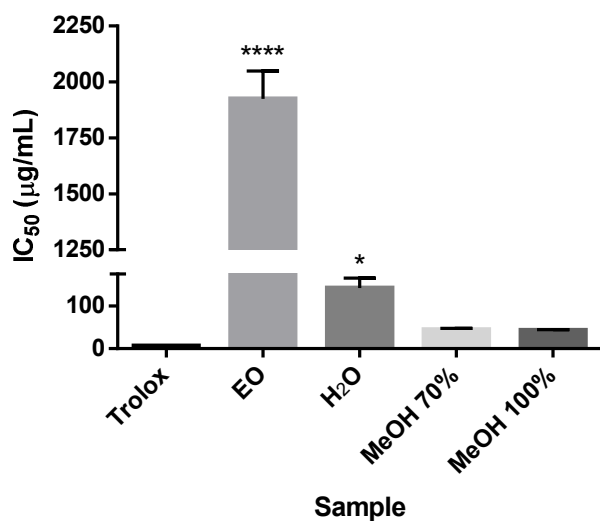
Figure 15 - Trolox Equivalent Antioxidant Capacity (TEAC) of the essential oil and phenolic-enriched extracts using ABTS cation radical scavenging assay. Results are shown as mean ± S.E.M of at least three independent assays, done in duplicate. Statistically significant differences when compared to Trolox (*$p<0.05$, ****$p<0.001$, using one-way ANOVA followed by Dunnett's multiple comparision test).

Moreover, studies evaluating the antioxidant potential through ABTS and DPPH of the major compounds of *D. carota* subsp. *carota* essential oil (geranyl acetate, α-pinene, 11αH-himachal-4-ene-1-β-ol and limonene) are absent and hence, any hypothetical contribution of these compounds for the activity of the sample cannot be inferred. However, studies using some major compounds of the extracts are available. In fact, Yuan and colleagues (Yuan *et al.*, 2012) evaluated the IC_{50} using the ABTS$^{·+}$ scavenging assay of several caffeyolquinic and dicaffeoylquinic acids and the values were in the range of 2.79 and 6.27 µM. The IC_{50} of 1,3-dicaffeoylquinic acid was 12 µM in the ABTS$^{·+}$ scavenging assay (Danino *et al.*, 2009). Rutin, a major flavonoid in the extracts, had an IC_{50} of 5.65 µg/mL in the DPPH assay, although this test was not performed in this study, it serves to elucidate that rutin is able to scavenge radicals via single-electron transfer. These studies are elucidative about the activity of the whole extracts. The differences observed are explained by the presence of synergic or antagonistic minor compounds in the extracts.

4.3.3. Oxygen Radical Antioxidant Capacity (ORAC) assay

Figure 16 shows the ORAC of the different tested samples expressed in TE (Trolox equivalents)/mg DW. Similarly to what has been described for the ABTS$^{·+}$ scavenging assay, the essential oil had the weakest performance with an activity of 7.13 ± 1.5 µmol TE/mg oil.

5. Anti-inflammatory activity

The MeOH 100% and H_2O extracts had a similar activity, 4.3 ± 1.1 and 4.5 ± 1.1 µmol TE/g DW, respectively. The extract made with 70% methanol had a slightly weaker activity however still stronger than the essential oil, 3.1 ± 0.7µmol TE/g DW.
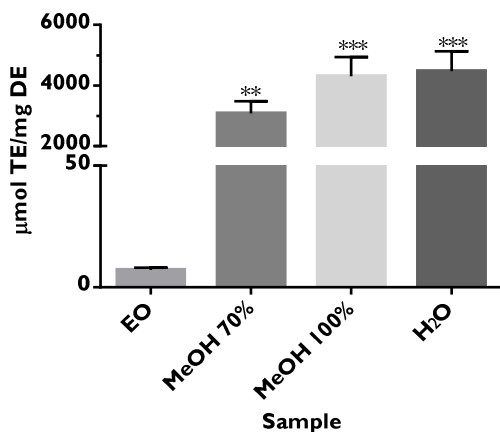
Figure 16 - Oxygen Radical Scavenging Capacity (ORAC) of the essential oil and phenolic-enriched extracts. TE was determined by the ratio of the slope of the linear regression of each sample with that of a Trolox standard curve. Results are shown as mean ± S.E.M of at least three independent assays done in duplicate. Significant differences compared to standard compound, Trolox (**$p < 0.01$, ***$p < 0.001$, using a one-way ANOVA followed by a Dunnett's multiple comparision test)

In previous assays, a methanol extract of carrot seed oil had an ORAC activity of 160 µmol TE/g oil (Yu *et al.*, 2005) wheareas a 80% ethanol extract had and ORAC activity ranging from 0.48 to 1.17 mmol Trolox/100 g FW (Singh *et al.*, 2012).

The flavonoid rutin was also tested for its ORAC and showed an IC_{50} of 12.041 mmol TE/g DW (Wang *et al.*, 2013). Although the activity of this phenolic compound is higher than the ones described by the studied extracts, the amount of rutin in our extracts is rather low thus the activity is not as preeminent as the isolated compound. In addition, the presence of minor phenolic compounds with antagonistic effect on rutin might also occur.

5. Anti-inflammatory activity

5.1. Introduction

The inflammation is a pathophysiological response of living tissue to either a parasitic invasion or an injury/trauma, as well as other hostile conditions not related to host defence or tissue repair in order to restore body homeostasis (Ashley *et al.*, 2012; Medzhitov, 2008; Vasudevan *et al.*, 2006). During this process an increase in the permeability of endothelial cells is verified, leukocytes migrate to the interstitium, an oxidative burst occurs and cytokines both pro- and anti-inflammatory are released. In addition, several enzymes are induced which leads to the metabolization of arachidonic acid into eicosanoids, prostaglandins (PGs) and thromboxane A2. Likewise, the expression of adhesion molecules, namely intracellular adhesion molecules (ICAM) and vascular cell adhesion molecules (VCAM) is also increased (Miguel, 2010).

Key players in the immune response are the activated macrophages, which are responsible for the production of numerous pro-inflammatory mediators, namely pro-inflammatory cytokines, nitric oxide (NO), PGs, and Reactive Oxygen Species (ROS) (Medzhitov, 2008). NO synthase is responsible for the formation of NO from L-arginine and molecular oxygen, while cyclooxygenase catalyses the production of PGs via the arachidonic acid pathway. These enzymes have inducible forms, iNOS and COX-2, respectively, which are activated by several inflammatory stimuli (e.g. LPS, cytokines, and nitric oxide). Their pathways are closely related and NO can stimulate COX-2 activity via interaction with the heme component (Dudhgaonkar *et al.*, 2004). It is known that the presence of high amounts of NO is an inflammation marker. Hence, in this context, the capacity to inhibit its production can be used to track new anti-inflammatory agents.

The expression of pro-inflammatory mediators is regulated by various transcription factors and signalling pathways. One transcriptional factor that regulates the expression of several genes is nuclear transcription factor kappa B (NF-κB), which is found in its inactivated state due to the presence of IκB inhibitor protein (seven members containing IκBα). Upon pro-inflammatory stimuli phosphorylation of IκBα occurs, causing NF-κB to be released and to be translocated to nucleus where it regulates gene transcription by binding to target genes. The binding of NF-κB to nuclear DNA leads to the expression of several genes that concomitantly induce the expression of pro-inflammatory cytokines, e.g. interleukine-1β and Tumor Necrosis Factor α (TNF-α), chemokines and other co-stimulatory molecules (Ashley *et al.*, 2012; Scheidereit, 2006; Wong *et al.*, 2009).

5. Anti-inflammatory activity

Impaired inflammatory responses are implicated in several chronic diseases such as cancer (Khatami, 2012; Porta *et al.*, 2009), obesity, diabesity (Schmidt *et al.*, 2003), rheumatoid arthritis, cardiovascular diseases, neurodegenerative diseases (Hunter *et al.*, 2010), inflammatory bowel disease (Predonzani, 2015) and aging. Currently, these diseases are a major burden despite the recent success in biopharmaceuticals. Absence of responsiveness and drug resistance, delivery problems and the cost of biopharmaceutical drugs validate the search for new anti-inflammatory agents and the development of new approaches to control these diseases. The perfect anti-inflammatory drug would be able to relieve pain and inflammation, along with the capacity to slow down, stop or, even better, prevent the disease progression (Zuzarte, 2012).

Traditional medicine has been using plants since immemorial times to alleviate inflammatory disorders and has been providing front-line pharmacotherapy for millions of individuals worldwide (Recio *et al.*, 2012). Medicinal plants are, therefore, a potential source of biologically active molecules, as well as a promising avenue for the discovery of new drugs (e.g. aspirin which is derived from salicylic acid from *Salix* species) (Recio *et al.*, 2012). In fact, several lines of investigation are based on the anti-inflammatory activity of essential oils and phenolic-enriched extracts and/or their constituents (Zuzarte, 2012).

Plant-derived pharmacological agents belong to a wide panoply of chemical compounds such as flavonoids, terpenoids, quinones, catechins, alkaloids, amongst others, which are known to modulate the pro-inflammatory signals (Recio *et al.*, 2012), scavenge free radicals, modulate the arachidonic acid metabolism as well as the production of cytokines (Zuzarte, 2012).

In what concerns the anti-inflammatory activity of *Daucus carota*, only a few studies have been conducted. Momin and colleagues (Momin *et al.*, 2003) demonstrated that *trans*-asarone, 2,4,5-trimethoxybenzaldehyde and oleic acid isolated from *Daucus carota* seed hexane extract were able to inhibit COX activity, being 2,4,5-trimethoxybenzaldehyde specific for COX-2. Moreover, the ethanolic extract of carrot seed was able to inhibit carrageenan-, histamine- and serotonin-induced paw oedema as well as formaldehyde-induced arthritis. In addition, this extract demonstrated analgesic and antinoceptive properties (Vasudevan *et al.*, 2006). A chromatographic fraction of purple carrot seed methanolic extracts was able to reduce NO, pro-inflammatory cytokines and iNOS levels. The authors associated the NO

reduction to the presence of polyacetylenes (falcarinol, falcarindiol and falcarindiol-3-acetate) in the fraction (Metzger _et al._, 2008).

Noteworthy many authors do not identify the subspecies used in their works and this is an important aspect to consider taking into account the high intraspecific polymorphism of this species, as well as the economic potential of the subspecies _carota_. Having this problematic in mind, the anti-inflammatory activity of the essential oil from _Daucus carota_ subs _carota_ was herein assessed for the first time.

5.2. Material and methods

The anti-inflammatory potential of the essential oil was performed using both a chemical (NO$^\bullet$ cavenging assay using SNAP as a NO donor) and a cellular model (LPS-stimulated RAW 264.7 macrophages). The anti-inflammatory assay was only considered for the essential oil as it was the most promising sample in both antifungal and antibacterial properties.

5.2.1. Essential oil
The essential oil used in these assays was the same used in the previous ones.

5.2.2. Materials
Reagents were purchased from Sigma Chemical Co. (Saint Louis, MO). Specifically, Dulbecco's Modified Eagle Medium and fetal bovine serum were obtained from Gibco (Invitrogen, Paisley, UK).

5.2.3. Nitric oxide scavenging assay in a chemical model
NO scavenging assay was performed using a chemical model in accordance with previous studies conducted by our team (Valente *et al.*, 2015). Briefly, 6 µL of serial double dilutions of essential oil (1.25 – 0.08 µL/mL) was added to 300 µL of DMEM in a 48-well plate. Then, 0.9 µL of S-nitroso-N-acetyl-D,L-penicillamine (SNAP, 100mM) was used as a NO donor and added to the wells. The plate was incubated at 37°C for 3h. At the end of reaction time, 170 µL of supernatant was collected to 96-well plates and 170 µL of Griess Reagent (Sulphanilamide 1% (m/v) on H_3PO_4 5% (m/v), *N*-(1-naphtyl)-ethyldiamine 0.1% (m/v); 1:1) was added and left to stand for 30 min in the dark, at room temperature. The absorbance was read at 550 nm on a plate reader without filters, against a negative control (only DMEM) and a positive control (DMEM + SNAP). The nitrite concentration was determined from a linear regression analysis using serial dilutions of sodium nitrite as standard (50 – 0.000 mM). All determinations were made in duplicate. The results are shown as mean ± standard deviation of three independent determinations.

5.2.4. Cell culture
RAW 264.7, a mouse leukaemic macrophage cell line (ATCC (TIB-71) was cultured in endotoxin-free Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) non-inactivated foetal bovine serum, 3.02 g/L sodium bicarbonate, 100 µg/mL streptomycin and 100 U/mL penicillin at 37°C, in an humidified atmosphere of 95% air and 5% CO_2. The cells were used when the confluence was 80-90%. Viable cells were counted on a

haemocytometer using Trypan blue dye. Morphological changes was monitored microscopically.

5.2.5. Cell Viability

To evaluate the cytotoxicity of the oil, the resazurin assay was used according to Riss (2013) with slight modifications Macrophages (0.3×10^6 cells/well) were plated in 48-well microplates and allowed to stabilize for 12h. Then, cells were either maintained in culture medium (control) or incubated with different concentrations (1.25 – 0.08 µL/mL) of the essential oil for 24h. After treatment, the resazurin solution (C=0.125 mg/mL) was added (1:10) and cells were incubated at 37°C for 30 min in a humidified atmosphere of 95% air and 5% CO_2. Quantification was performed using an ELISA microplate reader (SLT, Austria) at 570 nm, with a reference wavelength of 620 nm. A cell-free control was performed in order to exclude non-specific effects of the oils on resazurin (data not shown).

5.2.6. In vitro anti-inflammatory activity

To evaluate the anti-inflammatory potential of the oil, macrophages (0.3×10^6 cells /well) were cultured in 48-well microplates and allowed to stabilize for 12h. Following this period, cells were either maintained in culture medium (control) or pre-incubated with different concentrations of the essential oil for 1h, and later activated with LPS (1 µg/mL) for 24h. The production of NO was evaluated by measuring the accumulation of nitrites in the culture supernatants, using the colorimetric Griess reagent (Green et al., 1982). Briefly, an aliquot of culture medium was diluted with equal volume of the Griess reagent and maintained during 30 min, in the dark. The absorbance was measured using an ELISA microplate reader (SLT, Austria) at 550 nm. Culture medium was used as blank and nitrite concentration was determined from a linear regression analysis using serial dilutions of sodium nitrite as standard.

5.2.7. Statistical analysis

The statistical analysis comparing the control group with the different treatment groups was performed using an ordinary one-way ANOVA with Dunnett's multiple comparison test. These tests were applied using GraphPad Prism version 6 (GraphPad Software, San Diego, CA, USA).

5.3. Results and Discussion

5.3.1. Nitric oxide scavenging assay using a chemical model

The figure 17 shows the nitric oxide scavenging capacity of the essential oil expressed in mM of nitrites after the treatment of culture medium with essential oil following the addition of the NO donor, SNAP. It can be seen that for the tested concentrations (1.25 – 0.08 µL/mL), the essential oil showed no ability to scavenge the NO release triggered by SNAP.
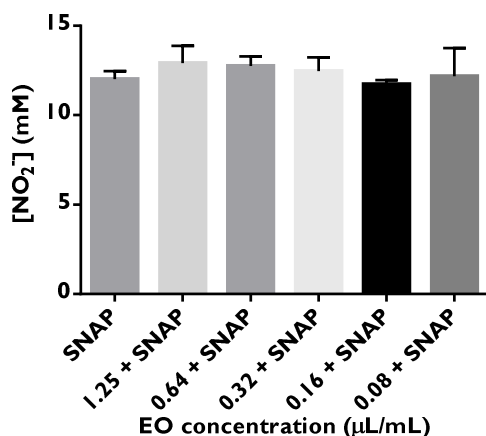
Figure 17 - Nitric oxide scavening of *Daucus carota* subsp. *carota* essential oil. Results are shown as mean ± S.E.M of three independent assays, done in duplicate. No statistically significant differences were found using a one-way ANOVA followed by a Dunnett's multiple comparison test.

Up to date, no studies regarding the nitric oxide inhibition capacity of the essential oils from *Daucus carota* subsp. *carota* have been conducted. However, Valente and colleagues (Valente *et al.*, 2015) performed some studies on *Daucus carota* subsp. *gummifer* and showed that the essential oil from this subspecies had significant NO scavenging activity (about 30% NO reduction at 0.08 µL/mL), using the same method. However, the essential oil of *D. carota* subsp. *gummifer* has a distinct chemical composition from that of *Daucus carota* subsp. *carota*, reported in this study, being the main compounds in subsp. *gummifer trans-β*-ocimene (31.3%), sabinene (29.0%) and *cis-β*-ocimene (12.3%). Moreover, the NO scavenging of sabinene was previously reported (about 30% NO reduction at 0.64 µL/mL) (Valente *et al.*, 2013), and this could partially contribute for the NO scavenging activity of subsp. *gummifer*. Regarding the subsp. *carota*, sabinene is not a major compound and no studies have been reported for the main compounds identified in the oil in the present study (geranyl acetate, α-pinene, 11αH-himachal-4-ene-1-β-ol and limonene).

5.3.2. Nitric oxide scavenging in a cellular model

As shown in Figure 18, incubation of macrophages (Fig. 18a) with LPS, for 24h, resulted in a significant increase in nitrite production compared to the control (1.29 µM vs 25.21 µM). Taking into account the toxicity of the oil presented in Figure 18b and described in the following section (6. Cytotoxicity), inhibition of NO production was only considered for non-toxic concentrations of the oil (i.e. up to 0.64 µL/mL). Therefore, the results obtained show that the treatment of LPS-stimulated macrophages with 0.64 µL/mL of *D. carota* subsp. *carota* essential oil caused a decrease around 19% on the production of NO (Fig. 18a; p<0.05).

Figure 18 - Anti-inflammatory effect of *Daucus carota* subsp. *carota* on LPS-stimulated RAW 264.7 macrophages: a) Nitric oxide production and b) cell viability. The cells were treated with essential oil (1.25 – 0.08 µL/mL) for 1h prior to LPS (1µg/mL) addition and incubated for another 24h before NO release evaluation and resazurin (0.125 mg/mL) was added to assess cell viability. Results are shown as mean ± S.E.M of at least three independent assays. Significant difference when compared to LPS-treated cells (*p<0.05; **p<0.01; ***p<0.0001 using one-way Anova followed by Dunnett's multiple comparison test).

Studies regarding the anti-inflammatory properties of *D. carota* essential oil are very scarce. Some authors have demonstrated the anti-inflammatory potential of other Apiaceae namely *Oenanthe crocata* (Valente *et al.*, 2013)*, Distichoselinum tenuifolium* (Tavares *et al.*, 2010) and *Anethum graveolens* (Kazemi, 2014). Moreover, the anti-inflammatory potential of *D. carota* subsp. *gummifer* essential oil was also evaluated using the same inflammation model (Valente *et al.*, 2015). This subspecies oil was also able to reduce NO production at 0.64 µL/mL although a higher inhibition was achieved (65.15%). This difference is probably explained by the different chemical composition of both oils, since *D. carota* subsp. *gummifer* is enriched in *trans-β-*

ocimene, sabinene and *cis-β*-ocimene which are known to be anti-inflammatory (Valente *et al.*, 2013).

The anti-inflammatory properties of some major compounds identified in the present study in the oil of *D. carota* subsp. *carota* have also been described. Rufino and colleagues assessed the anti-inflammatory properties of both α-pinene (68.5% of NO inhibition at 200 µg/mL) and limonene (IC_{50} of 85.3 µg/mL) (Rufino *et al.*, 2014, 2015). Kazemi (Kazemi, 2014) also evaluated the anti-inflammatory effect of limonene (45 µM reduces the NO production to 70.87%). Moreover, geranyl acetate is able to reduce the NO production from 125% (LPS-treated cells) to 100% at 0.64-0.32 µg/mL without showing toxicity (Gonçalves *et al.*, 2012). The activity of these major compounds might partially explain the activity of the *D. carota* subsp. *carota* essential oil although minor compounds may have an antagonistic effect, thus slightly reducing the antin-inflammatory effect

5.3.2. Nitric oxide scavenging in a cellular model

As shown in Figure 18, incubation of macrophages (Fig. 18a) with LPS, for 24h, resulted in a significant increase in nitrite production compared to the control (1.29 µM vs 25.21 µM). Taking into account the toxicity of the oil presented in Figure 15b and that described in the following section, inhibition of NO production was only considered for non-toxic concentrations of the oil (i.e. up to 0.64 µL/mL). Therefore, the overall obtained results allowed to conclude that the treatment of LPS-stimulated macrophages with the essential oil of *D. carota* subsp. *carota* caused a decrease on the NO production, by about 19% (Fig. 15a; $p < 0.05$).
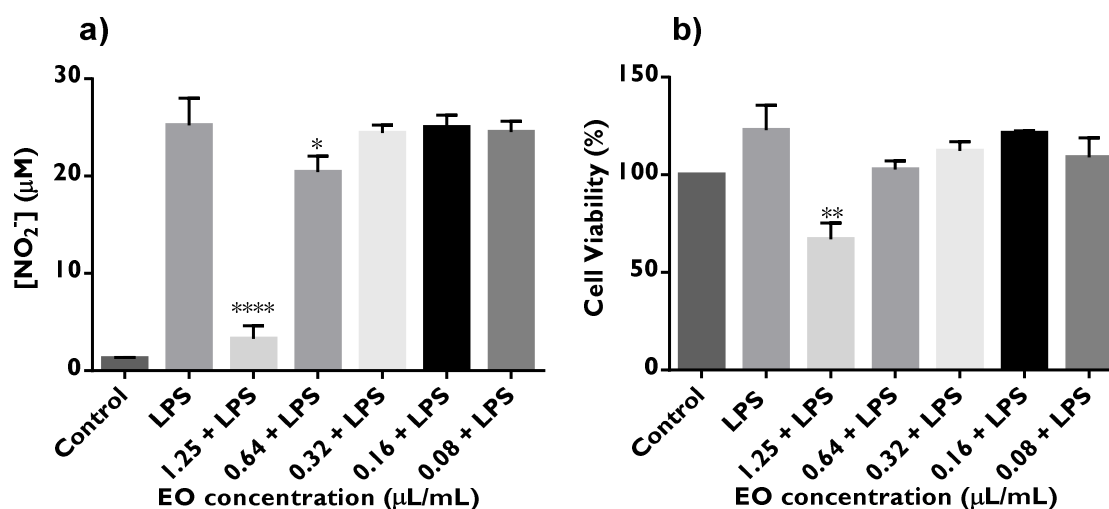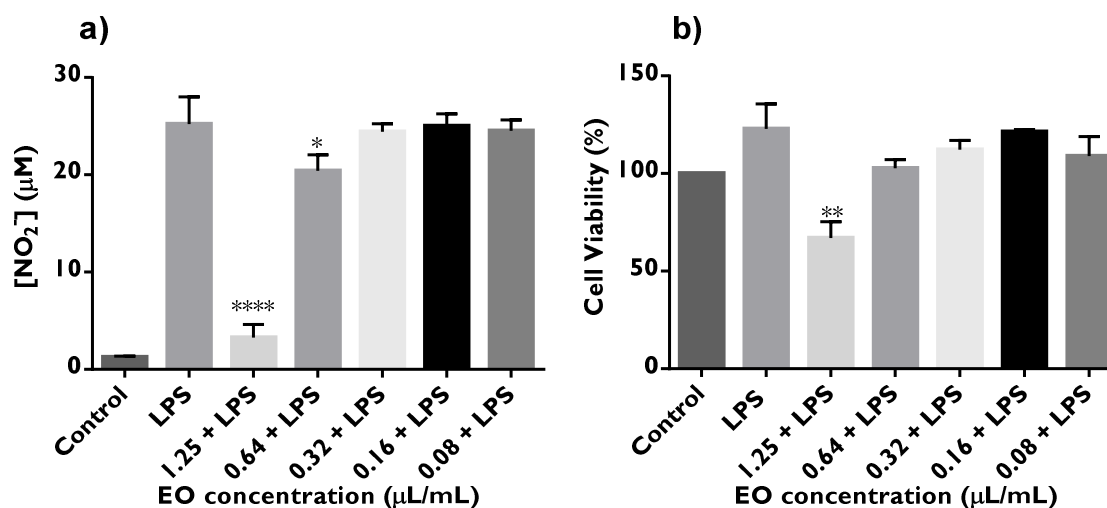

Figure 19 - Anti-inflammatory effect of *Daucus carota* subsp. *carota* on LPS-stimulated RAW 264.7 macrophages: a) Nitric oxide production and b) cell viability. The cells were treated with essential oil (1.25 – 0.08 µL/mL) for 1h prior to LPS (1µg/mL) addition and incubated for another 24h before NO release evaluation and resazurin (0.125 mg/mL) was added to assess cell viability. Results are shown as mean ± S.E.M of at least three independent assays. Significant difference when compared to LPS-treated cells (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ using one-way Anova followed by Dunnett's multiple comparison test).

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trans-β-ocimene, sabinene and *cis-β*-ocimene which are know to be anti-inflammatory (Valente *et al.*, 2013).

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6. Cytotoxicity

6.1. Introduction

Although medicinal and aromatic plants are long used in traditional medicine, many usages are based upon anecdotal evidence instead of scientific research thus the need to implement thoughtful and well-designated studies in order to avoid misleading reports as well as to elucidate their efficacy and safety. In addition to animal as well as clinical evidences, safety assays must also be considered. Lately, *in vitro* toxicity assays have been developed with the aim to substitute animal testing methods in early phases of drug development (Bhanushali *et al.*, 2010).

The usage of natural products in the food and health industry as a source of bioactive compounds has become more and more popular, in addition to their recognized usage in cosmetic and perfumery industries (Lahlou, 2004). Currently in aromatherapy, aromatic plants and their essential oils are greatly used due to their effect on the central nervous system, namely mood, anxiety and depression improvement (Edris, 2007). The application of essential oils is normally achieved via inhalation, topical application or oral administration (Reichling *et al.*, 2009). The evaluation of the sensitization, carcinogenicity and toxicity of essential oils is constantly carried out by several associations/programs, such as Research Institute for Fragrance Materials (RIFM), International Flavour and Fragrance Association (IFRA), Flavour Essence Manufacturers Association (FEMA) and National Toxicology Program (NTP) with the purpose of establishing their No Adverse Effect Level (NOAEL) (Vigan, 2010).

Toxicity assays are generally assessed to determine the cytotoxic effect of the essential oils and their isolated compounds using specific staining or fluorescent dyes. The most common assays performed include neutral red uptake (NRU) (Nathalie *et al.*, 2006; Söderberg *et al.*, 1996; Stammati *et al.*, 1999), 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) test (Chung *et al.*, 2007; Sun *et al.*, 2005; Yoo *et al.*, 2005), alamar blue (resazurin) test (O'Brien *et al.*, 2000), trypan blue exclusion test (Horvathova *et al.*, 2006; Slameňová *et al.*, 2007), propidium iodide test (Dušan *et al.*, 2006), and more recently, unscheduled DNA synthesis (to detect the presence and removal of adducts of DNA) (Burkey *et al.*, 2000). MTT assay and other tetrazolium salts (e.g. XTT) are characterized by the reduction into formazan, a coloured product that can be detected spectrometrically (Riss *et al.*, 2013). MTT conversion into purple formazan, which is insoluble in the culture medium, is thought to be mediated by a reduction reaction catalysed by either NADH or other reducing molecules that transfer electrons to MTT. Other spectulation explains this conversion by the envolvement of

mitochondrial enzymes, thus MTT is sometimes considered as a measurement of mitochondrial activity (Riss *et al.*, 2013). Resazurin is a cell permeable redox indicator which is metabolized by metabolically-active cells into resorufin, changing from deep-blue and non-fluorescent dye (resazurin) into a pink and fluorescent product (resorufin). Resazurin is thought to demonstrate toxicity to cells after long periods of incubation, thus the incubation time should be enough to provide sensitivity without showing toxicity (Riss *et al.*, 2013).

For those assays, cells lines are normally preferred as compared to the use of primary cell cultures because they are fully characterized and more easily cultured (Zuzarte, 2012). Overall, the majority of toxicity studies have been performed on human fibroblasts or skin epithelial cells, although tumour cell lines are also used (Reichling *et al.*, 2009). The cytotoxic effect of the essential oils is commonly estimated for a wide range of concentrations (5.0-1950 µg/mL), depending on the incubation time, which can vary from 1-96h (Reichling *et al.*, 2009).

Despite their usefulness, it is important to note that *in vitro* assays may overestimate the real *in vivo* toxicity since cell culture models do not take in account the tissue structure nor the biotransformation and transport processes that occur in vivo. Truly, these assays use monolayers of cells in which the essential oil/compound is in direct contact with the medium. Hence, this is the worst scenario possible and is unlikely to occur *in vivo* (Reichling *et al.*, 2009). Due to this difference between *in vitro* and *in vivo* toxicity, the need to find correlation models that predict the *in vivo* toxicity based upon *in vitro* assays have arisen (Forsby *et al.*, 2009). Moreover, the toxicity of essential oils is dependent on the composition, route of administration as well as the patient health (Vigan, 2010). The effects of these compounds varies from toxic effects (*e.g.* Hayes e Markovic, 2003; Prashar, Locke e Evans, 2004) to adverse reactions such as local irritation on skin and mucous membranes and allergic reactions (Schnuch *et al.*, 2004; Veien, 2004). The cytotoxic effect is thought to be due to the induction of either apoptosis or necrosis (Bakkali *et al.*, 2008).

Previously, the cytotoxicity of *Daucus carota* has been evaluated by a few authors. The essential oil of *Daucus carota* subsp. *halophilus* was tested against a foetal mouse skin dendritic cell line (Tavares *et al.*, 2008). The cytotoxicity of a pentane-based fraction of *Daucus carota* was also evaluated against two different cell lines from human breast adenocarcinoma (MDA-MB-231 and MCF-7; Shebaby *et al.*, 2014). A methanol:acetone extract from wild carrot was assessed against several acute myeloid leukaemia cell lines (HL-60, U937, ML1, ML2, Mono-Mac-1, Mono-Mac-6, KG-1, MV-4-11, TF1-vRaf, TF1-vSrc and TF1-HaRas) as well as human

peripheral blood mononuclear cells (PBMCs) (Tawil *et al.*, 2015). Against non-cancer cell lines, such as dendritic cell line (1.25 µL/mL; Tavares *et al.*, 2008) and PBMCs (>100 µg/mL; Tawil *et al.*, 2015) the oil demonstrated toxicity at higher concentrations, whereas for cancer cell lines the toxicity was demonstrated at lower concentrations (IC_{50} = 1 – 26.2 µg/mL; (Tawil *et al.*, 2015).

In the present study, the essential oil from *Daucus carota* subsp. *carota* was selected to perform toxicity assays since it demonstrated the most interesting antifungal and antibacterial effects and also showed an anti-inflammatory potential The cytotoxicity of the oil was assessed on several mammalian cell lines, namely keratinocytes (HaCat), alveolar epithelial cells (A549), macrophages (RAW 264.7) and the human hepatocellular carcinoma (HepG2), in order to mimetize the different routes of oil administration (oral, topical or through inhalation) and determine the practical relevance of the referred biological effects.

6.2. Material and methods

6.2.1. Essential oil
The essential oil used in these assays was the same used in the previous assays.

6.2.2. Reagents and Cell culture
Foetal calf serum was purchased from Biochrom KG (Berlin, Germany). DMEM (Dulbecco's modified Eagle's medium) and trypsin were from Gibco (Invitrogen, Paisley, UK) (XX, YY, ZZ). . All the remaining reagents and chemicals were purchased from Sigma-Aldrich Co. (St. Louis, USA).

The Human keratinocyte cell line, HaCaT (Figure 19a), obtained from DFKZ (Heidelberg, Germany) and the human alveolar epithelial cell line, A549 (Figure 19d), obtained from ATCC (number CCL-185) were cultured in DMEM medium supplemented with 10 % (v/v) inactivated foetal bovine serum, 3.5 g/L glucose, 3.02 g/L sodium bicarbonate, 100 µg/mL streptomycin and 100 U/mL penicillin at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

The Human hepatocellular carcinoma cell line, HepG2 (Figure 19b), obtained from ATCC (number HV-8065) was cultured in DMEM Low-Glucose medium supplemented with 10 % (v/v) inactivated foetal bovine serum, 1.5 g/L sodium bicarbonate, 100 µg/mL streptomycin and 100 U/mL penicillin at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

Mouse leukaemic monocyte macrophage cell line, RAW 264.7 (Figure 19c), purchased from ATCC (number TIB-71) was cultured in DMEM supplemented with 10% (v/v) non-inactivated foetal bovine serum, 3.5 g/L glucose, 3.02 g/L sodium bicarbonate, 100 µg/mL streptomycin and 100 U/mL penicillin at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

All cell lines were microscopically monitored in order to detect any morphological change during the experiments.
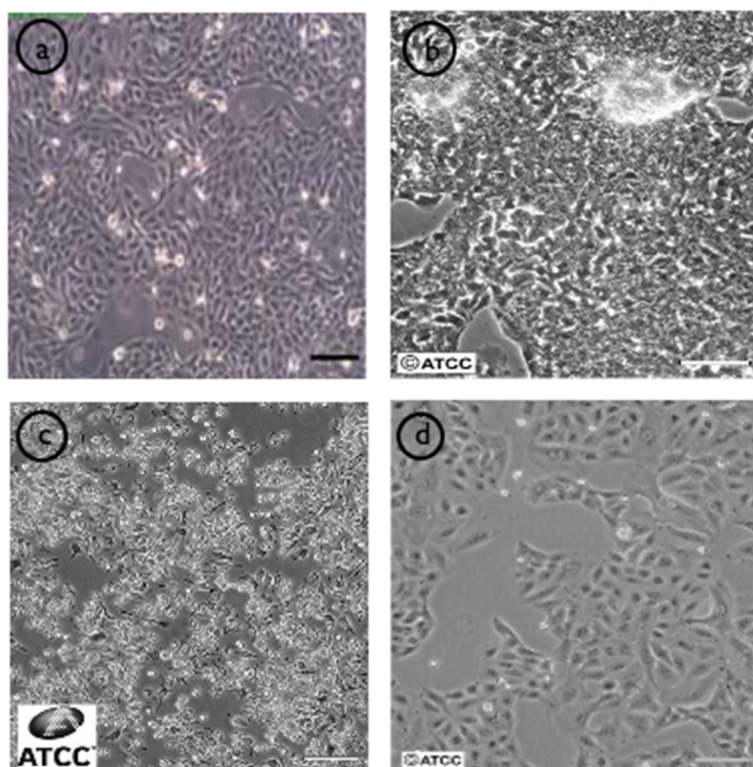
Figure 20 - Morphological appearance of the cell lines used in the cytotoxicity assay of *Daucus carota* subsp. *carota* essential oil: a - HaCat (DKFC). b - HepG2 (ATCC number HV-8065). c - RAW 264.7 (ATCC number TIB-71). d - A549 (ATCC number CCL-185). Bars = 100 µm

6.2.3. Cytotoxicity assay

RAW 264.7/HepG2/HaCaT/A549 were cultured on DMEM with the formulation described above for 48h at 37°C in humidified atmosphere of 95% air and 5% CO_2. When confluence reached 80-90% a cell suspension of 0.5×10^6 cells/mL was prepared for RAW264.7 and HepG2 while a cell suspension of 0.2×10^6 cells/mL was prepared for HaCaT and A549,. To a 48-well plate, 600µL of the cell suspension was added and the cells were left to stabilize for ±12h in the same conditions as above. The depleted medium was carefully removed and 588µL of fresh medium and following 12µL of essential oil (1.25 – 0.08 µL/mL) was added. The plates were then incubated for 24h. At the end, 60 µL of resazurin (C = 0.125 mg/mL) was added and the plates were incubated for 30 min for RAW 264.7, 60 min for HepG2. 120 min for HaCaT and 60 min for A549 in the same conditions. Cell viability was determined by reading the absorbance at 570 nm with a reference filter at 620 nm against a negative control (cells without EO) in an ELISA microplate reader (SLT, Austria). A cell-free control was performed so that unspecific effects of the oil on resazurin were excluded. All determinations

were made in duplicate. Results are shown as mean ± S.E.M of at least three independent assay. In this cytotocity assay the metabolic active cells reduce resazurin (blue dye) into resorufin (pink colored), therefore their number correlates with the magnitude of dye reduction and the viability is evaluated based on a comparison with untreated cells.

6.2.4. Statistical analysis

The statistical analysis comparing the control group with the different treatment groups was performed using an ordinary one-way ANOVA with Dunnett's multiple comparison test with significance levels $*p<0.05$, $**p<0.01$, $***p<0.001$ and $****p<0.0001$. These tests were conducted in GraphPad Prism version 6 (GraphPad Software, San Diego, CA, USA).

6.3. Results and discussion

The essential oil *Daucus carota* subsp. *carota* had no toxic effect on RAW 264.7 macrophages (Figure 20a) at concentrations ranging from 0.64 to 0.08 µLmL (92.8-154.6%). In fact, it even appears that the concentrations 0.32, 0.16 and 0.08 µg/mL promote either metabolic activity or cell growth. The A549 alveolar epithelial cells showed a susceptibility to the essential oil similar to that of RAW 264.7. No toxic effect was observed at concentrations up to 1.25 µL/mL and for 0.16-0.08 µL/mL, the oil showed a stimulatory effect on cell viability (Figure 20b). For HepG2 the concentrations 0.32 and 0.16 µL/mL had no effect on cell viability and at lower concentrations (0.08 µL/mL) the oil showed a stimulatory effect on cell viability (122.6%) (Figure 20c). Among all types of cells, keratinocytes were the most susceptible to *Daucus carota* subsp. *carota* essential oil, as only at 0.16 and 0.08 µL/mL no toxicity was observed (94.4% and 104.2%, respectively, Figure 20d).

6. Citotoxicity
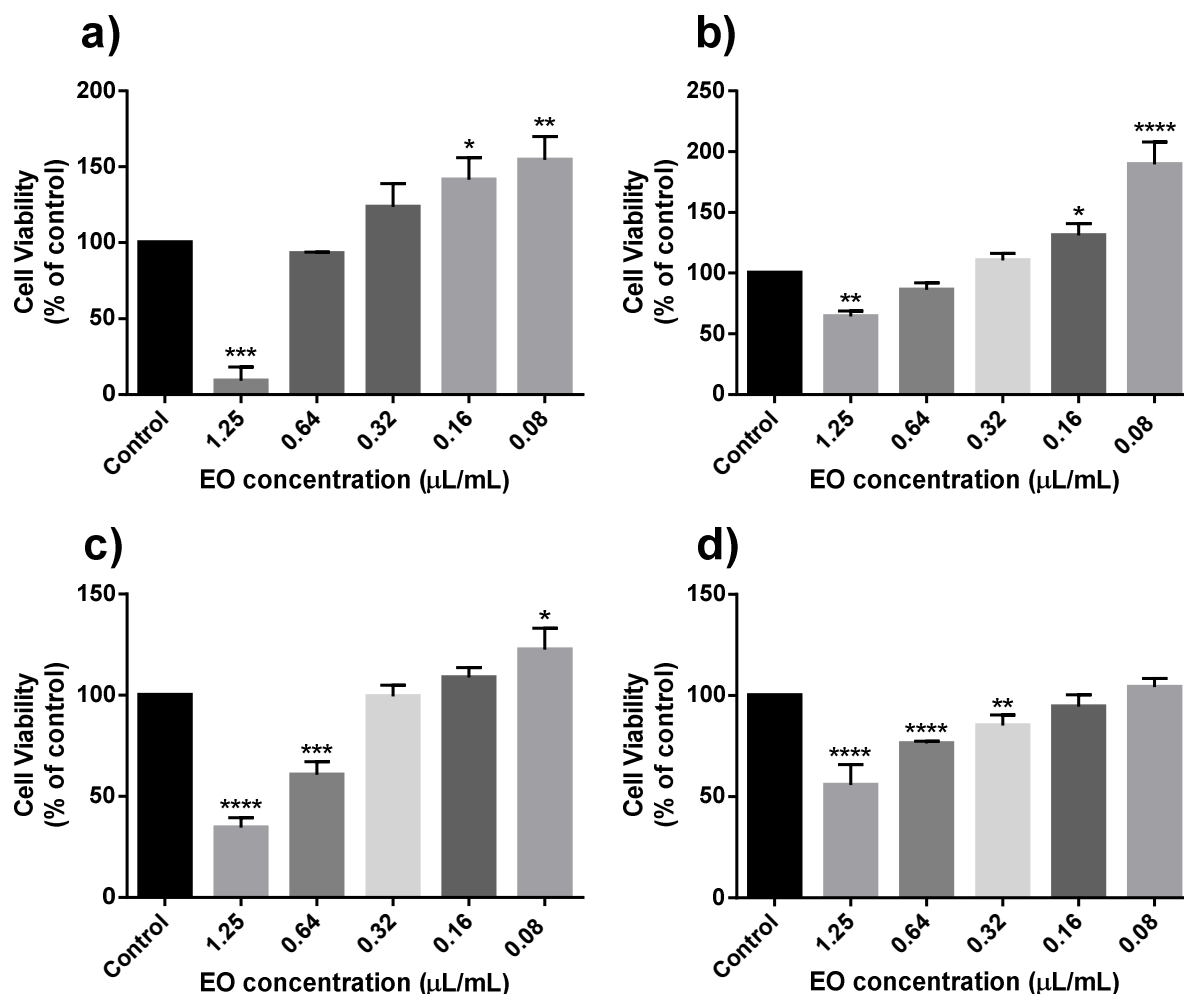
Figure 21- Effect of Daucus carota subsp. carota essential on the cell viability of a) RAW 264.7 macrophages, b) A549 alveolar cells, c) HepG2 hepatocytes and d) HaCaT keratinocytes. Cells where treated with essential oil (1.25 – 0.08 µL/mL) for 24h before addition of resazurin (0.125 mg/mL) was added. Results are expressed as percentage of resazurin metabolization by control cells. Each bar represents mean S.E.M of at least different experiments, performed in duplicate. Statistically difference compared to control cells (*p<0.05, **p<0.01, ***p<0.001, using a one-way ANOVA followed by a Dunnett's multiple comparison test)

The stimulatory effect of essential oils on cell viability was previously assessed by Gonçalves and colleagues (Gonçalves *et al.*, 2012) however a reason for this effect was not explained. Studies regarding the proliferative effect of *D. carota* subsp. *carota* essential oil should be conducted in order to elucidate the mechanisms behind such effect.

Regarding other subspecies of *D. carota* essential oil some cytotoxic studies have been conducted. In fact, the cytotoxicity of the essential oil from *D. carota* subsp. *halophilus* (Tavares *et al.*, 2008) has been tested on the foetal mouse skin dendritic cell line. However, since the authors have used a different cell line, no comparison is possible. Moreover, the cytotoxic

effect of the essential oil of *D. carota* subsp. *gummifer* has been tested against RAW 264.7, HepG2 and HaCaT cell lines. This oil was toxic at 1.25 µL/mL for RAW 264.7 (23%) and HaCaT (44.2%) whereas for HepG2 the oil had no significant effect on the cell viability. This difference might be explained by the different chemical composition between both essential oils (*trans-β*-ocimene (31.3%), sabinene (29.0%) and *cis-β*-ocimene (12.3%) of subsp. *gummifer* versus geranyl acetate (29%), α-pinene (27%), 11αH-himachal-4-en-1-β-ol (9.2%) and limonene (9%) of subsp. *carota*).

As far as we know no studies regarding the toxicity of *D. carota* subsp. *carota* have been conducted, thus the work presented here is the first of its kind. Regarding the cytotoxic effect of the major compounds of the studied essential oil (geranyl acetate, α-pinene and limonene) some studies have been conducted. Geranyl acetate was described as having a toxic effect on RAW 264.7 macrophages at 1.25 µL/mL (Gonçalves *et al.*, 2012), however other investigators have demonstrated that this compound had no effect on RAW 264.7 macrophages at 25 – 500 mg/mL (Lee *et al.*, 2009). At 100 µg/mL, α-pinene demonstrated a weak activity against hepatocyte (HepG2) cell line and no activity against alveolar epithelial cell line (A549) (Dar *et al.*, 2011). Limonene only demonstrated cytotoxic activity in RAW 264.7 macrophages at concentrations as high as 500 mg/mL (Lee *et al.*, 2009). By analysing these values, it would be expected a lower cytotoxic effect of the studied essential oil. However the cytotoxic effect of the essential oil was stronger than the isolated major compounds. This might be explained by the presence of minor compounds with synergisticeffects that overall lead to a decrease in cell viability.

In summary, the results shown in the present study suggest that Daucus *carota* subsp. *carota* essential oil is a promising bioproduct that can be further explored as an antifungal and anti-inflammatory drug. This is possible because at biological active concentrations the essential oil had no effector very low detrimental effects on cell viability.

VI. Conclusion and Future Perspectives

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6. Conclusion and Future perspectives

The work conducted in this thesis was performed using wild carrot (*Daucus carota* subsp. *carota*) growing spontaneously in Serra da Lousã, Portugal. The assessment of the biological activities: antioxidant, antifungal, antibacterial and anti-inflammatory as well as the toxicological profile, allowed a better understanding of the reasons why wild carrots have been widely used in folk medicine. In addition, it allowed a better valorisation of this species opening pathways to the possible economical exploitation of these plants, especially in the health and food sectors. The main conclusions of the present study as well as further research perspectives are pointed out below:

a. The antioxidant potential of *D. carota* subsp. *carota* essential oil and phenolic-enriched extracts [70% (v/v) and 100% (v/v) methanol and aqueous] were assessed by evaluating the scavenging capacity of free radicals (ABTS cation radical and ORAC). For polar extracts, the total phenolic content was determined using the Folin Ciocalteu assay. The essential oil had a weak performance on both antioxidant assays. This shows that the compounds in the essential oil are not good electron and H-atom donors. Conversely, 70% methanol extract had a higher activity in ABTS as well as in ORAC, showing that phenolic compounds have a tendence to be good donors of H-atom in addition to electrons. The total phenolic content of 70% methanol extract was 134.2 µg GAE/mg DW. The antioxidant activity of isolated compounds from the extracts seems to be responsible for the activity of the whole extract, nevertheless the extract´s activity is different than the isolated compounds due to the presence of minor compounds that might have a synergistic or antagonistic effect. Studies using the minor components and the conjugation between minor and major compounds should be conducted in order to establish the synergetic or anatogonistic effect between them. Also, different methods of NO scavenging should be considered. Moreover, the evaluation of the NO scavenging capacity of the phenolic-enriched extracts,as well as as well as different antioxidant assays, e.g. ferric reduction antioxidant power, DPPH radical scavenging, lipid peroxidation inhibition (TBARS, amongst others), should be conducted.

b. The antibacterial activity of the essential oil and phenolic-enriched extracts from *D. carota* subsp. *carota* have been assessed against several pathogenic bacteria. The essential oil had a higher antibacterial activity against the tested bacteria (MIC = 0.32 -

0.64 µL/mL) when compared with the 70% methanol extract (MIC = 5 - 10 mg/mL). The bacteria *E. coli* and *S. typhimurium* were resistant to both the essential oil and the phenolic extract at the tested concentrations. In the future, more assays against other pathogenic bacteria such as *C. jejuni, H. pylori¸ Pseudomonas* spp. or *Streptococcus* spp. should be conducted, along with the effect against biofilm-forming bacteria, e.g. *Streptococcus* spp. The antibacterial activity of both the essential oil and the hydromethanolic extract can be explained by the activity of isolated compounds and the differences seen between their activities is due to the presence of minor compounds that positively or negatively affect the major compounds. Nevertheless, studies evaluating the minor compounds activity as well as combination assays using both minor and major compounds should be conducted.

c. The antifungal properties of the essential oil and 70% methanol extract have been conducted against several yeasts, dermatophytes and *Aspergillus* strains. The essential oil was effective against dermatophytes (MIC = 0.31 – 0.62 µL/mL) and the yeast *Cryptococcus neoformans* (MIC = 0.16 µg/mL). All the tested yeasts from the genus *Candida, except C. guillermondi* (MIC of 0.32 µL/mL), were resilient to the action of the essential oil. However the essential oil was able to disrupt the preformed biofilm of *C. albicans* at concentrations lower than the MIC (1.25 µL/mL) thus making it promising for pharmacological application in the treatment of candidiosis. Regarding the effect on the tested *Aspergillus* strains, the essential oil demonstrated fungistastic effect against *A. fumigatus* and *A. niger* (MIC of 1.25 and 2.5 µL/mL, respectively) however without fungicidal effect (MLC higher than 10 µL/mL). The essential oil had no effect on *A. flavus* for the tested concentrations. The 70% methanol extract had a significant effect against all tested dermatophytes (MIC on the range of 1.25 and 10 mg/mL) except *T. verrucosum*. Nevertheless, it had no inhibitory effect against all tested yeast and *Aspergillus* strains. Further research regarding the antifungal activity of the essential oil and phenolic-enriched extracts should be carried out in order to further elucidate the mechanisms of action underlying these inhibitory effects. Other points that could be of interest to study is the inhibitory effect against other pathogenic fungi involved in human, animal or plant infections in order to outline a broader application of these compounds. The antifungal activity of both the essential oil and the hydromethanolic extract is explained by the activity of isolated compounds and any difference seen between their activities is due to the presence of minor compounds that positively or negatively effect the major ones. Nevertheless, studies evaluating the minor

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compounds as well as combination between minor and major compounds shouldbe conducted in order to clarify their contribution to the activity of the extracts.

d. The anti-inflammatory activity of the essential oil was carried out by evaluating it capability to inhibit NO release on LPS-stimulated RAW 264.7 macrophages. The essential oil was able to reduce by 19% the NO production in LPS-stimulated macrophages at 0.64 µL/mL without showing toxicity to the cells. Nevertheless, studies focusing on distinct inflammatory markers (e.g. TNF-α or IL-1β release) should also be conducted in order to elucidate the mechanism of action associated to this anti-inflammatory potential. Studies using phenolic-enriched extracts should also be performed, as phenolic compounds are also described as potential anti-inflammatory agents. The isolated major compounds of the essential oil are responsible for its anti-inflammatory activity however there are differences between their activities due to the antagonistic effects of other compounds.

e. The safety profile of the essential oil was evaluated on macrophages, keratinocytes, alveolar epithelial cells and hepatocytes. The essential oil was toxic for all cell lines only at higher concentrations (1.25 µL/mL). However at 0.64 – 0.08 µL/mL the toxicity was greatly dependent on the cell line being hepatocytes and keratinocytes slightly more susceptible. At 0.32 µL/mL the essential oil was able to inhibit the growth of *C. guillermondi*, *C. neoformans, E. floccosum* and *T. rubrum* without showing significant toxicity to keratinocytes, thus justifying a potential topical application. Also, at 0.64 µL/mL, the oil showed an anti-inflammatory potential without affecting macrophages viability. The cytotoxic effect of the essential oil at higher concentrations may be explained by the major compounds found as they are known to be toxic towards the studied cells lines. Research using phenolic-enriched extracts should be also performed together with individual components in order to evaluate which are responsible for the toxicity of both essential oil and extracts. In some cell lines, the essential oil had a stimulatory effect on them, further research should be conducted in order to clarify the reasons behind this effect. Also, cytotoxic effects on cancer cell lines should be evaluated thus elucidating the anti-cancer properties of the essential oil and phenolic-enriched extracts

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